Identification of the tetrad-sterility-causing *Solanum stoloniferum* cytoplasm in interspecific hybrids with *S. tuberosum*

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Abstract

Tetrad sterility, in which only clumps of four premature pollen grains are released from anthers, has been observed in some modern potato cultivars. It is a form of cytoplasmic male sterility caused by the cytoplasm derived from the Mexican tetraploid species *Solanum stoloniferum* Schlecht. et Bché., an important source of resistance to *Potato virus Y* in potato breeding. However, since *S. stoloniferum* is highly polymorphic, the source of tetrad-sterility-causing cytoplasm is unknown among diverse *S. stoloniferum* accessions. In this study, we directly crossed 24 *S. stoloniferum* accessions with pollen from 4x *S. tuberosum* and obtained 39 hybrids from 12 accessions. Nineteen hybrids from six accessions showed tetrad sterility, with either D/γ- or W/γ-type cytoplasm, and were triploid, tetraploid, or hexaploid. The W/γ-type cytoplasm was not necessarily associated with tetrad sterility. Sequence comparisons of 17 mitochondrial genes and their intergenic regions revealed a length polymorphism in the intergenic region between *rp15* and *rps10*, in which an amplified band of 859 bp was associated with tetrad sterility. This specific cytoplasm causing tetrad sterility is named TSC_{sto}. The 859-bp band would be a useful diagnostic marker for identifying TSC_{sto} in potato breeding.

Introduction

Cytoplasmic male sterility (CMS), resulting in the failure to produce normal pollen, is found in many higher plants, and it is thought to be caused by disruption of nuclear-cytoplasmic interactions (Hanson and Bentolia 2004). The mitochondrial genome is most likely involved as a cytoplasmic factor in this disruption, and through maternal inheritance, all progeny with the cytoplasm become male sterile. Several different types of male-sterility-causing cytoplasm have been identified in various crops and characterized at the molecular level (Schnable and Wise 1998; Budar et al. 2003; Hanson and Bentolia 2004).

As the common potato (*Solanum tuberosum* L., $2n=4x=48$) is propagated vegetatively and the edible parts are the tubers, the ability to produce pollen is not necessary for ensuring quality and productivity in commercial varieties. Indeed, many varieties cannot function as pollen parents because multiple nuclear genes interact with their cytoplasmic genome causing several different types of male sterility (Grun et al. 1977). However, since fertility is one of the most important traits for cross-breeding, male sterility is an unavoidable problem in potato breeding. Furthermore, in modern potato breeding, wild species have frequently been used to introduce useful traits such as resistance to pests and diseases into cultivars (Ross 1986; Plaisted and Hoopes 1989; Vos et al. 2015). With resistance breeding, however, the frequency of male-sterility-causing cytoplasm in the cultivated potato gene pool has increased, which has caused serious problems because available pollen parents are being gradually exhausted (Mihovilovich et al. 2015; Sanetomo and Gebhardt 2015).

In some modern potato varieties, pollen development stops at the tetrad stage, and pollen is only released as clumps of four premature pollen grains (Brown 1984; Ortiz et al. 1993; Lössl et al. 2000). This is called “tetrad sterility” (T-CMS). T-CMS was first observed in interspecific hybrids between the Mexican wild species *Solanum verrucosum* Schlecht. ($2n=2x=24$) and other diploid species such as *Solanum chacoense* Bitt. ($2n=2x=24$) (Buck 1960; Grun et al. 1962; Abdalla and Hermesen 1971, 1972). T-CMS is also known in all varieties possessing the extreme resistance gene to *Potato virus Y* (*Ry_{sto}*) (Song and Schwarzfischer 2008). The resistance gene was maternally derived from the Mexican wild tetraploid species *Solanum stoloniferum* Schlecht. et Bché. ($2n=4x=48$) (Cockerham 1943).

The cytoplasm of over 700 cultivars and breeding clones were discriminated into six types, namely, A, D, P, M, T, and W, using polymerase chain reaction (PCR) markers (Hosaka and Sanetomo 2012). In addition, mitochondrial DNA types can be determined as the α, β or γ type using the PCR primer pair ALM_4/ALM_5 (Lössl et al. 2000). Based on these classification systems, all of the varieties showing T-CMS have W/γ-type cytoplasm maternally descended from *S.*

stoloniferum (Lössl et al. 2000; Song and Schwarzfischer 2008; Hosaka and Sanetomo 2012; Sanetomo and Gebhardt 2015). However, the W/γ-type cytoplasm was not only found in *S. stoloniferum* but also in many wild potato species which do not occur male sterility in interspecific hybrids with *S. tuberosum* (Hosaka and Sanetomo 2012).

