Interspecific Potato Somatic Hybrids Between *Solanum Malmeanum* and *S. Tuberosum* Provide Valuable Resources for Freezing-Tolerance Breeding

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Abstract

Freezing stress affects the geographic distribution, growth, and development of potato, resulting in loss of its yield. Solanum malmeanum , a diploid wild species with strong freezing tolerance, was fused with a freezing sensitive dihaploid S. tuberosum by somatic hybridization. In our study, 980 calli were obtained, and 248 differentiate shoots from the calli. Parental-specific SSR markers were used to analyze the chromosome composition of the randomly selected 80 regenerated plants, resulting in 51 somatic hybrids. Among them, 44 somatic hybrids were tested with ploidy analysis in the years 2016 and 2020. During subculture, the genomic ploidy levels changed due to the composition of the unstable chromosome in 56.82% of the somatic hybrids. Compared with the cultivated parent, somatic hybrids showed better freezing tolerance. After freezing-tolerant somatic hybrids were selected to backcross with cultivars, we obtained some valuable breeding resources with enhanced freezing tolerance while similar tuberization capacity close to cultivars. The correlation analysis shows that freezing tolerance has no relation with tuberization capacity, which indicates that they are controlled by independent genetic loci. In all, we successfully conducted the protoplast fusion between S. malmeanum and S. tuberosum for the first time, which provided valuable resources for freezing-tolerant breeding.

Introduction

Potato (*Solanum tuberosum* L.), the fourth food crop and the most important tuber crop in the world, is cultivated extensively for low fat and rich nutrition value and consumed by more than one billion people (Bradeen and Kole 2016; Haan and Rodriguez 2016). While in the development of potato, it confronts a series of biotic and abiotic stresses. Especially the freezing stress, typical abiotic stress, makes disasters in the growth, development, production, and distribution of potato (Chinnusamy et al. 2007). Faced with the situation, it is significant to construct potato germplasm with freezing-tolerance by genetic modification. The cultivated potato is nearly all sensitive to frost, which has little genetic variation in both freezing tolerance and acclimation capacity (ACC). It will be frozen to death when the temperature decreases to -3°C (Chen and Li 1980; Seppänen et al. 2001). On the contrary, wild potato species possess higher freezing tolerance, including non-acclimated freezing tolerance (NA) and cold acclimation freezing tolerance (CA) (Palta and Simon 1993; Palta 1994; Gray et al. 1997; Seppänen et al. 2008). In previous studies, more than 30 wild species, such as *S. acaule*, *S. boliviense*, *S. chomatophilum*, *S. commersonii*, and *S. demissum*, were reported to possess strong freezing tolerance (Ross and Rowe 1965; Li 1977; Vega and Bamberg 1995). Our previous freezing tests in a large number of wild species also showed strong freezing tolerance in *S. acaule*, *S. albicans*, *S. commersonii*, *S. demissum*, and *S. malmeanum*, which all were significant germplasms for potato freezing-tolerant breeding.

The cross incompatibility is a strong barrier between interspecific hybridization. For example, the barriers act at the pollen-pistil (pre-zygotic) or the embryo and the endosperm levels (post-zygotic). If hybrids are formed, they can cause hybrid weakness, sterility, or a breakdown in segregating generations (Cardi et al. 1993; Camadro et al. 2004; Bryan et al. 2017). Protoplast fusion provides a new approach to utilize wild potato resources, especially for abiotic resistance. A series of efforts in genetic improvement arose after Shepard and Totten (1977) first employed somatic hybridization in potato research (Yamada et al. 1997; Bołtowicz et al. 2005; Bidani et al. 2007; Liu et al. 2016; Tiwari et al. 2018; Wang et al. 2020). In freezing tolerance research, wild potato species, such as *S. brevidens* and *S. commersonii*, were used to introgress freezing tolerance to

cultivated potato (Preiszner et al. 1991; Cardi et al. 1993; Nyman and Waara 1997; Bastia et al. 2000; Xu et al. 1993), which made the hybrids own a better resistance in cold stress. However, little follow-up was focused on the agronomic traits, tuberization, genetic improvements, and genetic stability of the obtained somatic hybrids.

In this study, we conducted the protoplast fusion between freezing-tolerant species *S. malmeanum* and *S. tuberosum*, and make further analysis on genetic stability after the periods of continuous tissue culture, freezing tolerance, and agronomic traits of the somatic hybrids. Backcross was conducted between somatic hybrids with freezing tolerance improved and tetraploid cultivars, resulting in that the BC1 progenies possessed greater freezing tolerance and better agronomic traits.

