Interspecific potato somatic hybrids between *Solanum malmeaanum* and *S. tuberosum* provide valuable resources for freezing-tolerance breeding

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Received: 7 March 2021 / Accepted: 19 May 2021 / Published online: 25 June 2021 © The Author(s), under exclusive licence to Springer Nature B.V. 2021

**Abstract**
Freezing stress affects the geographic distribution, growth, and development of potato, resulting in yield loss. *Solanum malmeaanum*, a diploid wild species with strong freezing tolerance, was fused with the freezing sensitive dihaploid *S. tuberosum* by somatic hybridization. In our study, 980 calli were obtained, and 248 differentiated shoots were obtained from the calli. Parental-specific SSR markers were used to analyse the chromosome composition of the 80 randomly selected regenerated plants, obtaining 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. The somatic hybrids showed better freezing tolerance than the cultivated parent. Then, freezing-tolerant somatic hybrids were selected to backcross with cultivars, and we obtained valuable breeding resources with enhanced freezing tolerance and tuberization capacity similar to that of cultivars. The correlation analysis showed that freezing tolerance has no relation with tuberization capacity, which indicates that they are controlled by independent genetic loci.

**Key message**
Freezing tolerance was transferred to cultivated potato from *S. malmeaanum* by protoplast fusion for the first time, and valuable resources for freezing tolerance breeding were obtained.

**Keywords** Potato · *Solanum malmeaanum* · Protoplast fusion · Freezing tolerance · Agronomic traits

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**Communicated by Ewa Grzebelus.**

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**Introduction**

Potato (*Solanum tuberosum* L.), the fourth-largest food crop and the most important tuber crop in the world, is cultivated extensively for its low fat content and high nutritional value and is consumed by more than one billion people (Bradeen and Kole 2016; Haan and Rodriguez 2016). Freezing stress, a typical abiotic stress, has strong adverse effects on the growth, development, production, and distribution of potato (Chinnusamy et al. 2007; Chang et al. 2014). Given this situation, it is important to construct potato germplams with freezing tolerance by genetic modification. Nearly all cultivated potatoes are sensitive to frost with little genetic variation in terms of both freezing tolerance and acclimation capacity, and the plants will die when the temperature decreases to −3 °C (Seppänen et al. 2001). In contrast, wild potato species exhibit higher freezing tolerance, including...
non-acclimated freezing tolerance and cold acclimation freezing tolerance (Chen and Li 1980; Palta and Simon 1993; Seppänen et al. 1998). In previous studies, more than 30 wild species, such as *S. acaule*, *S. boliviense*, *S. chomato-philum*, *S. commersonii*, and *S. demissum*, were reported to exhibit strong freezing tolerance (Chen and Li 1980; Vega and Bamberg 1995; Luthra et al. 2007). Our previous freezing tests conducted 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 are all significant germplasms for freezing-tolerant potato breeding.

The potato (*Solanum* spp.) germplasm resources include up to 107 wild species, which are potentially rich sources of biotic and abiotic stress resistance, while cultivated potatoes lack these features (Hawkes 1990; Spooner et al. 2014). Cross incompatibility, the pre-zygotic hybridization barrier that acts at the pollen-pistil level, acts as a strong hybridization barrier during introgression of prominent genes of wild species into cultivated potato. It prevents pollen tubes from reaching the eggs, leading to the failure of hybrid zygote formation in crosses between two fertile species (de Nettancourt 2001), which can appear at various sites of the pistil or in the ovary (Hayes et al. 2005). Cross incompatibility, controlled by genes independent of the S-locus or its S-haplotype recognition region in potatoes by crossing with different accessions (Maune et al. 2018), can occur either as unilateral incompatibility or bilateral incompatibility. In contrast, endosperm abortion is considered to be the most important post-zygotic barriers (Camadro et al. 2004). Even though hybrids are formed with post-zygotic barriers, these barriers can cause hybrid weakness, sterility, or a breakdown in segregating generations (Camadro et al. 2004; Bryan et al. 2017). Protoplast fusion provides a new approach for utilizing wild potato resources, especially for abiotic stress resistance. A series of efforts for genetic improvement arose after Shepard and Totten (1977) first employed somatic hybridization in potato research (Tiwari et al. 2010; Sharma et al. 2011; Sarkar et al. 2011; Chandel et al. 2015; 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 into cultivated potato (Preisznzer et al. 1991; Cardi et al. 1993; Xu et al. 1993; Nyman and Waara 1997), which made the somatic hybrids exhibit better resistance in cold stress. However, many protoplast fusion studies have mainly concentrated on the transfer of biotic or abiotic resistance from wild species into cultivated potato, and little follow-up has been performed on the agronomic traits, tuberization, genetic improvements, and genetic stability of the obtained somatic hybrids.