S. stoloniferum is one of the most common, widespread, and morphologically polymorphic species of wild potatoes in North and Central America (Spooner et al. 2004). It is an allotetraploid species with a genome formula of AABB (Matsubayashi 1955; Irikura 1976; Pendinen et al. 2008), while *S. tuberosum* is an autotetraploid or a segmental allotetraploid (AAA[†]A[†], Matsubayashi 1991). In addition, the endosperm balance number (EBN), which regulates interspecific cross compatibility (Johnston et al. 1980), is different between tetraploid cultivars (4EBN) and *S. stoloniferum* (2EBN), and it would theoretically be difficult to obtain interspecific hybrids through direct crosses. Interestingly, however, all cultivated varieties showing T-CMS were derived by direct crosses between *S. stoloniferum* as female parents and *S. tuberosum* as male parents (Song and Schwarzfischer 2008).

In this study, to identify the specific *S. stoloniferum* cytoplasm causing tetrad sterility, we crossed various accessions of *S. stoloniferum* with pollen from 4x*S. tuberosum*, and the obtained interspecific hybrids were characterized for tetrad sterility. We then sequenced 17 mitochondrial genes and their intergenic regions and found that an 859-bp band amplified from the intergenic region between *rpl5* and *rps10* was associated with tetrad sterility.

Materials And Methods

Plant materials

Forty *S. stoloniferum* accessions were used in this study (Table 1). All seeds were obtained from the Potato Introduction Station (NRSP-6), Sturgeon Bay, Wisconsin, USA. As male parents producing abundant fertile pollen, 4x cultivars and breeding clones of *S. tuberosum* (cv. Konafubuki, cv. Nagasaki Kogane, Saikai 35 and 10H17) were used for crossing. The clone DM 1-3 516 R44 (hereinafter, simply referred to as DM) was used to provide reference sequences (Potato Genome Sequencing Consortium 2011). Cultivars Alwara and Serrana Inta were used as standard genotypes showing T-CMS.

Crossing

All plants were grown in a pollinator-free greenhouse. Day length (16 h) was controlled using supplementary lights. All crosses were made in an ordinary manner; anthers and petals were removed from flower buds one or two days prior to opening, and freshly collected pollen was applied immediately to the stigmas. Berries were collected one month after pollination. After another month of maturation, berries were opened using a knife, and only plump seeds were collected, dried and stored at 4 °C until use.

Raising seedlings

Seeds were soaked in 2,000 ppm gibberellic acid (GA₃) (Nacalai Tesque, Inc., Kyoto, Japan) for 48 h, and rinsed with tap water, sown in a cell tray filled with potting soil, and covered with vermiculite. After the second leaves were well expanded (usually 3 weeks after seed sowing), the young seedlings were transplanted to black vinyl pots (10.5 cm diameter) and then into 15-cm-diameter or 21-cm-diameter pots for further growth. The greenhouse temperature was kept above 15 °C in winter, and in summer, the windows were opened to allow natural circulation.

Ploidy test and pollen viability

The ploidy level was determined by flow cytometry (CyFlow Ploidy Analyzer, Sysmex Corporation, Kobe, Japan). Approximately 1 cm² of fresh leaf was used for DAPI fluorescence staining according to the manufacturer's instructions. For pollen viability analysis, fresh pollen was collected at least twice in different flowering periods and stained with 1% acetocarmine. Over 300 pollen grains in each sample were counted under the microscope (Axio Imager A1, Carl Zeiss Co., Ltd.). The stainability percentage was calculated as the number of stained grains divided by the total number of pollen grains × 100.

Hybridity test and determination of cytoplasm type

A hybridity test was carried out using a combination of different marker systems: 11 RAPD markers (Hosaka and Hanneman 1994), two simple sequence repeat markers (STM3012 and STI50) (Milbourne et al. 1998; Feingold et al. 2005), and a multiplex PCR marker for resistance genes (Mori et al. 2011). The cytoplasm type (A, D, P, M, T, or W) was determined using the procedure described by Hosaka and Sanetomo (2012). Mitochondrial DNA type (α , β , or γ) was determined using the primer pair ALM_4/ALM_5 (Lössl et al. 1999).