Materials And Methods

Plant materials and growth conditions

Except MLM266-2 kindly-provided by Wageningen UR, the other materials were preserved in our lab. The dihaploid *S. tuberosum* (AC142) is freezing susceptible and diploid wild species *S. malmeanum* (MLM266-2) holds strong freezing tolerance. Another 21 tetraploid cultivars were used to make genetic improvement of the somatic hybrids in this study (Table 1), including Redsen, Adirondack, 393160-4, Huashu 1, etc. The fusion parents and regenerated plants were maintained in tissue culture on MS medium (Murashige and Skoog 1962) supplemented with 4% sucrose and 8% agar at 22 ± 2°C with a photoperiod of 16 h/day under a light intensity of 60 umol m^{-2} s^{-1}. The regenerated plants were numerically named by the order of callus and shoot formation. For example, M+A1-1 represented the first shoot regenerated from the first callus formed, which was fused from parents MLM266-2 and AC142.

Protoplast isolation to plant regeneration

The protoplasts were isolated and fused as described by Yu (2013), leaves from three-week-old plantlets were pretreated in the floatation medium (FM) solution at 25°C under 48 h darkness. The conditioned medium (CM) solution was used to incubate at 4°C in darkness for 24 h. The leaves were cut into pieces and incubated overnight in an enzyme solution, subsequently filtered through a nylon sieve (100 μm mesh). Then protoplasts were derived after being purified by centrifugation in solutions of 6.35% (w/v) mannitol + 0.2 mM $CaCl_2$. All the solutions mentioned above were prepared as described by Yu (2013). The protoplast of both parents was mixed with a ratio of 1:1 by volume and then was adjusted to the cell density of 2×10^5 ml^{-1} by electrical fusion solution. Then protoplasts were treated under an AC-field at 100 V cm^{-1} for 20 s, subsequently, under 1,100 V cm^{-1} DC voltage for 60 μs to achieve protoplast fusion (Eppendorf Multiporator 4308, Germany). Calli formed in the induced medium after the protoplast fusion, and regenerated shoots were derived in differentiation medium. The regenerated shoots were excised and cultured on MS medium for root development.

Ploidy determination and SSR analysis of the somatic hybrids

The ploidy level of somatic hybrids and BC1 progenies was determined by flow cytometry (BD FACS Calibur) and Cystain® UV Precise T Kit. Approximately 0.5 cm^2 of leaves from four-week-old grown plantlets were chopped in a plastic Petri dish containing 60 μl nuclei extraction solution for 30 to 60 s, subsequently stained in 500 μl Staining Buffer for 30 s. Leaf extracts were then filtered through a 50 μm CellTrics filter into a tube, and

the DNA content of nuclei was tested by a ploidy analyzer (BD FACS Calibur). Taking the diploid AC142 and tetraploid E3 as control, the distribution graph was analyzed using flow cytometry.

Root tips were used for chromosome counting, and the root length was 1-2 cm of the *in vitro* plantlets. The materials were treated in 0.2 mmol/L of 8-hydroxyqumoline for 3 h at room temperature, rinsed in distilled water, and fixed in a solution of ethanol: glacial acetic acid (3:1, v/v) for at least 2 h. The treated materials were maintained in 70% alcohol for further use. Then the samples were placed in an enzyme mixture for 1.5 h at 37°C, which contained 2% (w/v) pectinase and 2% (w/v) cellulase (Sigma, USA), dissolved in citrate buffer solution (a ratio of 123:77(v/v) between 0.1 M citrate buffer and 0.1 M citrate sodium). The root tips were rinsed in water for 10 min twice, subsequently transferred to a clean slide. Added with 1-2 drops of Carnoy's Fluid, and the root tips were squashed to spread the chromosomes. Then they were stained with 2 μg ml^{-1} DAPI in darkness, and fluorescent images of the chromosomes were captured by fluorescence microscope.

Total genomic DNA was extracted from leaves of the *in vitro* plantlets followed CTAB procedure. Two pairs of fusion parents-specific SSR markers were utilized to identify somatic hybrids, primer pairs S165 (localized on chromosome XI, FW: 5'-ACCTGTACCAGTCGGACCTT-3' and RW: 5'-GCACAAGGGGTTGCTTAACC-3') and S215 (localized on chromosome I/VII/VIII, FW: 5'-GTGGTGGTGGAATCGTCTTT-3'and RW: 5'-AGGCAGTGATGAGATCACAAA-3'). The amplification was carried out with a C1000 Thermal Cycler (Bio-Rad Inc., Hercules, CA, USA), and it was performed in a 20 μl reaction mixture containing 14.4 μl dd H_2O, 2.6 μl Taq mix, 2 mM $MgCl_2$, 1 μl 10 μM of each primer pair and 50 ng of genomic DNA. The thermal cycling profile was: 4 min pre-denaturing at 95°C, followed by 35 cycles (95°C for 40 s, temperature annealing 54°C for 40 s, and 72°C for 1 min), and a final extension for 10 min at 72°C. The amplification products were analyzed by 9 % polyacrylamide gel electrophoresis (PAGE) and silver staining. Images were captured by a digital camera.