Hawkes (1990) classified *S. malmeanum* as *S. commersonii* subsp. *malmeanum*, since *S. commersonii* subsp. *commersonii* and *S. commersonii* subsp. *malmeanum* had almost the same botanical characteristics, with the exception of some minor differences in lateral leaflet character, inflorescence branch, and corolla color. With the development of molecular biological technology and the clear origin and evolution of potato species, taxonomists and geneticists have further studied the subspecies of *S. commersonii*. Based on the analysis of numerous genotypes by using randomly amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), and simple sequence repeat (SSR) markers (Jacobs et al. 2008; Siri et al. 2009; Jacobs et al. 2011), Spooner et al. (2014) reclassified potato germplasms based on previous reports, leading to *S. malmeanum* being classified as an independent wild species. However, although the 1 endosperm balance number (EBN) wild species *S. malmeanum* shows desirable agronomic and disease resistance, studies on the freezing tolerance of this species are rare.

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

### Materials and methods

#### Plant materials and growth conditions

MLM266-2 kindly provided by Dr. Vleeshouwers Vivianne GAA from Wageningen University, and the other materials were preserved in our laboratory. The dihaploid *S. tuberosum* line (AC142) is freezing susceptible, and the diploid wild species *S. malmeanum* (MLM266-2) exhibits strong freezing tolerance. Another 21 tetraploid cultivars were used to genetically improve the somatic hybrids in this study (Supplement Table S1), including Redsen, Adirondock, 393160-4, Huashu 1, etc. The fusion parents and regenerated plants were always 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−1 under a light intensity of 60 µmol m−2 s−1. The regenerated plants were numerically named according to 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 the parents MLM266-2 and AC142.
Protoplast isolation to plant regeneration

The protoplasts were isolated and fused as described by Yu et al. (2013), and leaves from three-week-old plantlets were pretreated in floatation medium (FM) solution at 25 °C under 48 h of darkness. The conditioned medium (CM) solution was incubated at 4 °C in darkness for 24 h. The leaves were cut into pieces, incubated overnight in an enzyme solution, and subsequently filtered through a nylon sieve (100 μm mesh). Then, protoplasts were derived after being purified by centrifugation in a solution containing 6.35% (w/v) mannitol + 0.2 mM CaCl₂. All the solutions mentioned above were prepared as described by Yu et al. (2013). The protoplasts of both parents were mixed at a ratio of 1:1 by volume and then adjusted to a cell density of 2 × 10⁵ ml⁻¹ by using electrical fusion solution. Then, the protoplasts were treated under an AC-field at 100 V cm⁻¹ for 20 s and subsequently treated under 1100 V cm⁻¹ DC voltage for 60 μs to achieve protoplast fusion (Eppendorf Multiporator 4308, Germany). Calli were formed in the induced medium after protoplast fusion, and regenerated shoots were derived in differentiation medium. The regenerated shoots were excised and cultured on MS medium for root development.

