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Original article

**Two cytoplasmic male sterility phenotypes in beet (*Beta vulgaris* L.):
Implications of their simultaneous onset and divergent paths**

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Running title: Two cytoplasmic male sterility phenotypes in beet

Abstract

Cytoplasmic male sterility (CMS) is a mitochondrion-encoded trait specifically affecting anthers. Several male sterility-inducing mitochondrial types are known, many of which affect the development of anther tapetum cells, but no sound explanation for this tissue's vulnerability has been proposed. Insights into the cause of CMS can be obtained by the detailed phenotypic comparison of different male sterility-inducing mitochondrial types of the same plant species. In pursuit of this objective, we conducted an investigation into anther development in two sugar beet CMS lines. We compared an Owen type CMS line used for hybrid breeding with the G type CMS line derived from wild beet. Both CMS lines have the same nuclear background. The tapetum of the G CMS line exhibited hypertrophy in the microspore stage, as reported previously in Owen CMS lines. Ultrastructural analysis revealed mitochondrial abnormalities, including low electron density and aberrant cristae appearing in the tapetum after meiosis in both lines. The Owen CMS line lacked Ubisch bodies and had poorly developed bacula and tecta in the pollen cell walls,

whereas the G CMS line retained these features, but the pollen wall was highly deformed. Ultimately, microspores and the tapetum degenerated in both lines, and the male sterile phenotypes were eventually very similar. Although it had been hypothesized that mitochondrial activation was associated with CMS expression, mitochondria in the root apical meristem appeared normal in beet roots with G- and Owen type mitochondria. We propose that CMS expression includes at least two mechanisms: one triggers abnormal mitochondrial generation, and the other affects the type of developmental abnormality.

Keywords

Anther development, cristae, cytoplasmic male sterility, mitochondria, sugar beet, tapetum

Introduction

Cytoplasmic male sterility (CMS) is a maternally inherited trait that leads to pollen-production failure without affecting female- or vegetative organs (Schnable and Wise 1998). CMS has been associated with mitochondria: male-sterility inducing mitochondria (hereafter S mitochondria) have open reading frames (ORFs) encoding unique proteins that are absent from non-male sterility-inducing mitochondria (N mitochondria). Such ORFs, termed *S-orfs*, are a type of ‘de novo’ ORF that are composed of sequences of unknown origin and fragments of duplicated mitochondrial sequences (Hanson and Bentolila 2004; Kitazaki et al. 2023). The number of *S-orfs* reported to date exceeds 38 from various plant species, but they have no or minimal homology with each other (Kim and Zhang 2017). Phenotypes of CMS vary, such as the homeotic conversion of stamen into petals or carpels and the production of apparently normal pollen that cannot germinate (Schnable and Wise 1998; Hanson and Bentolila 2004). In many cases, however, the anther tapetum of CMS plants, the innermost cell layer in anther locules surrounding the pollen mother cells/microspores, has developmental abnormalities as revealed by cytological studies (Schnable and Wise 1998). The fundamental question of why the action of many *S-orfs* coincides with the defective development of the same tissue has remained unanswered for decades.

Mitochondria are cellular organelles thought of as the power plants of eukaryotic cells

because they provide cellular energy by producing ATP via oxidative phosphorylation (Scheffler 1999), but mitochondria are also involved in many other metabolic and biological processes (Ng et al. 2021). Impairment of mitochondrial metabolism in the tapetal tissue of anthers should limit pollen development because one of the roles of this tissue is to nurse microspores by providing the necessary substances for pollen development (Wu and Cheun 2000). Cytological and molecular biological studies have shown that mitochondria are highly activated in the anther tapetum, and this activity is associated with tapetum abnormalities in CMS germplasm (Schnable and Wise 1998). Programmed cell death (PCD) is another biological role of mitochondria (Hoeberichts and Woltering 2002) and functions in a crucial step in pollen development because mutants defective in tapetal PCD fail to shed functional pollen (Wilson and Zhang 2009).