Detection of mitochondrial polymorphism and sequencing

Based on the reference sequence of the mitochondrial genome in DM (S_tuberosum_Group_Phureja_mitochondrion_DM1-3-516-R44, Ver. 3), 51 primers were designed as mitochondrial gene-specific PCR primers. Two primers, ALM_1 and ALM_5 (Lössl et al. 1999), were used to amplify the intergenic regions around *atp6* and *rps10*, respectively. In addition, the Cob_shoRoutF and rp15rps14outF primers were newly designed with reference to a potato mitochondrial sequence including *rpl5*, *rps14*, and *cob* (GenBank ID: AF095274.1). A total of 28 primer sets amplifying 17 mitochondrial genes (*atp1*, *atp4*, *atp6*, *atp9*, *atp8*, *cob*, *cox1*, *cox2*, *cox3*, *nad1b*, *nad1e*, *nad3*, *nad4*, *nad6*, *rpl5*, *rps10*, and *18S rRNA*), four intergenic regions, and one intragenic (intron) region were used (Supplementary Table 1). Amplification was carried out in a volume of 10 μ l containing 2 μ l of genomic DNA (5 ng/ μ l), 5 μ l of 2× Ampdirect® Plus (Shimadzu, Japan), 0.25 U of heat-activated *Taq* DNA polymerase (BIOTAQ™ HS DNA Polymerase, Bioline Ltd., UK), and 0.3 μ M forward and reverse primers. The thermal profile was as follows: 95 °C for 10 min, followed by 35 cycles at 95 °C for 30 s, 60 °C for 1 min, and 72 °C for 1 min, and a final extension of 72 °C for 5 min. For amplification with primer pairs Cob_shoRoutF/ALM_5 and rp15rps14outF/ALM_5, the thermal profile was as follows: 95 °C for 10 min, followed by an initial cycle of 94 °C for 30 s, 65 °C for 30 s, and 72 °C for 1 min; a reduction in the annealing temperature by 1 °C during each cycle for the next five cycles; then, the annealing temperature at 60 °C for the remaining 25 cycles; and a final extension at 72 °C for 5 min. The amplification products were electrophoresed on a 2% agarose gel with 1× TAE buffer. If single bands were obtained from all samples, the PCR products were Sanger-sequenced by a commercial provider (Takara Bio Inc., Kusatsu, Japan). Sequences were compared with DM as reference sequences using Lasergene SeqMan Pro 13 software (DNASTAR Inc., Madison, Wisconsin, USA).

Results

Cytoplasm types in *S. stoloniferum*

To determine cytoplasmic diversity in *S. stoloniferum*, 40 accessions were analyzed, which revealed seven D/ α -, 20 D/ γ -, three W/ α -, and 10 W/ γ -type accessions (Table 1).

Interspecific hybrids from crosses of *S. stoloniferum* and 4x*S. tuberosum*

A total of 4,839 pollinations were performed between 24 accessions of *S. stoloniferum* and pollen of *S. tuberosum* (Table 2). Although 2,008 berries were formed, most of them were seedless or contained only aborted seeds. A total of

466 seeds were obtained from 35 cross combinations, with a range from one to 151 seeds per cross combination. Plants were grown from these seeds. However, most of the plants looked similar to the parent *S. stoloniferum*, possibly due to contamination by naturally selfed seeds. Plants with hybrid-like morphology were tested using molecular markers (Fig. 1), which confirmed the hybridity of 39 plants from 12 accessions of *S. stoloniferum*. The overall percentage of hybrids obtained per pollination was only 0.08%.

Characterization of interspecific hybrids between *S. stoloniferum* and *S. tuberosum*

Thirty-nine interspecific hybrids of *S. stoloniferum* and *S. tuberosum* were characterized for ploidy levels and pollen stainability (Table 3). Eight hybrids were not available for ploidy determination. Of the remaining 31 hybrids, 11 were hexaploids, eight were tetraploids, and 12 were triploids. Within the same cross combination, plants with different ploidy levels were identified: two hexaploids and one tetraploid from the cross PI 473534 × Saikai 35 (hybrids 3–5) and two triploids and three tetraploids from the cross PI 498035 × 10H17 (hybrids 33–37). Pollen shape and stainability were observed for 37 hybrids because two hybrids did not flower. Eleven hybrids derived from six accessions of *S. stoloniferum* produced normally stained pollen with more than 41.0% stainability (Fig. 2a). These hybrids had the D/α, D/γ, and W/γ cytoplasm types. Among the 26 remaining hybrids, tetrad pollen was observed in 20 hybrids that were derived from six accessions of *S. stoloniferum* (PI 255547, PI 497994, PI 498035, PI 498038, PI 558449, and PI 558450) (Fig. 2b), and their cytoplasm types were either D/γ or W/γ. These hybrids produced mostly unstained tetrad pollen. Tetrad pollen containing a single stained pollen grain (“partial” in Table 2) was also observed in hybrid 36 (Fig. 2c), and a mixture of unstained tetrad and unstained independent pollen (“mixed”) was observed in hybrids 20, 33 and 35 (Fig. 2d). In hybrids 7, 15, 25 and 34, each pollen grain was independent and unstained in the early flowering stage, but then these plants showed T-CMS at a later developmental stage or in a different season (“unstable”, Fig. 2e, 2f).

Plants producing normally stained pollen were either hexaploids or tetraploids (Table 3). Among hybrids showing T-CMS, six were triploids, seven were tetraploids, and four were hexaploids. Ten hybrids with the same cytoplasm derived from PI 497994 (hybrids 8–18) were all triploids and exhibited abnormal growth characteristics, such as thin and weak stems, creeping growth or no flowering. Of these hybrids four showed T-CMS and the others showed lower than 16.6% stainability. Seven hybrids with the same cytoplasm derived from PI 558450 (hybrids 21–27) were all hexaploids (three of them were undetermined), and all of them exhibited T-CMS.