Measurement of plant morphology

Four-week-old plantlets of the fusion parents, somatic hybrids, and BC1 progenies were transplanted into plastic pots (10 Í 10 cm) during a growth room at 22 ± 2°C with a photoperiod of 16 h/day under light. After three-week growth, they were transplanted into bigger size pots (21 Í 32 cm, each line for 5 pots) in a greenhouse under normal conditions favorable for potatoes, for further research on hybridization experiment and agronomic traits. The agronomic traits including plant morphology, leaf shape, tuber yield, and tuber traits followed the methods described by Gomez KA and Gomez AA (1984). Plant morphology and leaf shape were measured during the bloom stage and tuber yield and related traits were recorded after harvest (a week later). The cross method and data analysis were described by Luthra et al. (2016), containing hybrid combinations, times of hybridization, number of berries and seeds, and so on.

Determination of freezing tolerance

Freezing tolerance was assessed by using electrolyte leakage. Four-week-old plantlets were transplanted into plastic pots (10 Í 10 cm) in a growth room at 22 ± 2°C, the humidity of 50 ± 10%, and a photoperiod of 16 h/day under light. After three-to-four-week growth, they were tested by the semi-lethal temperature (LT_{50}). For the cold acclimation test, moving the sample plants into a low-temperature climatic chamber (a photoperiod of 14 h/day under light, at 4 ± 2°C, the humidity of 50 ± 10%) for two weeks of cold acclimation, then the plants were under the same procedure as freezing tolerance test, which was described by Kou (2018). The freezing

tolerance tests of BC1 progenies were the same procedure as above, and only the electrolytic leakage at -3°C was evaluated, not the LT_{50}.

Results

Protoplast fusion and plant regeneration

Somatic hybridization between AC142 and MLM266-2 was successfully conducted via protoplast fusion. Among 980 calli, 248 differentiate calli were selected for plant regeneration, and one vigorous shoot of each callus was transferred to MS medium for root development. The brown and dead shoots were removed during subculture, and in total 80 vigorous shoots with strong roots were used for the analysis of their ploidy and genetic constitution (Fig. 1).

Identification and ploidy analysis of somatic hybrids

Two pairs of specific SSR primers (S215 and S165) of MLM266-2 and AC142 were used to identify somatic hybrids from the 80 regenerated plantlets. Amplified by primers S165, we observed the specific bands of 110 bp in AC142 and 150 bp in MLM266-2 (Fig 2A). For primers S215, a specific band (270 bp) was found in AC142 and a 250 bp band was found in MLM266-2, respectively (Fig. 2B). As a result, 51 regenerated plantlets were identified as somatic hybrids among 80 tested plantlets, which led to the successful protoplast fusion rate to 63.75%.

The ploidy of 51 somatic hybrids determined by flow cytometry were diverse, which included 23 octoploids, 20 hexaploids, 7 tetraploids, and a mixoploid. Unexpectedly, somatic hybrids of protoplasts fusion between diploid AC142 and MLM266-2 were mostly hexaploids and octoploids with much fewer tetraploids (Fig. 3A).

After subcultured for 4 years, the ploidy of 44 somatic hybrids out of 51 was analyzed again by flow cytometry, together with chromosome counting. Results showed that 23 had ploidy changes, accounting for 52.27% among 44 hybrids (Fig. 3B). Compared with the ploidy analysis in the years 2016, 16 out of 17 were still hexaploids, while only one hybrid changed to aneuploid, which was the most consistent type. Among 6 tetraploids, one changed to a hexaploid. A chimera of hexaploid and octoploid changed to be a hexaploidy. The most unstable ploidy was octoploidy, in which all the 20 hybrids had ploidy changes, turning out to be a tetraploid, 5 pentaploids, 9 hexaploids, a heptaploid, and 4 aneuploids. In a word, hexaploids and tetraploids were more stable at ploidy level in somatic hybrids (Fig 3, Supplement Table S1).

Analysis of freezing tolerance and agronomic traits in somatic hybrids

Electrolyte leakage was used to evaluate the freezing tolerance of NA and CA in 44 somatic hybrids. The results showed that the mean value of their NA reached -2.85°C and the coefficient of variation (CV) was 47.36% (Table 2). Among them, 88.64% of hybrids (39/44) hold intermediate NA between AC142 (-2.38°C) and MLM266-2 (-5.10°C), which suggested somatic hybrids had improvement of NA compared with the cultivated parent. On the other hand, most hybrids had more obvious enhancement in ACC than AC142 (0.74°C) and greater variation than NA (Table 2).

We analyzed the agronomic traits of fusion parents and 44 somatic hybrids, including plant morphology, flowering habit, and tuber yield, etc. The cultivated parent AC142 was semi-erect, abundant in flowers and had excellent tuber characters. And the wild species MLM266-2 was semi-erect and abundant in flowers, with high pollen viability and low yield (a large number of small tubers per plant). The somatic hybrids grew well on the whole, which exhibited 24 semi-erect plants, 16 decumbent plants, and 4 erect plants. Among the 44 hybrids, 75% of them showed heterobeltiosis in plant height and 30 hybrids (68.18%) produced normal flowers. All the somatic hybrids can produce tuber normally, tuber shape including oval, flat oblong, and oblate, etc., with a wide range of distribution and great differences in tuber weight per plant and tuber number per plant (Table 2). However, like the wild parent, most of them had many small tubers per plant.