Most debates about the mechanism of CMS expression have assumed that *S-orf* is absolutely harmful for mitochondria, *e.g.*, causing ATP depletion. However, this notion raises the question of why the only visible phenotype appears in anthers, whereas the expression of many *S-orfs* is constitutive (Schnable and Wise 1998). If *S-orf* is harmful, phenocopies of CMS would be expected from other mutants associated with mitochondria. However, no known nuclear mutants associated with the respiratory complex reproduce the anther-specific phenotype (Touzet and Meyer 2014). Although mutants in the plant mitochondrial genome are very rare, mutants in RNA editing, a post-transcriptional process that converts specific cytidine residues into uridine in mRNA, are known. Defects in the nuclear-encoded editing factor are considered ‘surrogate’ mitochondrial mutants because the gene products of the unedited mRNA would be deleterious (Colas des Francs-Small and Small 2014). Interestingly, none of the 57 mutants with defects in their RNA editing reproduced the CMS phenotype (Takenaka et al. 2019). The mutants exhibited either developmental defects evident in the whole plant, *e.g.*, defects in seed or fruit development, or no morphological defects when grown in laboratory conditions. Recently, a mitochondrial mutant of *nad7* was obtained by mitochondrial genome editing, and its phenotype expressed a developmental defect in the whole plant (Ayabe et al. 2023), a finding reminiscent of a tobacco mutant with a defect in *nad7* (Pla et al. 1995). Thus, CMS is a unique class of mitochondrial variants.

Molecular diversity of *S-orf* has significantly complicated the study of CMS. If different *S-orfs* take advantage of tapetum's susceptibility to induce male sterility, examining phenotypic differences and similarities between different *S-orf* induced CMS's should provide insights into the function of the *S-orf* gene, the long-standing conundrum of this research field. Our idea was to compare two CMS lines with different mitochondrial types at the cellular and subcellular levels, focusing on the onset of CMS. For such a comparative study, different S cytoplasms should exist in the same nuclear genotype because the phenotype of CMS is affected by the nuclear background (Colhoun and Steer 1981).

Sugar beet (*Beta vulgaris* ssp. *vulgaris*) CMS was first reported by a U.S. geneticist, F.V. Owen, who identified CMS in the 'US-1' cultivar (Owen 1945). The cytoplasm is now known as the Owen cytoplasm. Mitochondrial DNA from the Owen cytoplasm has been sequenced to identify its *S-orf* (Sato et al. 2004). A variant of *atp6* with an NH₂-terminal extension of 387 amino acid residues is associated with the Owen mitochondria (Yamamoto et al. 2005). After translation of *atp6*, the NH₂-terminal extension, named *preSatp6*, is excised from the precursor and accumulates in the inner mitochondrial membrane (Yamamoto et al. 2005). Molecular analyses of *preSatp6* indicate it is the *S-orf* of Owen mitochondria (Kitazaki et al. 2015).

Several other S mitochondria are known in beet, of which a CMS type with several nonsynonymous mutations in *cox2* and *nad9* was found in wild beet (*B. vulgaris* ssp. *maritima*) in France (Ducos et al. 2001). None of the mutations are shared with the Owen mitochondria, nor any other S mitochondria reported to date, making this CMS type unique. In addition, this wild beet mitochondrial genome has another defect unique to this mitochondrial type, namely a missense mutation in the translational initiation codon of *cox1* that adds an NH₂-terminal extension (Darraq et al. 2011). A fusion protein of COXI with the extension was detected (Meyer et al. 2018). Unlike Owen mitochondria, the *atp6* in this mitochondrial type is identical to that of the N mitochondrial line (Darraq et al. 2011). This male sterility-inducing cytoplasm is known as the G cytoplasm (Ducos et al. 2001). Expression of Owen- and G cytoplasms is suppressed by nuclear genes, but the suppressors are different: for Owen cytoplasm, two suppressors were mapped onto chromosomes 3 and 4, whereas the suppressor for G cytoplasm was mapped onto

chromosome 8 (Pillen et al. 1993; Schondelmaier and Jung 1997; Hjerdin-Panagopoulos et al. 2000; Hagihara et al. 2005; Honma et al. 2014; Touzet et al. 2004). Collectively, the available evidence suggests that the two cytoplasms are genetically distinct.

