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M. Nyman · S. Waara

Characterisation of somatic hybrids between *Solanum tuberosum* and its frost-tolerant relative *Solanum commersonii*

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Abstract Somatic hybrids between three dihaploid *Solanum tuberosum* (2n = 2x = 24) genotypes and the frost-tolerant, diploid, relative *Solanum commersonii* (2n = 2x = 24) were analysed for variation in morphological traits, fertility and frost tolerance. The somatic hybrids were more vigorous than their parents and in many ways resembled a tetraploid *S. tuberosum.* All of the hybrids flowered profusely, although the male fertility was largely dependent on the *S. tuberosum* genotype used. In one hybrid combination all plants were both male- and female-fertile, while in the other two combinations the majority of plants were male-sterile but female-fertile. The somatic hybrids showed an increase in direct frost tolerance when compared with the dihaploid *S. tuberosum* parents, and to a varying extent had gained the capacity to cold acclimate. These somatic hybrids will be used in breeding programmes involving repeated cycles of anther culture and somatic hybridisation.

Key words Somatic hybrids · Fertility · Frost tolerance · *S. tuberosum* · *S. commersonii*

Introduction

Freezing temperatures cause a considerable reduction in crop yield in many parts of the world, and unseasonal frosts are considered to be one of the major factors limiting potato production (Chen and Li 1987). Little progress has been made by using traditional breeding methods for the improvement of frost tolerance in crop plants, a failure that in part is due to the lack of genetic diversity (Marshall 1982). The cultivated potato, *Solanum tuberosum*, possesses very little or no frost tolerance and does not exhibit any cold-acclimation capacity, making introgression of genes from wild frost-tolerant *Solanum* species desirable.

Solanum commersonii Dun, is a tuber-bearing, diploid, species that can withstand low temperatures and possesses the ability to cold acclimate. While the killing temperature for *S. tuberosum* has been reported to be around −3°C depending on the genotype, *S. commersonii* can survive temperatures as low as −11.5°C and −4.5°C, with and without proper acclimation, respectively (Chen and Li 1980). In addition, several *S. commersonii* accessions possess other characters of agronomic interest, such as resistance to common scab (*Streptomyces scabies*) and resistance to the Colorado beetle (*Leptinotarsa decemlineata*) (Hawkes 1990). The use of *S. commersonii* in potato breeding has, however, been problematic since it is not readily crossable with *S. tuberosum.* In the study of Novy and Hanneman (1991) hybrid plants where obtained from *S. tuberosum* haploids and a tetraploid *S. commersonii.* The hybrids where all triploid and female- as well as male-sterile. A way to circumvent the crossing barriers between species is to use somatic hybridisation. Successful production of somatic hybrids between *S. tuberosum* and various wild *Solanum* species (for a review see Waara 1996), including *S. commersonii* (Cardi et al. 1993 a, b), has been reported. However, only a few tetraploid hybrids where obtained in the study of Cardi et al. (1993 b), and only one of them proved to be both male- and female-fertile. This fertile hybrid was analysed for the capacity to withstand frost, and a slight increase in tolerance, compared to the *S. tuberosum* parent, was observed. Protoplast fusion, followed by an efficient

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M. Nyman (✉) · S. Waara[1]
Department of Plant Breeding, Swedish University of Agriculatural Sciences, Box 7003, S-750 07 Uppsala, Sweden
Fax: +46-18-673279
E-mail: Marie.Nyman@vf.slu.se

Present address:
[1] Department of Energy, Mälardelens Högskola, Box 883, S-721 23 Västerås, Sweden

flow cytometric enrichment of heterokaryons, enabled the production of a large number of tetraploid somatic hybrids between *S. tuberosum* and *S. commersonii* (Waara et al., submitted). These hybrid plants form the basis for the present study and have been evaluated with regard to morphology, fertility and frost tolerance.