Selection of excellent somatic hybrids and analysis of backcross efficiency with tetraploid cultivars

Nine somatic hybrids with strong freezing tolerance and relatively good agronomic traits were selected to backcross with 21 tetraploid cultivars to produce 46 backcross combinations. A total of 2175 seeds within 142 berries were obtained after 344 times of pollination (Supplement Table S2). The berry rates varied greatly for different somatic hybrids, ranging from 20% to 100%, and the average rate was 47.08% of the 9 somatic hybrids. In the backcross, the top three somatic hybrids of berry rate were the M+A74-1, M+A70-1, and M+A76-1. Meanwhile, M+A59-1, with the most backcross combinations, successfully backcrossed with 15 cultivars and obtained 755 seeds within 62 berries. However, a barrier of the post-zygotic development was observed in the backcross between somatic hybrids and cultivars, resulting in very few seeds per berry (Table 3).

Phenotype assessment of BC1 progenies

Three backcross combinations mentioned above were selected for further research and the progenies of M+A17-1 (6x) × Redsen (4x), M+A17-1 (6x) × Adirondack (4x) and M+A2-1 (6x) × 393160-4 (4x) were named FT069, FT070, and FT071 respectively. The ploidy level of the BC1 progenies ranged from 4x to 6x, and the majority were 5x (25/84) and 5x-6x (28/84), in which the proportion of mixoploids reached 50% by the female parent of M+A2-1 (Table 4).

The results of the electrolyte leakage test at -3°C showed that the freezing tolerance of three BC1 progenies largely varied (Table 4). Their progenies with NA (electrolyte leakage values) less than 50% accounted for 80.56%, 84.21%, and 70.37%, respectively; and with CA (electrolyte leakage values) less than 50% accounted for 83.78%, 90.91%, and 96.29%, respectively, which revealed the improved freezing tolerance in the materials compared with *S. tuberosum* (Table 4, Supplement Table S3).

The assessments of agronomic traits suggested that BC1 progenies had great variations in plant height, tuber number per plant, and tuber yield per plant (Fig. 4). The plant height varied from 30 cm to 110 cm, the number of tubers per plant ranged from zero to 135, and tuber yield per plant weighed from none to 534 g. The average tuber number per plant in FT070 was significantly less than that of the other two backcross combinations, while its tuber yield per plant was significantly higher (Fig. 4). The tuber traits of BC1 progenies were significantly improved compared with fusion parents, and some progenies had higher tuber yields, among which the tuber weight of FT069-16, FT069-32, FT070-1, FT070-22, and FT071-57 was close to that of the tetraploid cultivar (Supplement Table S3).

Correlation analysis between freezing tolerance and multiple traits, including ploidy, NA, CA, agronomic traits (tuber yield per plant and number of tubers per plant), were examined by 74 BC1 progenies of the three backcross combinations mentioned above. The results showed no significant correlation between ploidy with other traits and no significant correlation between NA or CA and agronomic traits (tuber yield per plant and number of tubers per plant). The results suggested that tuberization capacity and freezing tolerance were regulated by independent genetic loci with no interaction, which was more beneficial for the genetic improvement of freezing-resistant breeding materials (Table 5). To evaluate whether the aneuploids are useful for potato breeding, the differences in freezing tolerance and tuberization capacity between euploids and aneuploids were analyzed by two independent T-tests. There were no significant differences observed (Table 6), which proved that aneuploids did not affect freezing tolerance and yield-related traits.

Discussion

Hawkes (1990) divided *S. malmeanum* into *S. commersonii* subsp. *malmeanum* since *S. commersonii* subsp. *commersonii* and *S. commersonii* subsp. *malmeanum* had almost the same botanical characters except for some minor differences in lateral leaflet character, inflorescence branch, and corolla color. This division had been generally accepted for a long time so that previous researches on the freezing tolerance of potato were reported by *S. commersonii*, not distinguished from *S. malmeanum*. With the development of molecular biology technology and the clear origin and evolution of potato species, taxonomists and geneticists had further studied the subspecies of *S. commersonii*. Jacobs et al. (2008) analyzed about 5000 genotypes of 196 species, 15 subspecies, and 17 interspecific hybrids, by more than 200 AFLP markers, and constructed an NJ cladogram with 4929 genotypes. The results showed *S. commersonii* subsp. *malmeanum* was genetically distinguished from *S. commersonii* subsp. *commersonii* (Jacobs et al. 2011). Siri et al. (2009) used RAPD, AFLP, and SSR markers to analyze the genotype diversity of *S. commersonii* from 30 different sources. The results also showed that *S. malmeanum* and *S. commersonii* were clustered into two main clusters. Based on the above, Spooner et al. (2014) reclassified potato germplasm based on previous reports, which made *S. malmeanum* an independent wild species.