Determination of the ploidy of the somatic hybrids

The ploidy level of somatic hybrids and BC1 progenies was determined by flow cytometry (BD FACSVerse, BD Biosciences, San Jose, CA, USA) and a Cystain® UV Precise T Kit. Approximately 0.5 cm² leaves from four-week-old plantlets were chopped in a plastic Petri dish containing 60 μl of nucleus extraction solution for 30–60 s, followed by staining in 500 μl of Staining Buffer for 30 s. The leaf extracts were then filtered through a 50 μm CellTrics filter into a tube, and the DNA content of the nuclei was tested by a ploidy analyzer (BD FACSVerse). Taking diploid AC142 and tetraploid E3 as controls, the distribution graph was analysed using flow cytometry.

Root tips were used for chromosome counting, and the root length of the in vitro plantlets was 1–2 cm. The materials were treated in 0.2 mmol L⁻¹ 18-hydroxyquemoline 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 containing 2% (w/v) pectinase and 2% (w/v) cellulase (Sigma, USA) for 1.5 h at 37 °C, dissolved in citrate buffer solution containing 0.1 M citrate buffer and 0.1 M citrate sodium at a ratio of 123:77 (v/v)]. The root tips were rinsed in water for 10 min twice and subsequently transferred to a clean slide. Then, 1–2 drops of Carnoy’s Fluid were added, and the root tips were crushed to spread the chromosomes. Then, the slides were stained with 2 μg ml⁻¹ DAPI in darkness, and fluorescent images of the chromosomes were captured by fluorescence microscope.

SSR analysis of the somatic hybrids

Total genomic DNA was extracted from the leaves of the in vitro plantlets by following the CTAB procedure (Del-laporta et al. 1983). The somatic hybrids were confirmed by SSR analysis according to the presence of parent-specific diagnostic bands of the fusion parents. We obtained 8 parent-specific SSR markers (Supplement Table S2), which were screened from 98 SSR primer pairs from our previous reports (Naz et al. 2018). Amplification was carried out with a C1000 Thermal Cycler (Bio-Rad Inc, Hercules, CA, USA) in a 20 μl of reaction mixture containing 14.4 μl of dd H₂O, 2.6 μl of Taq mix, 2 mM MgCl₂, 1 μl of each primer pair (10 μM) and 50 ng of genomic DNA. The thermal cycling profile was as follows: 4 min of pre-denaturation at 95 °C, followed by 35 cycles of 95 °C for 40 s, annealing at 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 analysed by 9% polyacrylamide gel electrophoresis (PAGE) and silver staining. Images were captured by a digital camera.

Determination of plant morphology

The fusion parents, somatic hybrids, and BC1 progenies were cultured four weeks on MS medium supplemented with 4% sucrose and 8% agar at 22 ± 2 °C with a photoperiod of 16 h day⁻¹ under a light intensity of 60 μmol m⁻² s⁻¹. Subsequently, all the plantlets were transplanted into plastic pots (10 cm diameter) in a growth room at 22 ± 2 °C with a photoperiod of 16 h day⁻¹ under light. After 3 weeks of growth, they were transplanted into larger pots (32 cm diameter, each line for five pots) in a greenhouse under the normal favourable conditions to potatoes for further research on hybridization experiment and agronomic traits. The agronomic traits, including plant morphology, leaf shape, tuber yield, and tuber traits, were performed according to the methods described by Gomez and Gomez (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 mean values of morphology, including plant height, number of main stems, number of tubers per plant, and tuber yield per plant from three backcross combinations were compared using Student’s t test. The cross method and data analysis were performed as Luthra et al. (2016), including the hybrid combinations, times of hybridization, and number of berries and seeds.
Determination of freezing tolerance

Freezing tolerance was assessed by measuring electrolyte leakage. Four-week-old plantlets were transplanted into plastic pots (10 cm diameter) in a growth room at 22 ± 2°C with a humidity of 50 ± 10% and a photoperiod of 16 h day⁻¹ under light. After three to 4 weeks of growth, the plantlets were tested to determine the semi-lethal temperature (LT₅₀). For the cold acclimation test, the sample plants were moved into a low-temperature climatic chamber (with a photoperiod of 14 h day⁻¹ under light, at 4 ± 2°C, with a humidity of 50 ± 10%) for 2 weeks of cold acclimation. Then, the plants were subjected to the same procedure as that used in the freezing tolerance test, which was described by Kou et al. (2018). The freezing tolerance tests of BC1 progenies were performed as described above, and only the electrolytic leakage at −3°C was evaluated, not the LT₅₀. The freezing tolerance of each somatic hybrid and BC1 progeny was tested biologically three times.