Material and methods

Plant material

The plant material consisted of five different hybrid combinations between *S. commersonii* and *S. tuberosum*. The material used for fusion was *S. commersonii* 2:2 and 2:5 (two seeding clones from OKA 5040, PI 472834) and the anther-derived dihaploid *S. tuberosum* clones 536:2 (cv Matilda), 161:14 (cv Stina) and 67:9 (Norwegian breeding clone Y-67-20-40). The different fusion combinations are listed in Table 1. Procedures for the production and identification of the hybrids used will be reported elsewhere (Waara et al., submitted). All the hybrids assessed in this study were tetraploids according to a flow-cytometric analysis of the DNA content.

Morphology

Three plants from each of 125 tetraploid hybrid clones were transferred to the greenhouse. Tubers from these plants were further transplanted and grown to maturity. At the onset of flowering the following characters were recorded; plant height, plant morphology, leaf morphology, flowering capacity, flower morphology and anthocyanin pigmentation. Tubers were harvested and the size, shape and yield were noted.

Fertility

Pollen from eight flowers/clone were collected by shaking mature flowers with a battery operated vibrator. The pollen was stained with fluorescein di-acetate (FDA) (Heslop-Harrison and Heslop-Harrison 1970) and run through a FACStar flow cytometer (Becton-Dickinson, Electronics Laboratory) to determine pollen viability. Male-sterile plants were pollinated with pollen from a fertile hybrid from the 67:9 (+) 2:2 combination. Plants were considered female-fertile if they produced berries with seeds.

Frost tolerance

The non-acclimated frost tolerance and the cold-acclimation capacity of the hybrids, the parental clones, and the tetraploid cv Matilda were determined by the freezing of detached leaves, wrapped in Miracloth (Calbiochem), in a temperature-controlled ethanol bath. One hundred and twelve hybrids were tested for non-acclimated frost tolerance and 106 hybrids were tested for their ability to cold acclimate. To avoid possible epigenetic effects only tuber-derived plants were used in these analyses. Prior to flowering, three fully expanded leaves per clone were tested at each temperature. The procedure was essentially as described by Lång et. al (1989). Extracellular freezing was initiated at −0.5°C by touching the samples with a frosted wire. After 1 h the temperature was lowered at a rate of 2°C/h, and then held at the temperature to be tested for 1/2 h. The samples were thawed on ice overnight after which the freezing injury was determined using the electrolyte-leakage method, essentially as described by Sukumaran and Weiser (1972), with modifications according to Lång et al. (1989).

De-ionized water (20 ml) was added to each test tube. After 1 h on a shaker, the conductivity of the solution was measured. In order to obtain a value for 100% leakage, leaves were submerged in liquid nitrogen, put back into the original solution and, after an additional hour on the shaker, the conductivity was measured once more.

The temperature at which over 50% leakage occurred was considered as being the lethal temperature (LT_{50}), i.e. the temperature that would kill all the foliage.

The capacity to cold acclimate was evaluated by keeping the plants at 4°C/2°C day/night, for 2 weeks before their ability to tolerate frost was determined as described above.

Results

Morphology

In the first generation, i.e. plants derived from in vitro culture, there was a pronounced morphological variation between the hybrids, with the most morphologi cally deviating plants exhibiting a stunted growth (Fig. 1 c) and producing only a few buds that fell off before flowering. By contrast, the phenotypes of the tuber-derived hybrids were very similar within and between the hybrid populations (Fig. 1 a). Only the description of the tuber-derived somatic hybrids is presented below.

The anther-derived dihaploid clones did not flower under the prevailing conditions. However, the tetraploid anther donor clone cv Matilda (dihaploid 536:2) produced a few white flowers while the flowers of the tetraploid cv Stina (dihaploid 161:14) were red-purple. The dihaploid clone 67:9 has in an earlier study produced anthocyanin-pigmented flowers with rudimentary petals (Waara et al. 1992). Stems and leaves were pure green in 536:2, and slightly anthocyanin pigmented in 67:9 and 161:14 *S. commersonii* produced a large number of stellated purple flowers and the plants showed pronounced anthocyanin pigmentation on stems and leaves.