We raised the question of how the two CMS phenotypes differed and conducted a comparative study. In this report, we show that the phenotypes of the two CMS are generally similar. Abnormal mitochondria appear almost simultaneously in anther tapetal tissues. Although the later developmental processes are distinctive, the ultimate phenotypes are similar. The root apical meristem is also a mitochondrion-activated tissue, but no abnormalities were observed. We favor the notion that there is a cue to trigger abnormal mitochondrial generation after meiosis, and other mechanisms to affect the mode of developmental abnormality.

Materials and methods

Plant materials

Wild beet accession FR4-31 is from a germplasm collection in France from which male sterile plants were found to possess mitochondrial genomes indistinguishable from other beets with G cytoplasm (Kawanishi et al. 2010). Thus, in this study, we considered the FR4-31 cytoplasm to have G cytoplasm. Kawanishi et al. (2010) crossed FR-4-31 with TK-81mm-O, a Japanese sugar beet line, to obtain male sterile hybrids. These male sterile hybrids were backcrossed four times with TA-33BB-O, another Japanese sugar beet line developed by the Hokkaido Agricultural Research Center, National Agriculture and Food Research Organization (Matsuhira et al. 2022), to generate TA-33BB-CMS(G). TA-33BB-CMS is a sugar beet line having Owen cytoplasm, but its nuclear genotype is identical to TA-33BB-O (Matsuhira et al. 2022). In this study, the line is identified as TA-33BB-CMS(Owen) to emphasize the origin of its cytoplasm. Plants were grown in a Hokkaido University greenhouse. Anther contents were visualized by squashing the anthers on a glass slide and staining with Alexander's dye (Alexander 1969).

Specimen embedding in paraffin and sectioning

Flower buds of various sizes were collected from greenhouse-grown plants. Procedures for

section preparation generally followed those described in Arakawa et al. (2019) with some modifications. Briefly, excised organs were immersed in an FAA solution [50% (v/v) ethanol, 3.7% (v/v) formaldehyde, 5% (v/v) acetic acid] overnight at 20°C. After removing the FAA solution, specimens were dehydrated in an ethanol series [50, 60, 70, 85, 95% (v/v)] for 30 min each and submerged overnight in 95% ethanol containing 2% (w/v) Eosin Yellow (Wako Pure Chemical, Osaka, Japan). After the Eosin Yellow-containing solution was removed and replaced with fresh 95% ethanol, each specimen was submerged in a series of tert-butanol: ethanol solutions of 1:3, 1:1, and 3:1 (v/v) for 30 min each and finally placed in tert-butanol overnight at 37°C. The next day, the sample temperature was increased to 60°C and melted Paraplast Plus (Sigma-Aldrich Japan, Tokyo, Japan) was gradually poured on the specimens to replace the tert-butanol. After evaporating the tert-butanol, each specimen was embedded in fresh Paraplast Plus. Paraplast blocks were sectioned (8 µm thickness) using an HM360 rotary microtome (Carl Zeiss, Oberkochen, Germany). Sections on glass slides were stained with Toluidine Blue [0.05% (w/v); Sigma-Aldrich Japan].

Specimen embedding in resin and sectioning

Primary roots with lengths of less than 20 mm and no lateral roots or root hairs were excised from sugar beet seedlings germinated in plastic Petri dishes. Sugar beet roots were immersed in a pre-fixation solution [3% (w/w) glutaraldehyde, 50 mM phosphate buffer (pH 7.2)] in a vacuum chamber and incubated for 20 h at 4°C. Each specimen was washed twice in a washing solution [0.14 M sucrose, 0.1 M phosphate buffer (pH 7.2)] for 15 min at 4°C. Samples were fixed in an osmium tetroxide solution [1% (w/v) osmium tetroxide, 0.21 M sucrose, 50 mM phosphate buffer (pH 7.2)] for 2 h at 20°C, followed by two washes with phosphate buffer [50 mM (pH 7.2)] for 15 min at 4°C. Each specimen was dehydrated in an ethanol series [50, 70% (w/w)] for 10 min at 4°C. Further dehydration was done in 90% ethanol for 10 min at 20°C. Finally, each specimen was immersed twice in 99.5% ethanol for 10 min and then immersed twice in anhydrous ethanol for 10 min at 20°C. The ethanol was replaced with propylene oxide (Nisshin EM, Tokyo, Japan) and was incubated for 10 min at 20°C. The incubation step was repeated twice with fresh