As the endosperm balance number (EBN) of *S. malmeanum* is 1, it was difficult to cross with cultivars directly, and few reports were about the utilization of *S. malmeanum*. In our research, the protoplasts of *S. malmeanum* and a dihaploid cultivar were fused to obtain somatic hybrids, which was the first successful transfer of freezing tolerance from *S. malmeanum* to cultivar. The study greatly enriched the freezing tolerance gene pool and provided resources for the genetic improvement of cultivars in freezing tolerance and further research of the genetic mechanism of *S. malmeanum* under freezing stress.

The ploidy level of potato interspecific somatic hybrids would change a lot in the process of continuous subculture. Guo et al. (2010) obtained somatic hybrids fused by protoplasts of a diploid wild species *S. chacoense* and a tetraploid *S. tuberosum*. Flow cytometry analysis showed 68 out of 108 interspecies hybrids changed in ploidy level after 4 years of continuous subculture (2003-2007). Compared with the analysis in 2003, 14 hybrids out of 54 hexaploids changed in ploidy level. The hexaploids showed the highest ploidy stability among all the somatic hybrids with different ploidy. An obvious transformation was observed that the chimeras and the aneuploids tended to become euploids. In this study, somatic hybrids fused by protoplasts of a dihaploid *S. tuberosum* and a diploid wild species *S. malmeanum* were obtained. Their ploidy levels were

largely varied, mainly in hexaploidy and octaploid, while tetraploid accounted for a relatively small proportion (7/51), which indicated the fusion was complex, random, and uncontrollable. In the results of two ploidy tests, it was found that somatic hybrids were still accompanied by a high frequency of chromosome loss and copy number variation during the subculture. The whole trend of somatic hybrids changed from an unstable high ploidy to a low ploidy, and finally, most of the progenies were stable to tetraploid and hexaploid. In our study, most of the 20 octaploids changed into stable tetraploid and hexaploid, which was consistent with previous reports.

Most somatic hybrids obtained in this study grew well and were fertile, which could be used for further genetic improvements. The selected 9 somatic hybrids with better traits were backcrossed with tetraploid cultivars, and the berry rate was more than 40% in 5 somatic hybrids. The electrolyte leakage rate of more than 80% BC1 progenies was less than 50% at -3°C both in NA and CA (Table 3, Supplement Table S3), which indicated that the freezing tolerance of wild species *S. malmeanum* had been successfully transferred to *S. tuberosum* by protoplast fusion, and the trait of somatic hybrids was inherited normally. The correlation analysis between freezing tolerance and tuberization capacity of BC1 progenies showed no significant difference, which was consistent with a previous report (Chen et al 1999). It indicated that freezing tolerance and tuberization capacity were controlled by independent genetic loci, so it was feasible to introduce freezing tolerance into cultivars without affecting yield. Besides, we identified excellent progenies with strong freezing tolerance and significantly improved comprehensive agronomic traits, such as FT069-16, FT069-32, FT070-1, FT070-22, FT071-57, and FT071-69, from the BC1 progenies. These progenies would be useful for subsequent genetic breeding and resource construction to improve the freezing tolerance ability of potato cultivars (Table 3).

Declarations

Authors' Contributions: B S and X C conceived and supervised the study. Y Z, Q Z, J Y, and J W performed the experiments. W T and J D wrote the manuscript; J D revised the manuscript. All authors read and approved the manuscript.

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Compliance with ethical standards

Conflicts of Interest: The authors declare no conflict of interest.

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Tables

Table 1 Materials

Materials	Ploidy	Species	Materials	Ploidy	Species
MLM266-2	2x	*S. malmeanum*	AC Red Island	4x	*S. tuberosum*
AC142	2x	*S. tuberosum*	Adirondack	4x	*S. tuberosum*
03H95-5	4x	*S. tuberosum*	Amsel	4x	*S. tuberosum*
08CA9512-05	4x	*S. tuberosum*	Congo	4x	*S. tuberosum*
08CA9635-19	4x	*S. tuberosum*	E3	4x	*S. tuberosum*
08HE042-2	4x	*S. tuberosum*	F01002	4x	*S. tuberosum*
09HE039-1	4x	*S. tuberosum*	F04023	4x	*S. tuberosum*
10FM33-8	4x	*S. tuberosum*	Huacai 1	4x	*S. tuberosum*
10CH36-1	4x	*S. tuberosum*	Huashu 1	4x	*S. tuberosum*
11EM47-40	4x	*S. tuberosum*	Huashu 10	4x	*S. tuberosum*
12FM29-1	4x	*S. tuberosum*	Huashu 11	4x	*S. tuberosum*
393160-4	4x	*S. tuberosum*			