All hybrids were taller than the parental clones and largely resembled a tetraploid *S. tuberosum* plant. They flowered profusely with semi-stellated purple flowers (Fig. 1 b) and the anthocyanin pigmentation was as in *S. commersonii*, distributed over stems and leaves. The size of the flowers was larger than those of *S. commersonii*.

All hybrids produced a large number of tubers, with a shape resembling their *S. tuberosum* parent, respectively, i.e. round for those containing 161:14 or 67:9 and oval for those that had 536:2 as the *S. tuberosum* parent. The tuber size and the yield were, with a few exceptions, larger than those of the parental clones. A typical *S. commersonii* character, notted skin, was also appearent on most of the hybrid tubers, and all of them like their wild parent, produced long stolons (Table 1).

In a parallel experiment, asymmetric hybrids were produced between cv Matilda and X-irradiated

S. commersonii protoplasts (data not shown). The tubers from some of these plants suffered severely from common scab, while the symmetric hybrids never showed any sign of this disease.

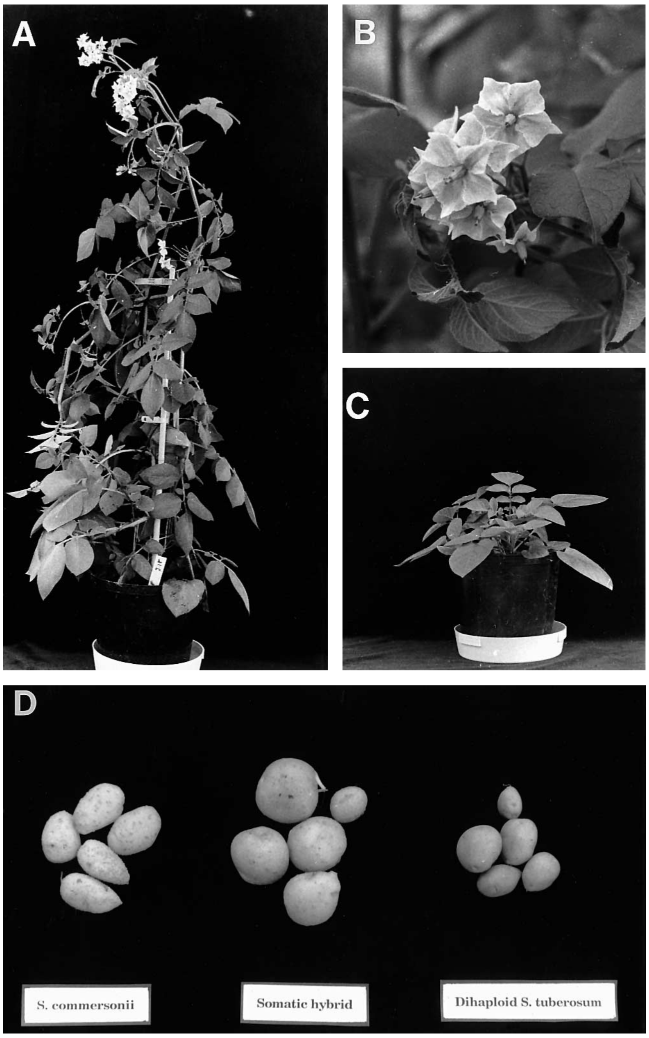

Fig. 1a–d **a** A somatic hybrid from the tuber generation. **b** Flowers from a fertile somatic hybrid. **c** The same somatic hybrid as in **a**, planted in greenhouse directly from in vitro culture without an intervening tuber phase. **d** Tubers from *S. commersonii*, a somatic hybrid, and dihaploid *S. tuberosum*

Fertility

S. commersonii flowered profusely and produced pollen that showed 80% viability, while the dihaploid *S. tuberosum* clones did not flower at all.