Mitochondrial genome polymorphism

To detect polymorphisms in the mitochondrial genome of *S. stoloniferum*, 15 accessions of *S. stoloniferum* (two D/α-, six D/γ-, two W/α-, and five W/γ-type accessions), together with the T-CMS variety Alwara, were subjected to PCR amplification using 28 primer pairs designed for 17 mitochondrial genes and their intergenic regions (Supplementary Table 1). All the primer pairs except Cob_shoRoutF/ALM_5 and rp15rps14outF/ALM_5 amplified single bands in all samples. These amplified products were Sanger-sequenced and compared with the reference sequence of DM. A total of 14 polymorphisms were detected in nine genes (Table 4), of which 11 polymorphisms were those between DM and the others (Table 5). The *S. stoloniferum* accessions were distinguished into four types by three polymorphisms. PI 498027 was the most different from the others in terms of *atp6* and *atp1*. Alwara was similar to three of the 15 *S. stoloniferum* accessions and different from DM for most polymorphisms.

Using the Cob_shoRoutF/ALM_5 primer pair, which was designed to amplify the intergenic region between *cob* and *rps10*, we detected multiple bands across the 16 samples (Fig. 3a). Alwara lacked a major band of approximately 1,650 bp that was observed in all other *S. stoloniferum* accessions. Instead, a single band of approximately 850 bp was detected in Alwara and shared with most other accessions. However, in PI 558455, the 850-bp band was faint, and there was an extra band of approximately 700 bp.

Using the rp15rps14outF/ALM_5 primer pair, which was designed to amplify the intergenic region between *rp15* and *rps10*, we identified a band of approximately 860 bp in seven accessions of *S. stoloniferum* and Alwara (Fig. 3b). In addition, a larger band of approximately 4 kbp was found in three accessions. The 860-bp band was sequenced, which revealed that all eight samples had similar sequences of 859 bp (Supplementary sequencing data).

The relationships between mitochondrial genome polymorphisms and T-CMS

The mitochondrial genome polymorphisms identified in this study were investigated in terms of T-CMS. All except for the 859-bp band amplified by the rp15rps14outF/ALM_5 primer pair were uncorrelated with T-CMS. The 859-bp band was detected in 27 interspecific hybrids derived from four D/γ- and two W/γ-type *S. stoloniferum* accessions (Table 3, Fig. 5). All the hybrids showing T-CMS had the 859-bp band. In contrast, T-CMS was not observed in any of the hybrids lacking the 859-bp band. The 859-bp band was observed in two T-CMS varieties, Alwara and Serrana Inta, but not in Early Rose (T-type cytoplasm), Tunika (D-type cytoplasm), DM (P-type cytoplasm), or PW-363 (male-fertile clone with a *S. stoloniferum*-derived resistance gene to *Potato virus Y*; Flis et al. 2005).

Geographical distribution

All the 40 accessions of *S. stoloniferum* were analyzed using the primer pair rp15rps14outF/ALM-5. The 859-bp band was amplified from 13 D/γ-type and six W/γ-type accessions of *S. stoloniferum* (Table 1). Collection sites of these *S. stoloniferum* accessions were plotted on the map (Fig. 5). The accessions that caused T-CMS in the interspecific hybrids were shown with red dots in the map. Four cytoplasm types, the T-CMS-causing cytoplasm, or accessions amplifying the 859-bp band were not localized to a specific region, but spread widely across the distribution area.

Discussion

A specific *S. stoloniferum* cytoplasm type causes T-CMS

Since all varieties and breeding clones showing T-CMS have been observed to contain only W/γ-type cytoplasm, this cytoplasm type was thought to cause T-CMS (Lössl et al. 2000; Yermishin et al. 2017). However, we showed for the first time that T-CMS was not necessarily associated with W/γ-type cytoplasm but with a specific cytoplasm with the γ-type mitochondrial genome of *S. stoloniferum*. We name this T-CMS-causing cytoplasm “TSC_{sto}”, which actually induces T-CMS by interaction with the nuclear gene(s) of *S. tuberosum*.

Characterization of TSC_{sto}

Although T-CMS is a stable trait among potato varieties, the interspecific hybrids of *S. stoloniferum* showed some range of variation in T-CMS, from completely unstained tetrad pollen to partially stained tetrad pollen and a mixture of sterile tetrad and sterile independent pollen. In addition, these morphologies changed depending on the time of observation and the plant growth stage. Sensitivity of male sterility to the environment has often been reported in plants, with 44% of cases known to be temperature-sensitive and 12% to be photoperiod-sensitive (Kaul 1988). Similarly, the *S. stoloniferum*-derived T-CMS may be related to environmental factors that affect the growth stage or subtle temperature differences at the pollen maturation stage, although we could not notice any prominent difference in the growing conditions.