Statistical analysis

The homogeneity and normality assumptions were tested for the independent variables before univariate ANOVA. Ploidy verification was tested by the McNemar Chi-square test to assess the null hypothesis (H₀) that there was no difference among chromosome counts. We used Bivariate Correlate Analysis in the SPSS 20.0 program to evaluate the relationship between the freezing tolerance and tuber traits of the BC1 progenies and used the Spearman correlation coefficient to estimate the significance of the correlation.

Results

Protoplast fusion and plant regeneration

Somatic hybridization between AC142 and MLM266-2 was successfully conducted via protoplast fusion. Among 980 calli, 248 differentiated 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 analysis of their ploidy and genetic constitution (Fig. 1).

Identification and ploidy analysis of somatic hybrids

Somatic hybrids were tested for hybridity using 8 selected SSR markers that could demonstrate parent-specific polymorphic bands in the hybrids. Regenerates showing one or more parent-specific bands, which were tested by any

Fig. 1 Procedure of plant regeneration by protoplast fusion. a Fused protoplast within 24 h, b the first mitosis after protoplast fusion, c cell mass after several cell divisions, d calli on propagation medium, e shoot formation from calli, f regenerated plantlet
one or any combination of the SSR markers, were considered somatic hybrids. For example, upon amplified with the primer S165, we observed specific bands of 110 bp in AC142 and 150 bp in MLM266-2 (Fig. 2a). With the primer S215, a specific band (270 bp) was found in AC142, and a 250 bp band was found in MLM266-2 (Fig. 2b). As a result, 51 regenerated plantlets were identified as somatic hybrids among the 80 tested plantlets, indicating a successful protoplast fusion rate of 63.75% (51/80).

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

After subculturing for 4 years under the same conditions as those used for fusion parents, the ploidy of 44 somatic hybrids out of the 51 was analysed again by flow cytometry, together with chromosome counting. The results showed that 23 had ploidy changes, accounting for 52.27% of 44 hybrids, while the fusion parents (MLM266-2 and AC142) invariably remained diploid (Fig. 3b, Supplement Table S3). Compared with the ploidy analysis conducted in 2016, 16 out of 17 remained hexaploid, while only one hybrid changed to

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

Fig. 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) as a diploid control, d MLM266-2 (2x), e E3 (4x) as a tetraploid control, 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
aneuploid, which was the most consistent type. Among the six tetraploids, one switched to hexaploid. A chimera of hexaploid and octoploid switched to hexaploid. The most unstable ploidy was octoploid, in which all 20 hybrids showed ploidy changes, turning into one tetraploid, 5 pentaploids, 9 hexaploids, one heptaploid, and 4 aneuploids. In summary, hexaploids and tetraploids were more stable at the ploidy level in somatic hybrids (Fig 3, Supplement Table S3).

Analysis of freezing tolerance and agronomic traits in somatic hybrids

Electrolyte leakage was used to evaluate the freezing tolerance of non-acclimated freezing tolerance and cold acclimation freezing tolerance in 44 somatic hybrids. The results showed that the mean value for non-acclimated freezing tolerance was -2.85 °C, and the coefficient of variation (CV) was 47.36% (Table 1). Among the somatic hybrids, 88.64% (39/44) had intermediate non-acclimated freezing tolerance between that of AC142 (−2.38 °C) and that of MLM266-2 (−5.10 °C), which suggested that somatic hybrids showed improvement in non-acclimated freezing tolerance compared with the cultivated parent. On the other hand, most hybrids showed more obvious enhancement in acclimation capacity than AC142 (0.74 °C) and greater variation than that observed for non-acclimated freezing tolerance (Table 1).