In the hybrid combinations using 67:9 as the *S. tuberosum* parent, all plants produced flowers that were considered to be male- as well as female-fertile. The viability of the pollen varied considerably between different clones, from about 1 up to 90%. Twenty one plants showed less than 25% pollen viability, one plant between 25 and 50%, nine plants between 50 and 75%, and five plants more than 75%. However, even those plants that produced less than 5% viable pollen produced berries with seeds.

The hybrids produced from the combinations involving the dihaploids 161:14 or 536:2 produced phenotypically normal flowers with well developed anthers, but most of these anthers did not contain any pollen. Microscopical studies revealed a few degenerated structures in the anthers, but no tetrads. However, after pollination with one of the 67:9 (+) *S. commersonii* hybrids, most of them produced berries and were thus regarded as female-fertile. Three 536:2 (+) *S. commersonii* hybrids produced pollen with a viability between 20 and 25%. However, the amount of pollen in these plants was very low, i.e. 100–200 pollen grains/8 flowers, was collected with the method described above, compared with between 5–20 000 grains for the 67:9 (+) *S. commersonii* hybrids.

Frost tolerance

All of the hybrids showed a higher degree of non-acclimated frost tolerance than their dihaploid *S. tuberosum* parents, and had to a varying extent gained the capacity to cold acclimate. The dihaploid *S. tuberosum* parents, 161:14, 67:9 and 536:2, had a lethal temperature of −1°C, while the frost killing temperature for the tetraploid cv Matilda was −2°C. None of the *S. tuberosum* genotypes were able to cold acclimate. The lethal temperature for *S. commersonii* proved to be −10.5°C and −4.5°C with or without a hardening pre-treatment, respectively. There was a variation in frost tolerance between the different hybrids. With

Table 1 Characters of the parental clones *S. cmm* (*S. commersonii*), 536:2, 67:9 and 161:14 (dihaploid *S. tuberosum*), and somatic hybrids. Figures in brackets indicate the number of hybrids tested

Characters	536:2	67:9	161:14	*S. cmm*	*S. cmm* (+) 536:2 (25)	*S.cmm* (+) 67:9 (50)	*S. cmm* (+) 161:14 (50)
Long stolons	No	No	No	Yes	Yes	Yes	Yes
Anthocyanin production	Absent	Low	Low	High	High	High	High
Flowering	Absent	Absent	Absent	Present	Present	Present	Present
Pollen production	–	–	–	Abundant	Low	Abundant	None
Shape of tubers	Oval	Round	Round	Oval	Oval	Round	Round
Acclimation capacity	No	No	No	Yes	Yes	Yes	Yes

Table 2 The number of non-acclimated somatic hybrids with a LT_{50} value at a given temperature

Temperature	67:9 (+) 2:2	67:9 (+) 2:5	161:14 (+) 2:2	161:14 (+) 2:5	536:2 (+) 2:5
− 2°C	10	3	17	12	15
− 3°C	15	12	6	13	9
Total	25	15	23	25	24

Table 3 The number of acclimated somatic hybrids with a LT_{50} value at a given temperature

Temperature	67:9 (+) 2:2	67:9 (+) 2:5	161:14 (+) 2:2	161:14 (+) 2:5	536:2 (+) 2:5
− 6°C	3	2	1	3	7
− 7°C	5	6	3		6
− 8°C	10	7	10	10	5
− 9°C	5	1	7	10	5
Total	23	16	21	23	23

respect to non-acclimated frost tolerance, 57 plants had, like the tetraploid *S. tuberosum* tested, a lethal temperature around −2°C and 55 plants died at −3°C (Table 2). The estimated LT_{50} of the hybrids after cold acclimation ranged from −6°C down to −9°C (Table 3). As an additional test, visual observations of the leaves after freezing were performed. These observations were in agreement with the results from the electrolyte-leakage method, since loss of turgor was always correlated with the estimated LT_{50} value.