The callose in the cell wall of a tetrad is decomposed by callose- and pectin-degradation enzymes secreted by tapetum cells (Dawson et al. 1993; Taylor et al. 1998; Sanders et al. 1999; Wilson et al. 2001). If this separation fails due to incomplete degradation of the callose wall (Rhee and Somerville 1998) or if the pectic components persist around the microspores (Scott et al. 1991), tetrad sterility might occur. Shishova et al. (2019) detected specific metabolic changes

for sporopollenin biosynthesis and exine formation in mature anthers of T-CMS potato varieties, resulting in failed disintegration of tetrads into microspores. Further analyses at molecular level are necessary to understand the process from a tetrad to independent pollen grains.

Mitochondrial genome associated with TSC_{sto}

None of the four cytoplasm types (D/α, D/γ, W/α, and W/γ) identified among 40 accessions of *S. stoloniferum* were associated with T-CMS. Although 17 mitochondrial genes and their intergenic regions, with a total sequence length of 29 kbp, were almost identical among 15 accessions of *S. stoloniferum*, three polymorphisms in three genes and length polymorphisms in two intergenic regions were detected. Among these polymorphisms, an 859-bp band amplified from the intergenic region between *rp15* and *rps10* was associated with T-CMS. The *rp15* intergenic region containing partial *cob*, pseudo-*rps14*, and *rps10* has been reported to be rich in mutations (Scotti et al. 2004), and the primer pair ALM_4/ALM_5 that discriminates the α, β, and γ cytoplasm types was also designed to amplify this region (Lössl et al. 1999). Male sterility in rice, known as wild-abortive CMS (WA-CMS; Bentolila and Stefanov 2012), was detected as a mitochondrial structural mutant with over 100 bp of insertions up- and downstream of *rp15-rps14* (Zhu et al. 2015). Although the CMS mechanism in potato has not yet been elucidated at the molecular level, we tentatively suggest that the region around *rp15* is a causative region for tetrad sterility in the case of TSC_{sto}.

Interspecific hybrids with different ploidy levels

Crosses between 4x (2EBN) *S. stoloniferum* and 4x (4EBN) *S. tuberosum* produced an extremely low number of hybrid seeds due to an imbalanced EBN relationship (Johnston et al. 1980). A balanced EBN relationship in this cross can be achieved by fertilizing 2n eggs of *S. stoloniferum* with normal pollen from *S. tuberosum*, which actually produces hexaploid hybrids. In this study, however, triploid hybrids were also observed in the cross of *S. stoloniferum* and 4x*S. tuberosum* (Table 3). The *S. tuberosum* germplasm was certainly incorporated into the triploid hybrids because marker analysis revealed *S. tuberosum* parent-specific bands in the hybrids (Fig. 1). To the best of our knowledge, triploids have rarely been reported from 4x × 4x crosses in potato. Interestingly, however, diploid hybrids have been reported from the crosses of *S_vS_v*-lines (2x F₂ hybrids between 2x*S. tuberosum* and *S. verrucosum*, Polyukhovich et al. 2010) × *S. stoloniferum* and *S. stoloniferum* × 2x*S. tuberosum* (Vorontkova et al. 2007; Yermishin et al. 2017). Two probable mechanisms were proposed by Vorontkova et al. (2007): (1) generation of twofold reduced gametes (monoploid, *n*=*x*=12), carrying one of the genomes of *S. stoloniferum*, (2) preferential chromosome elimination, which was previously proposed as a mechanism of dihaploid formation in potato (Clulow et al. 1991). A certain set of chromosomes (half of *S. tuberosum* chromosomes, B-genome chromosomes of *S. stoloniferum*, or a combination of these chromosomes) might be preferentially eliminated during embryogenesis. Although the details remain unknown, the T-CMS trait was certainly inherited by some of the triploid hybrids.

Nuclear genes interacting with TSC_{sto}

Male fertility can be restored by a nuclear-encoded fertility restorer (*Rf*) gene(s) in many CMS plants (Hanson and Bentolila 2004). In potato, a dominant restorer gene (*Rt*) has been reported for the cytoplasm of *S. tuberosum* (Iwanaga et al. 1991). Some wild species are highly self-fertile (Hawkes 1990), but the hybrids became male sterile, as in the case of *S. stoloniferum*. In this case, the original species might have a fertility restorer gene, and the expression of the gene is suppressed by some factor in the nuclear genome of the other parent, which causes cytoplasmic male sterility (Yamagishi and Bhat 2014). If a fertility restorer gene exists in the *S. stoloniferum* nuclear genome, it should be active in hexaploid hybrids that probably have the whole *S. stoloniferum* genome (AABB), and the fertility of these hybrids should be restored. However, we found that the hexaploid hybrids also showed T-CMS (Table 3). In other words, even if the fertility restorer gene from *S. stoloniferum* exists in interspecific hybrids, T-CMS may be caused by a dominant

factor in the nuclear genome from *S. tuberosum*, suppressing the expression of the restorer gene. Indeed, the hexaploid, tetraploid, and even triploid interspecific hybrids showed T-CMS, indicating that incorporation of *S. tuberosum* germplasm did cause T-CMS. A similar idea has been proposed for tetrad sterility in interspecific hybrids of *S. verrucosum*, in which the suppressor of the restorer gene is regarded as a dominant tetrad sterility-causing gene (*Tr*) and interacts with the cytoplasm of *S. verrucosum* (Buck 1960; Abdalla and Hermesen 1972).