propylene oxide. Agar New Spurr Resin (EM Japan, Tokyo, Japan) was used to prepare a resin solution consisting of 4.8 g of LV Resin, 1.6 g of LV Hardener VH1, 3.6 g of LV Hardener VH2, and 0.25 mL of Accelerator. An equal volume of the resin solution was added to the specimens in propylene oxide to yield a final concentration of 50% resin solution. The propylene-oxide/resin-immersed specimens were rotated for 2 h at 20°C. Next, fresh resin solution was added to increase the resin concentration to 75% with a rotation time of 16 h at 20°C. A second portion of fresh resin solution was added to yield a resin concentration of 90% resin, and the samples were rotated for 6 h at 20°C. Next, the resin was discarded, fresh resin was added, and the specimens were rotated for 16 h at 20°C. Finally, the specimens were transferred to another volume of fresh resin solution and rotated for 6 h at 40°C. Each specimen was put into a mold with fresh resin solution and incubated for 48 h at 60°C to advance polymerization. Sections of 1 µm were cut with a Reichert-Nissei Ultracut S ultramicrotome (Leica Microsystems, Wetzlar, Germany) with a glass knife made by LKB 7800 Knife Maker (Ted Pella, Redding CA, U.S.A.) and stained with Toluidine Blue [0.05% (w/v); Sigma-Aldrich]. Sections of 60-80 nm were cut using a Reichert-Nissei Ultracut S ultramicrotome equipped with an SYM0545 diamond knife (JEOL, Tokyo, Japan) and collected on 75-mesh formvar-coated copper grids. The grids were first stained with TI Blue (Nisshin EM), followed by staining with lead citrate (Reynolds 1963).

Microscopic observation

Light microscopic images were collected using a BX50 light microscope (Olympus, Tokyo, Japan) equipped with a DP21 CCD camera (Olympus). For transmission electron microscopy (TEM) observations, a JEM-3200FS electron microscope (JEOL) was used with an acceleration voltage of 100 kV.

Results

Phenotype of TA-33BB-CMS(G)

As the phenotype of G-type CMS beet has not been detailed, we investigated TA-33BB-CMS(G) plants. The plants were grown in a green house and confirmed their male sterility (Fig. 1A). Their

anthers were thin, white and somewhat translucent. This phenotype was indistinguishable from those of TA-33BB-CMS(Owen) (Fig. 1B). Anthers of TA-33BB-CMS(G) contained no functional pollen grains but contained undeveloped pollen grain residues (Fig. 1C). The anther content of TA-33BB-CMS(Owen) was very similar in appearance (Fig. 1D).

We employed light microscopy to identify the developmental stages in which abnormalities first appear in anther sections of TA-33BB-CMS(G). According to Arakawa et al. (2019), we defined the meiosis stage as beginning when pollen mother cells (PMCs) enter meiosis. In this developmental stage, tapetum cells were intensely stained and clearly distinguishable from other cells or anther wall tissues (Fig. 2A). PMCs were aggregated in the center of the locule. In the tetrad stage, when meiosis was complete, tapetal cells were still deeply stained and distinctive from other tissues (Fig. 2B). Tetrads were enclosed by callose that brought them closer together. Later, the callose layer was thinner and situated the microspores even closer (Fig. 2C). The thickness of the tapetum had slightly decreased, and the cells appeared to be stuck together. Some, but not all, tapetal cells were slightly vacuolated (Fig. 2C). In the early microspore stage (corresponding to the microspore Sa stage in Arakawa et al. (2019)), callose disappeared, but many microspores were still closely situated (Fig. 2D). Some microspores contained vacuoles or were irregularly shaped (Fig. 2D). Tapetal cells were attached to form layers that either entirely adhered to or were detached from the anther wall (Fig. 2D). Many of the tapetal cells were vacuolated (Fig. 2D). Some tapetal cells showed the onset of swelling (Fig. 2D). In the later stages, the precise developmental stage could not be identified because of several morphological abnormalities in the microspores. Figures 2E and 2F are images of anthers at a later time point in the microspore stage than the image shown in Fig. 2D. The tapetum cells were extremely swollen and highly vacuolated (Fig. 2E). Some anther locules were not round but deformed, perhaps due to uneven hypertrophy of the tapetum (Fig. 2F). As shown in Figs 2E and 2F, the tapetal cells were less stained than those in the earlier stages. Microspores were still recognizable, and some were even spherical, but they seemed to be brought together in the center of the anther locules by the swollen tapetum. In the later stages shown in Figs 2G and H, anther locules were highly deformed or flattened, resulting in squashed microspores that became part of the aggregated