We analysed 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 and abundant in flowers and had excellent tuber characters. The wild species MLM266-2 was decumbent and abundant in flowers, with high pollen viability and low yield (a large number of small tubers per plant). Most of the somatic hybrids grew well, exhibiting 24 semi-erect plants, 16 decumbent plants, and 4 erect plants. Among the 44 hybrids, 75% showed heterobeltiosis in plant height and 30 hybrids (68.18%) produced normal flowers. All the somatic hybrids showed normal tuber production and tuber shapes such as oval, flat oblong, and oblate, etc., with a wide range of distributions and great differences in tuber weight per plant and tuber number per plant (Table 1). However, similar to the wild parent, most of the hybrids had many small tubers per plant. To analyse the ploidy variation in relation to the morphology variation of the somatic hybrids, correlation analysis between the ploidy level and multiple morphology traits was evaluated. The results showed that the ploidy level was not related to freezing tolerance, however, there was a significant correlation between ploidy level and tuber yield per plant (Supplement Table S4).

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 for backcrossing with 21 tetraploid cultivars to produce 46 backcross combinations. A total of 2175 seeds within 142 berries were obtained after 344 pollinations (Supplement Table S5). The berry rates varied greatly for different somatic hybrids, ranging from 20% to 100%, and the average berry rate was 47.08% of the nine somatic hybrids. In the backcross, the top three somatic hybrids in terms of berry rate were M+A74-1, M+A70-1, and M+A76-1. Moreover, M+A59-1, with the highest number of backcross combinations, was successfully backcrossed with 15 cultivars, and 755 seeds within 62 berries were obtained (Table 2). However, a barrier to postzygotic development was observed in the backcross between somatic hybrids and cultivars, resulting in very few seeds per berry (Table 2).

Table 1 Identification of freezing tolerance and morphology of somatic hybrids and fusion parents

| Characters                              | AC142       | MLM266-2   | Somatic hybrids |
|----------------------------------------|-------------|------------|-----------------|
| Non-acclimated freezing tolerance (°C) | −2.38 ± 0.01| −5.10 ± 0.15| −2.85 ± 1.35, CV=47.36% |
| Acclimation capacity (°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), abivate (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% |

Non-acclimated freezing tolerance: plants were grown at 22 °C for 4 weeks before determination of LT50. Cold acclimation freezing tolerance: plants were grown at 22 °C for 4 weeks, followed by 4 °C for 14 days before determination of LT50. Acclimation capacity: the values of non-acclimated freezing tolerance plus minus cold acclimation freezing tolerance. CV represents coefficient of variation. Values are means ± SD.
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 3).

The results of the electrolyte leakage test at −3°C showed that the freezing tolerance of the three BC1 progenies varied greatly (Table 3). Their progenies with non-acclimated freezing tolerance (electrolyte leakage values) less than 50% accounted for 80.56%, 84.21%, and 70.37% of the total, respectively, and those with cold acclimation freezing tolerance (electrolyte leakage values) less than 50% accounted for 83.78%, 90.91%, and 96.29% of the total, respectively, which revealed the improved freezing tolerance of these materials compared with that of S. tuberosum (Table 3, Supplement Table S6).

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 to 110 cm, the number of tubers per plant ranged from 0 to 135, and the tuber yield per plant ranged from 0 to 534 g. The average tuber number per plant of FT070 was significantly lower 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 those of the fusion parents, and some progenies had higher tuber yields, among which the tuber weights of FT069-16, FT069-32, FT070-1, FT070-22, and FT071-57 were close to that of the tetraploid cultivar (Supplement Table S6).