Table 2 Identification of freezing tolerance and morphology of somatic hybrids and fusion parents

Characters	AC142	MLM266-2	Somatic hybrids
NA (°C)	-2.38 ± 0.01	-5.10 ± 0.15	-2.85 ± 1.35, CV=47.36%
ACC (°C)	0.74 ± 0.17	2.97 ± 0.15	1.35 ± 0.73, CV=54.07%
Growth habit	Semi-erect	Decumbent	Decumbent (15/44), Semi-erect (25/44), Erect (4/44)
Plant height (cm)	20.33 ± 1.15	25.33 ± 5.13	32.17 ± 4.68, CV=14.55%
Stem diameter (mm)	3.60 ± 0.46	3.06 ± 0.30	9.82 ± 1.08, CV=11.00%
Flowering degree	Moderate	Profuse	Profuse (13/44), Moderate (9/44), Low (8/44)
Tube shape	Elliptic	Oblate	Elliptic (34/44), Fusiform (3/44), Ovate (2/44), Abvate (1/44), Oblate (3/44), Clavate (1/44)
Number of tubers per hill	12.00 ± 4.24	69.00 ± 16.70	24.20 ± 14.68, CV=60.66%
Production of tubers per hill (g)	55.43 ± 17.29	7.67 ± 2.48	14.70 ± 5.73, CV=38.98%

NA: plants were grown at 22°C for 4 weeks before determination of LT_{50}.

CA: plants were grown at 22°C for 4 weeks, followed by 4°C for 14 days before determination of LT_{50}.

ACC: the values of NA-CA. CV represents coefficient of variation. Values are means ± SD.

Table 3 Analysis of backcross efficiency between somatic hybrids and tetraploid cultivars

Somatic hybrids	Ploidy	Flowering degree	No. of backcross combinations	Pollinating flowers	Berries	Berry rate (%)	Seeds	Seeds/ berry
M+A2-1	6x	profuse	1	12	3	25.00	55	18.33
M+A17-1	6x	profuse	9	41	19	46.34	236	12.42
M+A18-1	6x	moderate	10	118	42	35.59	659	15.69
M+A28-1	aneuploid	profuse	3	18	7	38.89	45	6.43
M+A45-1	6x	moderate	1	5	1	20.00	22	22.00
M+A59-1	6x	profuse	15	148	62	41.89	775	12.50
M+A70-1	6x	profuse	5	17	10	58.82	337	33.70
M+A74-1	6x	profuse	1	2	2	100.00	10	5.00
M+A76-1	6x	profuse	1	7	4	57.14	36	9.00

The NA values are means ± SD.

Table 4 Ploidy and freezing tolerance of BC1 progenies

Cross	Female	Male	Ploidy				NA (%)	CA (%)
			4x	5x	5x-6x	6x		
FT069	M+A17-1	Redsen	7	12	7	9	32.74 ± 21.69	29.37 ± 4.11
FT070	M+A17-1	Adirondack	3	7	8	5	35.62 ± 20.42	28.25 ± 12.50
FT071	M+A2-1	393160-4	1	6	13	6	40.90 ± 18.16	29.26 ± 9.31

NA: plants were grown at 22°C for 4 weeks the range values are electrolyte leakage rate (%) at -3°C. CA: plants were grown at 22°C for 4 weeks, followed by 4°C for 14 days, the range values are electrolyte leakage rate (%) at -3°C. Values are means ± SD.

Table 5 Correlation analysis of traits in BC1 progenies

	Ploidy	NA	CA	Number of tubers per hill	Production of tubers per hill
Ploidy	1				
NA	0.178	1			
CA	0.185	0.001	1		
Number of tubers per hill	0.037	-0.159	-0.078	1	
Production of tubers per hill	0.005	0.04	-0.087	0.522**	1

Asterisks indicate significant difference between each other by using

Student's t-test (**P < 0.01; *P < 0.05).

Table 6 Traits comparison of euploids and aneuploids in BC1 progenies

Traits	Euploid	Aneuploid	P value
NA	0.35 ± 0.19	0.40 ± 0.21	0.1475
CA	0.29 ± 0.14	0.30 ±0.13	0.8287
Number of tubers per hill	22.67 ± 19.17	35.04 ± 35.32	0.059
Production of tubers per hill (g)	167.82 ± 138.01	197.13 ± 108.41	0.3771

Significant difference between each other by using Student's t-test (**P < 0.01; *P < 0.05).

Values are means ± SD.