cellular residue from degenerated tapetum cells. The residue was separated from (Fig. 2G) or attached to (Fig. 2H) the anther wall. Just before anthesis, the mass of residue held in the anther locule was greatly reduced (Fig. 2I). Radial bars of thickenings were observed in some endothecium cells (Fig. 2I). No traces of anther dehiscence were observed.

Anther development in TA-33BB-O and TA-33BB-CMS(Owen) at the light microscopic level was reported previously by Arakawa et al. (2019). Compared with our observations, no morphological differences were noted among TA-33BB-O, TA-33BB-CMS(G), and TA-33BB-CMS(Owen) during meiosis. In the late tetrad stage, vacuolation of some tapetal cells of TA-33BB-CMS(G) may be a unique phenotype of this line, but we are not convinced. The first obvious abnormality in anther morphology, characterized by tapetum vacuolation and hypertrophy, appeared in the early microspore stage in TA-33BB-CMS(G) and TA-33BB-CMS(Owen). In the later stages, the two CMS lines exhibited similar morphologies, but the ratio of round microspores seemed to be slightly higher in the microspore stage of TA-33BB-CMS(G). Ultimately, the phenotypes of the two CMS lines were very similar (Fig. 2I of this study and Fig. 4W of Arakawa et al. 2019).

Anther ultrastructure is indistinguishable among TA-33BB-O, TA-33BB-CMS(G), and TA-33BB-CMS(Owen) during the meiosis stage

As the *S-orfs* of the two CMS lines are different, we expected to see some difference in the fine structure of their anthers. We investigated anther ultrastructure using three sugar beet lines. In TA-33B-O anthers, PMCs were aggregated and varied in size and shape. One of the PMCs is shown in Fig. 3A. The electron density of the cytoplasm was high, the nucleus occupied most of the PMC, and there were plastids of various shapes. The PMCs of TA-33BB-CMS(G) and TA-33BB-CMS(Owen) were similar to those of TA-33BB-O (Figs. 3B and 3C). The tapetum of TA-33BB-O, TA-33BB-CMS(G) and TA-33BB-CMS(Owen) contained a very electron-dense cytoplasm (Figs. 3D, 3E and 3F) and plastids of various shapes. Unlike TA-33BB-O, a small number of highly electron-dense particles that appeared to be lipids were observed in TA-33BB-CMS(G) and TA-33BB-CMS(Owen) (Figs. 3E and 3F). In the tapetum of TA-33BB-O, there were

many mitochondria, one of which is shown in Fig. 3G. The mitochondria were oval, 400-700 nm in size and were generally very electron dense. Inside the mitochondria, many cristae that were narrow and straight but rather short were evident. The tapetal mitochondria of TA-33BB-CMS(G) and TA-33BB-CMS(Owen) were very similar to those of TA-33BB-O (Figs. 3H and 3I). The outer layer of the tapetum was an endothecium with large vacuoles and plastids filled with starch. These observations were similar for TA-33BB-O, TA-33BB-CMS(G), and TA-33BB-CMS(Owen) (Figs. 3J, 3K and 3L).

Abnormalities during the post-meiotic stage

In our ultrastructural analysis, we defined the developmental stages as follows: the early tetrad stage is when microspores with probacula are surrounded with callose; the late tetrad stage is when microspores with thin bacula and tecta are enclosed by callose; and the early microspore stage is when microspores with thick tecta and bacula occur without any callose.