Correlation analysis between freezing tolerance (non-acclimated freezing tolerance, cold acclimation freezing tolerance) and multiple traits, including ploidy and agronomic traits (tuber yield per plant and number of tubers per plant), was performed by using 74 BC1 progenies of the three backcross combinations mentioned above. The results showed no significant correlation between ploidy and other traits and no significant correlation between non-acclimated freezing tolerance or cold acclimation freezing tolerance and agronomic traits (tuber yield per plant and number of tubers per plant) (Table 4). 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. To evaluate whether aneuploids are useful for potato breeding, the differences in freezing tolerance and tuberization capacity between euploids and aneuploids were

Table 2 Analysis of backcross efficiency between somatic hybrids and tetraploid cultivars

| Somatic hybrids | Ploidy | Flowering degree | No. of backcross combinations | Pollinating flowers | No. of 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        |

Table 3 Ploidy and freezing tolerance of BC1 progenies

| Cross   | Female  | Male      | Ploidy | Non-acclimated freezing tolerance (%) | Cold acclimation freezing tolerance (%) |
|---------|---------|-----------|--------|--------------------------------------|----------------------------------------|
|         |         |           | 4x     | 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  |

Non-acclimated freezing tolerance: plants were grown at 22 °C for 4 weeks, the range values are electrolyte leakage rate (%) at −3 °C. Cold acclimation freezing tolerance: 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
analysed by two independent T-tests. There were no significant differences observed (Supplement Table S7), which indicated that aneuploids did not affect freezing tolerance or yield-related traits.

**Discussion**

It is well known that cultivated potato (*Solanum tuberosum* L.) is frost sensitive, while some wild species exhibit freezing tolerance far superior to that of cultivated species (Chen and Li 1980; Vega and Bamberg 1995). *S. malmeanaum* exhibited strong freezing tolerance in our previous research; however, as the endosperm balance number (EBN) of *S. malmeanaum* is one, it was difficult to cross it with cultivars directly, and there have been few reports on the utilization of *S. malmeanaum*. In our research, the protoplasts of *S. malmeanaum* and a dihaploid cultivar were fused to obtain somatic hybrids, representing the first successful transfer of freezing tolerance from *S. malmeanaum* to cultivar. The study greatly enriched the freezing tolerance gene pool and provided resources for the genetic improvement of cultivars in terms of freezing tolerance and for further research of the genetic mechanism of *S. malmeanaum* under freezing stress.

Although somatic hybridization could overcome sexual barriers and transfer desirable genes from wild *Solanum*
species into *S. tuberosum* cultivars, the genetic stability of somatic hybrids remains unsatisfactory. Tiwari et al. (2021) identified a large number of genes and genomic variants (SNPs, InDels and Copy Number Variations) in somatic hybrids via genome sequence. The ploidy level of potato interspecific somatic hybrids changes considerably during the process of regeneration and continuous subculture. In this study, the ploidy levels of the somatic hybrids varied greatly, mainly exhibiting hexaploid and octaploid, while tetraploid accounted for a relatively small proportion (7/51), which indicated that the fusion was complex, random, and uncontrollable. The results of the two ploidy tests (2016, 2020) showed that the somatic hybrids were still accompanied by a high frequency of chromosome elimination and copy number variation during subculture. The whole trend of somatic hybrids changed from an unstable high ploidy to a low ploidy, and finally, most of the progenies showed stable to tetraploid and hexaploid. Similarly, flow cytometry analysis of the somatic hybrids by protoplast fusion between *S. tuberosum* (4x) and *S. chacoense* (2x) showed that the ploidy level of 68 out of the 108 somatic hybrids changed after 4 years of continuous subculture (2003–2007) (Guo et al. 2010). Compared with the analysis conducted in 2003, 14 hybrids out of the 54 hexaploids showed a change in ploidy level. The hexaploids showed the highest ploidy stability among all the somatic hybrids with different ploidy, and the chimeras and aneuploids tended to become euploids. Similar results were reported in somatic hybrids obtained by fusion of the protoplasts of *S. pinnatisectum* and diploid breeding lines of *S. tuberosum* (Menke et al. 1996). In our study, most of the 20 octaploids changed into stable tetraploid and hexaploid, which was consistent with previous reports (Guo et al. 2010; Menke et al. 1996). The phenomenon of ploidy level instability in mixoploids and hybrids with higher ploidy might be attributed to the loss of fragments following genomic rearrangement, and the genotype affected the integrity of the genome (Aversano et al. 2009). On the other hand, the chromosome loss in the somatic hybrids probably resulted from different cell cycles of the remotely fused parents, mutations during fused cell growth and regeneration after protoplast fusion and interactions between cytoplasmic genomes and nuclear genomes (Wang et al. 2007). In addition, DNA methylation may specifically suppress the expression of genes involved in centromere function or the regulation or maintenance of embryogenesis (Wang et al. 2007; Abid et al. 2011), which may also result in chromosome elimination.