S. tuberosum is generally not considered chilling sensitive (Chen and Li 1980). However, when the asymmetric hybrids [cv Matilda (+) X-ray irradiated *S. commersonii*] were subjected to the acclimation conditions, severe chlorosis and necrotic lesions developed on the leaves of most of the plants, except for those that were able to cold acclimate (data not shown). None of the symmetric hybrids showed any symptoms of chilling injury.

Discussion

When regenerated plants are transferred from in vitro conditions to the greenhouse they can exhibit epigenetic changes. These types of transient, non-genetic, changes appear to be a direct effect of the culture conditions and are physiological in origin (Lindsey and Jones 1989). In our material the morphological variation seen between hybrids in the first generation, i.e. in vitro derived hybrids, disappeared in the tuber-derived plants and was then stable in the following generation, suggesting a pronounced epigenetic effect. Since tissue culture-derived plants are not always allowed to go through a second generation before analysis, many of the reports dealing with extensive somaclonal variation in the material might in fact be the result of a more transient effect.

The results from the frost-tolerance tests clearly showed that many of the hybrids expressed a higher non-acclimated frost tolerance than *S. tuberosum*, and that all of them had obtained the capacity to acclimate. There was no correlation between these two traits, in that hybrids that showed a good acclimation capacity automatically also had an increased non-acclimated frost tolerance. In the study of Cardi et al. (1993 b) only the fully fertile hybrid was evaluated for frost tolerance. This particular hybrid showed the following LT_{50} values (with those after acclimation shown in brackets): *S. tuberosum*, 0°C (− 0.8°C); *S. commersonii*, − 3.8°C (−9.8°C); somatic hybrid, − 0.7°C (−3.3°C). In the present study many hybrids had an LT_{50} value more close to that of *S. commersonii* for both acclimated and non-acclimated frost tolerance, with the most tolerant plants showing a decrease in the lethal temperature by 8°C and 1°C with and without acclimation, respectively, compared with *S. tuberosum*.

Most studies have concluded that freezing tolerance is a complex, polygenetically inherited trait (Stushnoff et al. 1984). Furthermore, Stone et al. (1993) reported evidence for independent genetic control of non-acclimated freezing tolerance and acclimation capacity in sexual hybrids between *S. commersonii* and *S. cardiophyllum*. The fact that there was no correlation between the two frost-survival strategies and that the hybrids had obtained the acclimation capacity to a varying degree implies, in agreement with the studies mentioned above, that several genes are involved and that these traits have independent genetic control mechanisms.

Cold acclimation has been correlated with an increase in total lipids, phospholipids and unsaturated fatty acids (Li 1984). Palta and Weiss (1993) reported that *S. commersonii* showed an increase in the unsaturation of membrane fatty acids during low-temperature treatment, while the non-acclimating species *S. tuberosum* did not. The fact that none of the hybrids in this study could reach the acclimation level of *S. commersonii* might therefore be a result of the mixed membrane composition of the hybrids. Another possible explanation is that the hybrids might not have developed the double palisade layers associated with the frost-hardiness of tuber-bearing *Solanum* species, such as *S. commersonii* (Palta and Li 1979).

Low, non-freezing, temperatures during the vegetative period limit the use of the potato in alpine and arctic regions. By comparing Andean populations of different potato species with European populations, Kristjansdottir (1989) concluded that the Andean population showed a general capacity to maintain growth at 10°C/4°C, day/night, while the European populations grew more slowly. All the plants in the Andean populations were free of chlorosis during the earlier part of the growth period, while no plants in the European populations where entirely without chlorosis. In the present study, we observed severe chlorosis and necrotic spotting on *S. tuberosum* and most of the asymmetric hybrids under acclimation conditions, while the symmetric somatic hybrids where unaffected, suggesting a increased chilling tolerance in the hybrid material.