MICROSPORES AND POLLEN CELL WALLS

In the early tetrad stage, round microspores were seen in all three lines (Figs. 4A, 4B and 4C). Whereas probacula were visible in the pollen walls of TA-33BB-O and TA-33BB-CMS(G) (Figs. 4D and 4E), few probacula were obvious in TA-33BB-CMS(Owen) and were thin and wavy (Fig. 4F). The electron density of the plasma membrane was higher in TA-33BB-CMS(Owen) than the other two lines (Fig. 4F).

In the late tetrad stage, microspores of TA-33BB-O were round (Fig. 4G). Their pollen cell walls contained bacula, tecta, and nexine (Fig. 4J). In close proximity to the plasma membrane, a layer of low electron-dense material was seen (Fig. 4J). TA-33BB-CMS(G) microspores were oval compared to those of TA-33BB-O (Fig. 4H). The thickness of TA-33BB-CMS(G) pollen walls was uneven compared to TA-33BB-O and were occasionally very thin (Fig. 4K), although bacula and tecta could be identified (Fig. 4K). The plasma membrane could also be identified, but a layer of low electron-dense material was obscure. In TA-33BB-CMS(Owen), microspores were oval (Fig. 4I). Their pollen walls were uneven in thickness. The plasma

membrane, bacula, and tecta could be identified, but the bacula were fewer, shorter, and thinner than those of TA-33BB-O and TA-33BB-CMS(G) (Fig. 4L). A layer of low electron density was rarely identified in the close vicinity of the plasma membrane.

In the early microspore stage, TA-33BB-O had round microspores (Fig. 4M). In their pollen walls, plasma membrane, bacula, and tecta could be identified (Fig. 4P). The tecta were thicker than those in the late tetrad stage. Deformed microspores were observed in TA-33BB-CMS(G) (Fig. 4N). Pollen wall thickness was uneven, but the plasma membrane, bacula, and tecta were identified (Fig. 4Q). The bacula and tecta were thicker than those from the previous stage; however, the tecta were still thinner than those of TA-33BB-O. In this stage, a low electron-dense layer was prominent (Fig. 4Q). The microspores of TA-33BB-CMS(Owen) were deformed (Fig. 4O), and the plasma membrane was distorted (Fig. 4R). The tecta were poorly developed compared with those of TA-33BB-O and TA-33BB-CMS(G) and were insufficient to cover the entire pollen wall. The bacula of TA-22BB-CMS (Owen) were uneven in thickness and were wavy (Fig. 4R).

MITOCHONDRIA IN TAPETAL CELLS

The mitochondria in the early tetrad stage of TA-33BB-O were round or oval and 350-550 nm in size (Fig. 5A). The electron density of the mitochondrial interior was very high, making identification of cristae difficult (Fig. 5A). The mitochondria of TA-33BB-CMS(G) were round and 300-500 nm in diameter (Fig. 5B). The electron density of their interiors was low. The cristae were straight (Fig. 5B) or obscure. The mitochondria of TA-33BB-CMS(Owen) were round or oval and measured 300-400 nm (Fig. 5C). Both high- and low electron-dense mitochondria were observed; the tapetal cells tended to have either type of mitochondria. An example of a low electron-dense mitochondrion is shown in Fig. 5C. The cristae were straight and occasionally circular (Fig. 5C).

In the late tetrad stage, oval mitochondria with dimensions of 350-500 nm were seen in TA-33BB-O, and their boundary membranes were slightly deformed (Fig. 5D). The electron density of their interiors was generally high (Fig. 5D); mitochondria of low density with straight

cristae were rarely found. TA-33BB-CMS(G) had mitochondria ranging in size from 200-500 nm with slightly deformed boundary membranes (Fig. 5E). The electron density seemed to be greater than that of the early tetrad stage (Fig. 5E). The cristae were straight and short, curved, or circular (Fig. 5E). The mitochondria of TA-33BB-CMS(Owen) were 250-500 nm in size. They were round or oval with occasionally deformed boundary membranes (Fig. 5F). The electron density of their interiors was either high or low, the latter of which accompanied multilayered, concentric cristae (Fig. 5F). We note that such cristae have been described as 'onion-like' in some published reports dealing with dysfunctional mitochondria (*e.g.*, Klecker and Westermann 2021).