Conclusion

We revealed that a specific cytoplasm type in *S. stoloniferum*, TSC_{sto}, caused tetrad sterility in interspecific hybrids with *S. tuberosum*. This cytoplasm was widely distributed across the distribution area of *S. stoloniferum*, indicating that TSC_{sto} was present before geographical differentiation of *S. stoloniferum*. We suggest that TSC_{sto} was derived from the maternal diploid ancestor, *S. verrucosum* (Spooner and Castillo 1977; Rodríguez and Spooner 2009; Sanetomo and Hosaka 2013), because the cytoplasm of *S. verrucosum* also causes T-CMS (Grun et al. 1962; Abdalla and Hermesen 1971, 1972). The 859-bp band of the mitochondrial genome was associated with T-CMS and might be a candidate region responsible for tetrad sterility. Thus, the 859-bp band can be used as a diagnostic marker for TSC_{sto}, which is useful for distinguishing T-CMS-causing cytoplasm in breeding. Without accurate information on the cytoplasm that breeders are using, male-sterile genotypes are increased unconsciously, and only a limited number of pollen parents are available to breeders (Hosaka and Sanetomo 2012; Mihovilovich et al. 2015; Sanetomo and Gebhardt 2015).

Declarations

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Authors' contributions

AN and RS carried out the crossing experiment and sequencing analysis. RS collected and analyzed all data and wrote the manuscript. All authors read and approved the final manuscript.

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Tables

Table 1 *Solanum stoloniferum* accessions used and their cytoplasm types

<i>S. stoloniferum</i> accession	Cytoplasm type
PI 184770, PI 186555, PI 283102, PI 338617, PI 338621, PI 473534, PI 498027	D/α
PI 251062, PI 251740, PI 255547*, PI 275162, PI 275248*, PI 310964*, PI 458418, PI 497994*, PI 498000, PI 498030*, PI 498038*, PI 545726*, PI 545788*, PI 545789, PI 558395*, PI 558447*, PI 558450*, PI 558451*, PI 558484, PI 607843*	D/γ
PI 186544, PI 195167, PI 545723	W/α
PI 161178*, PI 249929, PI 283101, PI 283108*, PI 498035*, PI 498238, PI 545780*, PI 558446, PI 558449*, PI 558455*	W/γ

*Possessing the 859-bp band amplified using the primer pair rp15rps14outF/ALM-5

Table 2 Crosses of 24 *S. stoloniferum* accessions with pollen of *S. tuberosum*

Female accession	No. of plants	Cyto-plasm type	No. of male genotypes ^a	Flowers	Berries	Seeds	Hybrids
PI 184770	8	D/α	2	161	32	2	2
PI 338617	16	D/α	1	71	30	6	0
PI 338621	4	D/α	3	106	43	1	0
PI 473534	16	D/α	1	158	81	11	3
PI 498027	6	D/α	2	220	36	6	1
PI 251740	19	D/γ	2	371	138	190	0
PI 255547	18	D/γ	3	465	286	16	1
PI 275248	10	D/γ	3	427	187	9	0
PI 310964	8	D/γ	3	196	39	0	-
PI 497994	8	D/γ	3	194	118	22	11
PI 498000	7	D/γ	2	110	46	7	1
PI 498038	8	D/γ	2	254	126	8	1
PI 558450	12	D/γ	3	205	48	12	7
PI 607843	3	D/γ	2	77	0	-	-
PI 186544	4	W/α	3	131	63	1	0
PI 195167	7	W/α	2	245	96	3	0
PI 545723	3	W/α	3	42	14	8	0
PI 249929	13	W/γ	2	229	114	131	3
PI 283101	11	W/γ	2	184	108	5	2
PI 498035	8	W/γ	3	134	79	11	6
PI 545780	9	W/γ	3	193	64	3	0
PI 558446	2	W/γ	2	20	13	0	-
PI 558449	11	W/γ	3	374	113	4	1
PI 558455	9	W/γ	3	272	134	10	0
Total				4839	2008	466	39

^aKonafubuki, Nagasaki Kogane, Saikai 35, and 10H17 were used as pollen parents

Table 3 Characterization of interspecific hybrids between *S. stoloniferum* and *S. tuberosum*