Figures

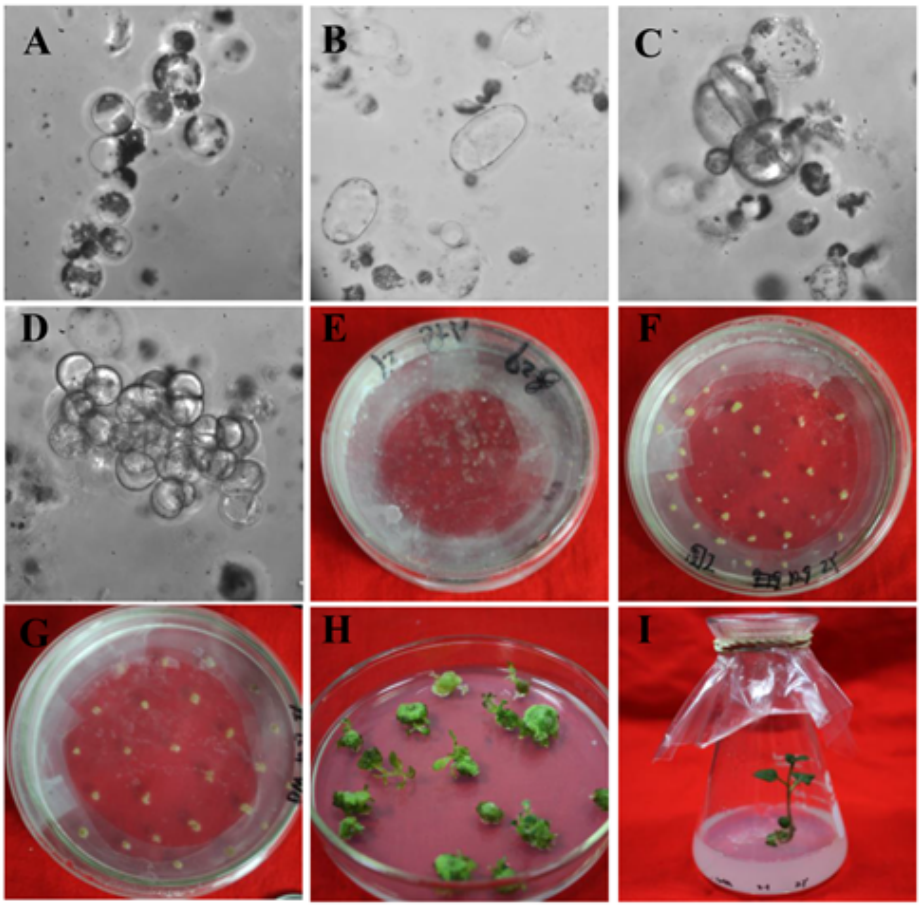

Figure 1

Procedure of plant regeneration from protoplast fusion. a Fused protoplast within 24 h, b oval fused cells formation with disintegration of chloroplast, c the first mitosis after protoplast fusion, d cell mass after several cell divisions, e double layer culture and formation of micro calli, f calli on propagation medium, g calli on differentiation medium, h shoot formation from calli, i regenerated plantlet

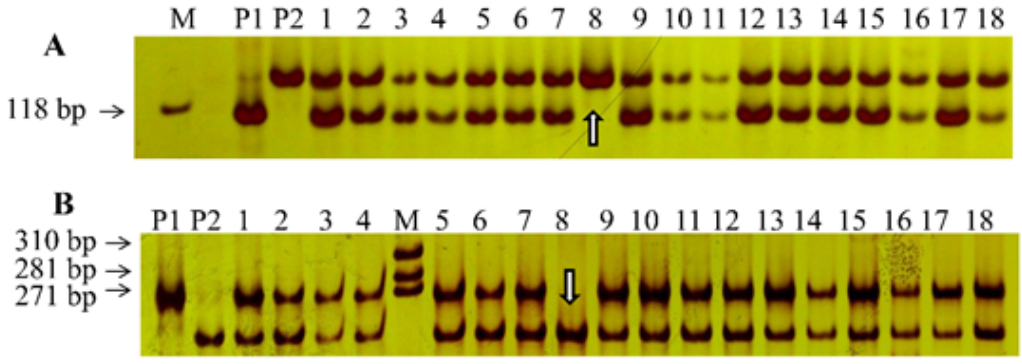

Figure 2

SSR analysis of the fusion parents and somatic hybrids. a Amplification of primers S165, b amplification of primers S215. P1 refers to the parent AC142, P2 refers to the parent MLM266-2, M marker uX174 DNA-HaeIII, 1-18 were regenerated plants. The white arrow indicates No. 8 clone was not somatic hybrid.