During the early microspore stage, the round mitochondria of TA-33BB-O were 300-400 nm in diameter (Fig. 5G). The electron density of their interiors was high, but some of the mitochondria had patches of low electron density (Fig. 5G). Cristae were straight or hardly visible. In TA-33BB-CMS(G), mitochondria were round or oval and 250-300 nm in size with deformed boundary membranes (Fig. 5H). Their electron density was comparable to previous developmental stages or lower (Fig. 5H). Circular and onion-like cristae were seen (Fig. 5H). In TA-33BB-CMS(Owen), mitochondria were round but generally smaller than those of the other two lines (100-250 nm). Occasionally, elongated mitochondria of 450 nm were seen (Fig. 5I). Their electron density was low. Onion-like and circular cristae were also present (Fig. 5I).

UBISCH BODIES

In the early tetrad stage, aggregates of 100 nm, electron-dense globular objects were seen in the tapetal cell walls of TA-33BB-O (Fig. 6A). The structures were pro-Ubisch bodies, the precursors of Ubisch bodies. In the anther walls of TA-33BB-CMS(G), pro-Ubisch bodies containing small amounts of lipids were present (Fig. 6B). In TA-33BB-CMS(Owen), no pro-Ubisch bodies were present, but electron-dense objects, likely lipids, of 300-400 nm were seen (Fig. 6C).

In the late tetrad stage, pro-Ubisch bodies were seen as 250- to 200-nm globular objects composed of highly electron-dense materials in TA-33BB-O (Fig. 6D). The structures were arrayed on the surface of tapetal cells (Fig. 6D). In TA-33BB-CMS(G), pro-Ubisch bodies of 100 nm were evident (Fig. 6E). In addition, highly electron-dense unidentified objects accumulated

on the surface of tapetum tissues (Fig. 6E). No pro-Ubisch bodies were seen in TA-33BB-CMS(Owen) at this stage, but electron-dense material had accumulated (Fig. 6F).

In the early microspore stage, tapetal cell walls had degenerated, and Ubisch bodies were arrayed on the surface of tapetal cells in TA-33BB-O (Fig. 6G) and TA-33BB-CMS(G) (Fig. 6H). In TA-33BB-CMS(Owen), the tapetum was in close contact with the microspores, but no Ubisch bodies were present (Fig. 6I).

Root apical meristems of TA-33BB-O, TA-33BB-CMS(G), and TA-33BB-CMS(Owen) are morphologically indistinguishable

According to the hypothesis that assumes that ATP depletion is the cause of CMS, mitochondria in cells with a high energy demand would also exhibit morphological abnormalities in CMS plants. The root apical meristem is known to be a highly respiring tissue (e.g., Gong et al. 2019), so we investigated root apical meristem mitochondria.

Vertical sections of roots sampled from TA-33BB-O, TA-33BB-CMS(G), and TA-33BB-CMS(Owen) are shown in Figs. 7A, 7B, and 7C. Root caps, epidermis, cortexes, and steles were identified: no differences were seen among the three lines. The root apical meristem was identified as an internal zone consisting of small cells about 250 μm distal from the root tip. In the images, we drew a horizontal line passing through the zone, counted the number of cells on the line, and found that 26-29 cells were present on the line. We next made transverse sections of the roots (Figs. 7D, 7E, and 7F) and counted the number of cells in the section. The diameter of the root transverse section should contain 26-29 cells if the section encompasses the zone. The number of cells on the diameter axis of our sections was 27-29, indicating that the section contained the root apical meristem. We could identify steles, cortexes, and the epidermis and saw no difference between the three CMS lines (Figs. 7D, 7E, and 7F).

Cells typical of the root apical meristem are shown in Figs. 7G, 7H, and 7I. The electron density of the cytoplasm was relatively high; each cell had a prominent nucleus, small vacuoles, and plastids of various shapes. Very few endoplasmic reticula were observed. These features were shared among TA-33BB-O, TA-33BB-CMS(G), and TA-33BB-CMS(Owen), and

no differences were observed. Many mitochondria were present in all three lines. The organelles were oval or round, with sizes ranging from 300-600 nm (Figs. 7J, 7K, and 7L), and the electron density of the interior was generally high. Many straight, relatively short cristae were observed. Some but not all the cristae in TA-33BB-CMS(G) and TA-33BB-CMS(Owen) appeared to be slightly wider than those of TA-33BB-O.