Hybrid identity	Female	Male	Clonal identity	Cyto-plasm type	Ploidy	Pollen stainability (%)	Notes ^a	859-bp band ^b
1	PI 184770	10H17	17H129	D/α	nd	41.0		-
2	PI 184770	Konafubuki	17H130	D/α	6x	91.7		-
3	PI 473534	Saikai 35	10H6-1	D/α	6x	2.9		-
4			10H6-5	D/α	6x	64.3		-
5			10H6-6	D/α	4x	52.7		-
6	PI 498027	10H17	17H131	D/α	6x	66.4		-
7	PI 255547	10H17	15H156	D/γ	4x	0.0	T-CMS (unstable)	+
8	PI 497994	10H17	19H71-2	D/γ	3x	0.0	T-CMS	+
9			19H71-5	D/γ	nd	nf		+
10			19H72-1	D/γ	3x	0.0	T-CMS	+
11			19H72-2	D/γ	3x	3.8		+
12			19H72-4	D/γ	3x	nf		+
13			19H73-1	D/γ	3x	12.7		+
14			19H73-2	D/γ	3x	0.0	T-CMS	+
15			19H73-3	D/γ	3x	0.0	T-CMS (unstable)	+
16			19H74-1	D/γ	3x	16.6		+
17			19H74-2	D/γ	3x	0.0		+
18			19H74-3	D/γ	3x	0.9		+
19	PI 498000	Konafubuki	17H125-1	D/γ	nd	59.3		-
20	PI 498038	10H17	19H81-2	D/γ	4x	<0.3 ^c	T-CMS (mixed)	+
21	PI 558450	10H17	17H120-1	D/γ	6x	0.0	T-CMS	+
22			17H120-2	D/γ	6x	0.0	T-CMS	+
23			17H120-3	D/γ	nd	0.0	T-CMS	+
24			17H120-4	D/γ	6x	0.0	T-CMS	+
25			17H120-5	D/γ	6x	0.0	T-CMS (unstable)	+
26			17H120-6	D/γ	nd	0.0	T-CMS	+
27	PI 558450	Konafubuki	17H121	D/γ	nd	0.0	T-CMS	+
28	PI 249929	10H17	15H150-1	W/γ	6x	86.8		-
29	PI 249929	Konafubuki	17H117-1	W/γ	nd	83.4		-
30			17H117-9	W/γ	6x	77.5		-
31	PI 283101	Konafubuki	17H118-3	W/γ	6x	73.9		-
32			17H118-4	W/γ	nd	78.7		-
33	PI 498035	10H17	19H75-1	W/γ	3x	0.0	T-CMS (mixed)	+
34			19H75-2	W/γ	4x	0.0	T-CMS (unstable)	+
35			19H76-1	W/γ	4x	<19.4 ^c	T-CMS (mixed)	+
36			19H76-2	W/γ	4x	0.0	T-CMS (partial)	+
37			19H76-3	W/γ	3x	0.0	T-CMS	+
38	PI 498035	Konafubuki	19H77	W/γ	4x	0.0	T-CMS	+
39	PI 558449	Konafubuki	19H69	W/γ	4x	0.0	T-CMS	+

^aRegarding tetrad sterility (T-CMS), one of the pollen grains in the tetrad was stained (partial), tetrad pollen and unstained independent pollen were mixed (mixed), or tetrad pollen and unstained independent pollen varied over time (unstable).

^b Presence (+) or absence (-) of the 859-bp band amplified using the primer pair rp15rps14outF/ALM-5

^cTetrad pollen was erroneously counted as one unstained pollen grain; thus, pollen stainability was lower than this percentage.
nd=not determined, nf=not flowered

Table 4 Polymorphisms detected in the mitochondrial genome

Gene	Primer (forward/reverse)	Amplified region in DM	Polymorphism	Polymorphism and its position ^a
<i>nad3</i>	nad3-F/nad3-R	Segment 31 (3796-4816 bp)	nad3	Deletion of T at or overlap from 113 (exon)
<i>nad4</i>	nad4-exon2F/nad4-exon2R	Segment 18 (21492-22240 bp)	nad4	T→C at 37 (intron)
<i>atp6</i>	atp6-1F/atp6-1R	Segment 8 (3070-4086 bp)	atp6-a atp6-b atp6-c	T→G at 395 (exon) T/G overlap at 868 (exon) G→T at 1118 (intron)
<i>atp1</i>	ALM_1/Band1-F27 atp1-F1/atp1-R1	Segment 8 (2082-3528 bp) Segment 29 (7449-8508 bp)	atp1-a atp1-b	Insertion of TAAAA between 89 and 90 (intron) C→A at 713 (exon)
<i>atp9</i>	atp1-F2/atp1-R2 atp9-1F/atp9-1R	Segment 29 (8285-9340 bp) Segment 12 (4367-5300 bp)	atp9	T→C at 479 (intron)
<i>cox3</i>	cox3-F/cox3-R	Segment 24 (225-1299 bp)	cox3	Insertion of G between 177 and 178 (intron)
<i>rps10</i>	rps10F/cox1_R	Segment 11 (5934-7316 bp)	rps10-a rps10-b rps10-c	T→A at 226 (exon) C/T overlap at 1033 (intron) G→A at 1214 (intron)
<i>cox1</i>	cox1-F2/cox1-R2	Segment 11 (4877-6045 bp)	cox1	C→T at 271 (exon)
<i>cox2</i>	cox2-intronF/cox2-intronR	Segment 15 (29539-30515 bp)	cox2	Overlap from 497 (intron)