Interspecific hybrids between potato species generally have a low male fertility or else are completely male-sterile (Ehlenfeldt et al. 1987; Mattheij et al. 1992; Cardi et al. 1993 b; Rokka et al. 1995). This is a problem since these plants often contain many unwanted traits from the wild species, making several backcrosses with the cultivated species necessary. It is therefore of utmost importance to obtain fertile hybrids, to allow for an introgression of germplasm from wild species into a breeding programme. Conversely, alloplasmic male sterility is a desirable trait for the production of true potato seed (Perl et al. 1990).

All of the hybrids where 67 : 9 was used as the *S. tuberosum* parent were fully fertile, while the other combinations resulted in only three pollen-producing hybrids which produced only a small amount of pollen. Nuclear-cytoplasmic interactions are known to be an important factor in male fertility (Hanson and Conde 1986). Studies on sexual hybridisation between *S. tuberosum* and *S. commersonii* points to the fact that this is also the main problem in trying to produce fully fertile hybrids involving *S. commersonii* (Novy and Hanneman 1991). Moreover, Cardi et al. (1993 b) reported that only one fully fertile plant was produced after somatic hybridisation between these two species.

In the present study *S. tuberosum* plants with different cytoplasmic backgrounds were used. The dihaploids 161 : 14 and 536 : 2, which in combination with *S. commersonii* produced completely male-sterile hybrids, or in a few cases hybrids with very low pollen production, have a *S. tuberosum* cytoplasm. By contrast, 67 : 9, which in combination with *S. commersonii* produced nearly 100% fully fertile plants, has a *S. stoloniferum* cytoplasm, suggesting that the cytoplasm from the potato parent plays an important role in producing male-fertile hybrids. These results were not expected, since the tetraploid anther donor plant (Y67-20-40) is male-sterile, with pollen development arrested at the tetrad stage.

The cv Matilda is very susceptible to common scab, while cv Stina is fairly resistant and Y67-20-40 completely resistant. The absence of this disease on the tubers of all the symmetric hybrids, in contrast to the asymmetric hybrids growing in the same greenhouse, suggests that, besides frost tolerance, other important resistance properties have been transferred to the hybrids.

In conclusion, a population of fully fertile, as well as female-fertile/male-sterile *S. tuberosum* (+) *S. commersonii*, somatic hybrids, with a varying degree of frost tolerance and the ability to cold acclimate has been produced. These plants are currently being used in breeding programmes involving repeated cycles of anther culture and somatic hybridisation as proposed by Rokka et al. (1995).

References

Cardi T, Puite KJ, Ramulu KS, D'Ambrosio F, Frusciante L (1993 a) Production of somatic hybrids between frost-tolerant *Solanum commersonii* and *S. tuberosum*: protoplast fusion, regeneration and isozyme analysis. Am Potato J 70 : 753–765

Cardi T, D'Ambrosio F, Consoli D, Puite KJ, Ramulu KS (1993 b) Production of somatic hybrids between frost-tolerant *Solanum commersonii* and *S. tuberosum*; characterization of hybrid plants. Theor Appl Genet 87 : 193–200

Chen THH, Li PH (1987) In vitro induction of cold acclimation in potato. In: YPS Bajaj (ed) Biotechnology in agriculture and forestry 3 – Potato. Springer-Verlag, Berlin Heidelberg New York, pp 256–267

Chen THH, Li PL (1980) Characteristics of cold acclimation and de-acclimation in tuber-bearing *Solanum* species. Plant Physiol 65 : 1146–1148

Ehlenfeldt MK, Helgeson JP (1987) Fertility of somatic hybrids from protoplast fusions of *Solanum brevidens* and *S. tuberosum*. Theor Appl Genet 73 : 395–402

Hanson MR, Conde MF (1986) Function and variation of cytoplasmic genomes: lessons from cytoplasmic-nuclear interactions affecting male sterility in plants. Int Rev Cytol 94 : 213–267