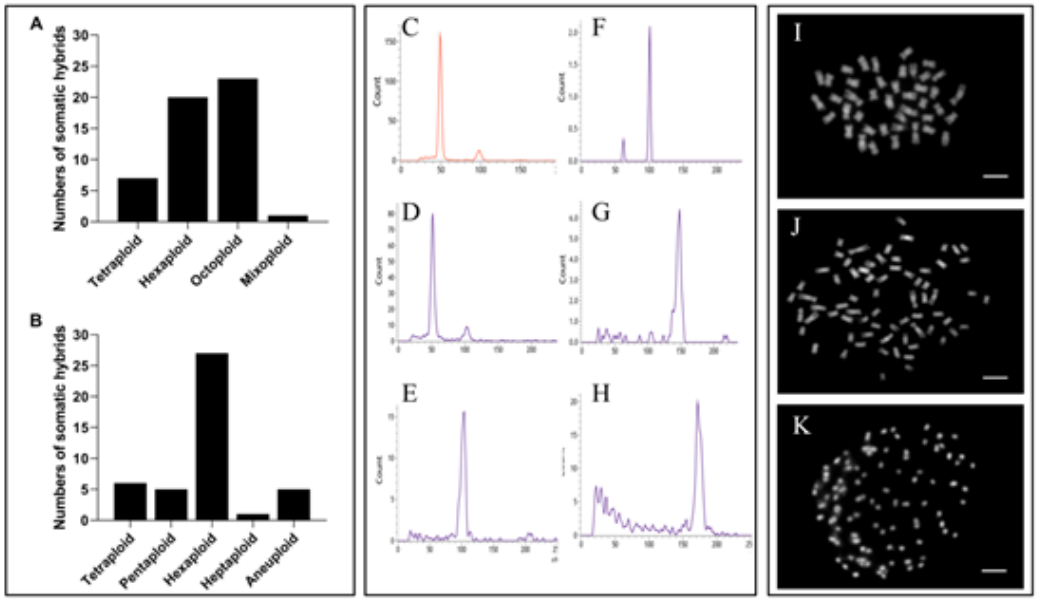

Figure 3

Ploidy analysis of fusion parents and somatic hybrids. a Ploidy analysis of 51 somatic hybrids in 2016, b ploidy analysis of 44 somatic hybrids in 2020, c-h ploidy analysis of partial somatic hybrids by flow cytometry, c AC142 (2x) control as diploid, d MLM266-2 (2x), e E3 (4x) control as tetraploid, f M+A59-1 (4x), g M+A27-1 (6x), h M+A75-1 (7x), i-k chromosome counting of partial progenies, i M+A59-1 (4x), j M+A27-1 (6x), k M+A75-1 (7x). Bars = 5 μm

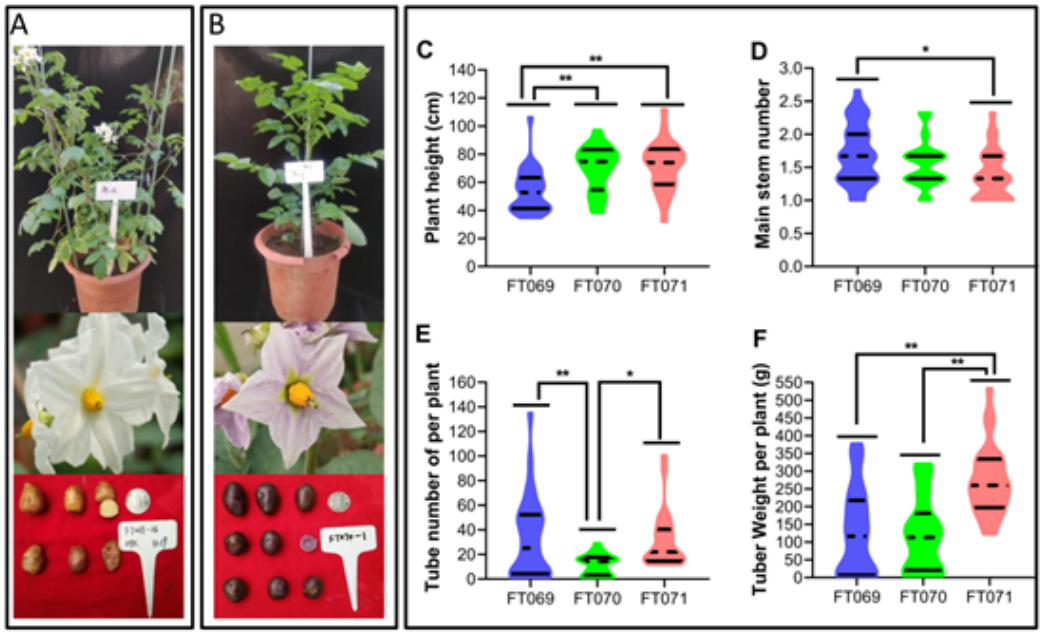

Figure 4

Assessments of agronomic traits in BC1 progenies. a FT069-16, b FT070-1, c plant height of BC1 progenies, d number of main stems in BC1 progenies, e number of tubers per plant in BC1 progenies, f tuber yield per plant in BC1 progenies. In the violin plot, the black solid line in the upper and lower is quartile line, and the black

dotted line is the median line. Asterisks indicate significant difference between each other by using Student's t-test (**P < 0.01; *P < 0.05).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- SupplementTableS1.xlsx
- SupplementTableS2.xlsx
- SupplementTableS3.xlsx