^aPositions are indicated in bp from the start of the forward primer

Table 5 Sequence polymorphisms in Alwara and 15 accessions of *S. stoloniferum* in comparison with DM

Genotype	Cytoplasm	nad3	nad4	atp6-a	atp6-b	atp6-c	atp1-a	atp1-b	atp9	cox3	rps10-a	rps10-b	rps10-c	cox1	cox2
DM	P	T	T	T	T	G	-	C	T	-	T	C	G	C	A
PI 184770	D/α	T	C	G	T/G	T	TAAAA	C	C	G	A	C/T	A	T	N
PI 498027	D/α	T	C	G	T	T	TAAAA	A	C	G	A	C/T	A	T	N
PI 251740	D/γ	T	C	G	T/G	T	TAAAA	C	C	G	A	C/T	A	T	N
PI 255547	D/γ	N	C	G	T/G	T	TAAAA	C	C	G	A	C/T	A	T	N
PI 275248	D/γ	T	C	G	T/G	T	TAAAA	C	C	G	A	C/T	A	T	N
PI 310964	D/γ	-	C	G	T/G	T	TAAAA	C	C	G	A	C/T	A	T	N
PI 498000	D/γ	T	C	G	T/G	T	TAAAA	C	C	G	A	C/T	A	T	N
PI 558450	D/γ	N	C	G	T/G	T	TAAAA	C	C	G	A	C/T	A	T	N
PI 186544	W/α	-	C	G	T/G	T	TAAAA	C	C	G	A	C/T	A	T	N
PI 195167	W/α	T	C	G	T/G	T	TAAAA	C	C	G	A	C/T	A	T	N
PI 249929	W/γ	T	C	G	T/G	T	TAAAA	C	C	G	A	C/T	A	T	N
PI 283101	W/γ	T	C	G	T/G	T	TAAAA	C	C	G	A	C/T	A	T	N
PI 545780	W/γ	T	C	G	T/G	T	TAAAA	C	C	G	A	C/T	A	T	N
PI 558449	W/γ	N	C	G	T/G	T	TAAAA	C	C	G	A	C/T	A	T	N
PI 558455	W/γ	N	C	G	T/G	T	TAAAA	C	C	G	A	C/T	A	T	N
Alwara	W/γ	N	C	G	T/G	T	TAAAA	C	C	G	A	C/T	A	T	N

N, overlapping signals; -, deleted

Figures

Figure 1

Hybridity test using molecular markers: a SSR primer STM3012, b RAPD marker (5'-GTCCCGTGGT-3') , c multiplex PCR marker for resistance genes (Mori et al. 2011). Interspecific hybrids are shown by their hybrid identity number given in Table 3. A, progeny from PI 497994×10H17; B, progeny from PI 498038×10H17; C, progeny from PI 249929×Konafubuki; D, progeny from PI 558450×10H17. DNA ladder markers in the first lane.

Figure 2

Pollen stainability and morphology in F1 hybrids. a Normal pollen from hybrid 28 (*S. stoloniferum* PI 249929×10H17) and b tetrad sterility in hybrid 21 (*S. stoloniferum* PI 558450×10H17). c One of the pollen grains in the tetrad was stained in hybrid 36 (*S. stoloniferum* PI 498035× 10H17). d Tetrad pollen and unstained independent pollen were mixed in hybrid 20 (*S. stoloniferum* PI 498038×10H17). e Hybrid 34 (*S. stoloniferum* PI 498035 × 10H17) showed unstained independent pollen, but f T-CMS appeared in this hybrid 30 days later.

Figure 3

Banding patterns of Alwara and 15 accessions of *S. stoloniferum* for the regions amplified by Cob_shoRoutF/ALM_5 (a) and rp15rps14outF/ALM_5 (b). A 1-kb Plus DNA ladder was used as a size marker in the first lane.

Figure 4

Banding patterns of the regions amplified by rp15rps14outF/ALM_5. A 1-kb Plus DNA ladder was used as a size marker in the first lane.

Figure 5

Geographical distribution of *S. stoloniferum* with different cytoplasm types. PI 473534 was not plotted because the collection site data was not available. Accessions shown with red and blue dots are those causing T-CMS and those not causing T-CMS (normal), respectively, in interspecific hybrids with *S. tuberosum*.