Hawkes JG (1990) The potato; evolution, biodiversity and genetic resources. Belhaven Press, London

Heslop-Harrison J, Heslop-Harrison Y (1970) Evaluation of pollen viability by enzymatically induced fluorescence: intracellular hydrolysis of fluorescein di-acetate. Stain Technol 45 : 115–120

Krisjansdottir IS (1989) Influence of suboptimal temperature on biomass production of potato populations of Andean and European origin. Euphytica 44 : 23–35

Li PH (1984) Sub-zero temperature stress physiology of herbaceous plants. Hortic Rev 6 : 373–416

Lindsey K, Jones MGK (1989) Plant biotechnology in agriculture. Open University Press, Milton Keyes

Lång V, Heino P, Palva ET (1989) Low-temperature acclimation and treatment with exogenous abscisic acid induce common polypeptides in *Arabidopsis thaliana* (L.) Heynh. Theor Appl Genet 77 : 729–734

Marshall HG (1982) Breeding for tolerance to heat and cold. In: Christianson MN, Lewis CF (eds) Breeding plants for less-favourable environments. Wiley-Interscience, New York, pp 47–70

Mattheij WM, Eijlander R, de Koning JRA, Louwes KM (1992) Interspecific hybridization between the cultivated potato *Solanum tuberosum* subspecies *tuberosum* L. and the wild species *S. circaeifolium* subsp. *circaeifolium* Bitter exhibiting resistance to *Phytophthora infestans* (Mont.) de Bary and *Globodera pallida* (Stone) Behrens. 1. Somatic hybrids. Theor Appl Genet 83 : 459–466

Novy RG, Hanneman RE Jr (1991) Hybridization between Gp. *tuberosum* haploids and 1EBN wild potato species. Am Potato J 68 : 151–169

Palta JP, Li PH (1979) Frost-hardiness in relation to leaf anatomy and natural distribution of several *Solanum* species. Crop Sci 19 : 665–671

Palta JP, Weiss LS (1993) Ice formation and freezing injury: an overview on the survival mechanisms and molecular aspects of injury and cold acclimation in herbaceous plants. In: Li PH, Christersson L (eds) Advances in plant cold hardiness. CRC Press, Boca Raton, Florida, USA, pp 143–176

Perl A, Aviv D, Galun E (1990) Protoplast fusion-derived CMS potato cybrids: potential seed-parents for hybrid, true potato seed. J Hered 81 : 438–442

Rokka VM, Valkonen JPT, Pehu E (1995) Production and characterization of haploids derived from somatic hybrids between *Solanum brevidens* and *S. tuberosum* through anther culture. Plant Sci 112 : 85–95

Stone JM, Palta, JP, Bamberg JB, Weiss LS, Harbage JF (1993) Inheritance of freezing resistance in tuber-bearing *Solanum* species: evidence for independent genetic control of non-acclimated freezing tolerance and cold-acclimation capacity. Proc Natl Acad Sci USA 90 : 7869–7873

Stushnoff C, Fowler B, Bruele-Babel A (1984) Breeding and selection for resistance to low temperature. In: Vose PB, Blixt SG (eds) Crop breeding: a contemporary basis. Pergamon Press, New York, pp 115–136

Sukumaran NP, Weiser CJ (1972) An excised leaflet test for evaluating potato frost tolerance. HortScience 7 : 467–468

Waara S (1996) The potentials of using dihaploid/diploid genotypes in breeding potato by somatic hybridization. In: Jain SM, Sopory SK, Veilleux RE (eds) In vitro haploid production in higher plants, vol 2. Kluwer Academic Publishers, pp 319–336

Waara S, Pijnacker L, Ferwerda MA, Wallin A, Eriksson T (1992) A cytogenetic and phenotypic characterization of somatic hybrid plants obtained after fusion of two different dihaploid clones of potato (*Solanum tuberosum* L.). Theor Appl Genet 85 : 470–479