Somatic hybrids were analysed with respect to ploidy level variation and morphological variation. The ploidy level of somatic hybrids was analysed with non-acclimated freezing tolerance and cold acclimation capacity, and the results showed that the ploidy level was not related to freezing tolerance (Supplement Table S4). However, there was a significant correlation between ploidy level and tuber yield per plant (Supplement Table S4), which may indicate that there was an interaction between growth habits and genome dosage. Variations in the morphology and ploidy level of the somatic hybrids derived from any of the fusion parent proved to be very common in *Solanum* (Szczerbakowa et al. 2011; Polzerová et al. 2011). Our result corresponds to the varied freezing tolerance and morphology of somatic hybrids, which might be related to genome dosage effects, chromosome instability and preferential elimination of certain chromosomes in wild species (Thieme et al. 1997) and to somaclonal variations (Sree Ramulu 1986).

Most of the somatic hybrids obtained in this study grew well and were fertile, indicating that they could be used for further genetic improvement. The nine selected somatic hybrids with better traits were backcrossed with tetraploid cultivars, and the berry rate was more than 40% in 5 of the somatic hybrids. The electrolyte leakage rate of more than 80% of the BC1 progenies was less than 50% at −3 °C in terms of non-acclimated freezing tolerance and cold acclimation capacity (Table 3, Supplement Table S6), which indicated that the freezing tolerance of the wild species *S. malmeaunum* had been successfully transferred to *S. tuberosum* by protoplast fusion and that the freezing tolerance of the somatic hybrids was inherited normally. Analysis of the correlation between the freezing tolerance and tuberization capacity of the BC1 progenies showed no significant difference, which was consistent with a previous report (Chen et al. 1999). This result 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 will be useful for subsequent genetic breeding and resource construction to improve the freezing tolerance of potato cultivars (Table 3).

In the present study, we succeeded in introgression of freezing tolerance from the 1 EBN *Solanum* wild species *S. malmeaunum* into *S. tuberosum*. We also obtained valuable breeding resources with elevated freezing tolerance as well as a tuberization capacity close to that of cultivars by using somatic hybrids backcrossed with cultivars. These findings will be important for improvement of the freezing tolerance of potato and will provide informative insights into the genetic control mechanism underlying this important phenomenon.

**Supplementary Information** The online version contains supplementary material available at https://doi.org/10.1007/s11240-021-02106-2.
Acknowledgements We greatly appreciate the help of Vice Prof. Yuhong Yao and Ting Liu in revising the manuscript. This research was funded by the Earmarked Fund for Modern Agro-Industry Technology Research System of China (Grant No CARS-09-P07) and the National Natural Science Foundation of China (grant No 31871685).

Author contributions BS and XC conceived and supervised the study. YZ, QZ, JY, and JW performed the experiments. WT and JD wrote the manuscript; JD revised the manuscript. All authors read and approved the manuscript.

Declarations

Conflict of interest The authors declare no conflict of interest.

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