Discussion

Wild beets with G cytoplasm inhabit the Atlantic coast from France to Morocco (Meyer et al. 2018), suggesting that the fitness penalty rendered by the G cytoplasm is limited. As repeated backcrossing was successful, G cytoplasm does not affect female reproductive organs. Therefore, G cytoplasm belongs to the typical CMS class even though its mitochondrial genome has several nonsynonymous substitutions, one of which truncates *cox2* (Ducos et al. 2001).

Although G- and Owen cytoplasm are genetically distinct, their phenotypes are generally similar; for example, the morphology of their anthers cannot be distinguished (Fig. 1). Anther developmental processes of the two CMS lines are also similar (Arakawa et al. 2019 and this study). Ultrastructural comparison of their anthers indicates that the first obvious abnormality in tapetal mitochondria occurs in the early tetrad stages. Mitochondrial morphology contrasts between the meiosis and the tetrad stages in both G- and Owen CMS. Therefore, the *S-orf* of the G cytoplasm (likely the variant *cox1* with an NH₂-terminal extension; Meyer et al. (2018)) and the *S-orf* of the Owen cytoplasm (*preSatp6*) may simultaneously induce male sterility. During anther development, the demand for mitochondrial activity is apparently high in beet as N mitochondria are electron-dense, an indication of activated mitochondria (Scheffler 1999) (Figs. 3 and 5). The abnormality in G- and Owen mitochondria is characterized by low electron density, a decreased number of cristae, and deformed cristae such as curved, circular, or onion-like structures. Such abnormal mitochondria with malformed cristae are likely functionally defective as there are close relationships between cristae morphology and mitochondrial function (Klecker and Westermann 2021). Because expression of the *S-orfs* is constitutive (Yamamoto et al. 2005; Meyer et al. 2018), the *S-orf* alone is insufficient to cause a mitochondrial abnormality, given that

mitochondria in the meiotic anthers of the two CMS lines were apparently normal. Therefore, a factor affecting mitochondrial function and cristae morphology of S mitochondria but not N mitochondria should occur after meiosis. Whether the factor is shared between G- and Owen CMS lines or each CMS line has its own factor should be investigated in the future. Another possibility is that the requirement of mitochondrial activity at the tetrad stage is higher than at the meiosis stage, and that *S-orfs* prevent mitochondrial activity to increase and meet the energy needs. In this case the hypothetical factor mentioned above is unnecessary.

We examined young roots in this study because roots are known to be highly respiring organs (Balk and Leaver 2001; Gong et al. 2019). We showed that the mitochondria in the root apical meristem are highly electron dense and have well-developed cristae (Fig. 7), as seen in activated mitochondria (Klecker and Westermann 2021). Nevertheless, no obvious abnormality was seen in the mitochondria of young roots of plants with the G- or Owen type of CMS, indicating that their S mitochondria can support tissue development requiring high mitochondrial activity, although the requirement for mitochondrial activity may be much higher in the tapetum than in the roots. Unlike the root apical meristem, however, the tapetum undergoes PCD in which tapetal mitochondria are closely associated (Balk and Leaver 2001). Hence, the functions of tapetal mitochondria are more complicated than those of the root apical meristem. Tapetal PCD involves the release of cytochrome *c* from mitochondria to the cytoplasm, as is the case for animal PCD (Balk and Leaver 2001). Although it is unknown how cytochrome *c* is released in the tapetum, the release occurs between the meiosis- and the tetrad stages in sunflower (Balk and Leaver 2001); hence, a developmental cue to release cytochrome *c* may be provided at this point. At the same time, the mitochondria need to maintain their activity, perhaps to meet the demands for pollen development, as seen in the mitochondria of TA-33BB-O (Figs. 5A, 5D, and 5G). Therefore, the mechanism for cytochrome *c* release in plants may be more complicated than in animals. Provided that the developmental cue to release cytochrome *c* for tapetal PCD evokes an improper response by S mitochondria, the cue satisfies the characteristics of a factor triggering the mitochondrial abnormality mentioned above. This hypothesis is worth additional investigation to determine why abnormal mitochondria occur simultaneously in two different CMS lines.

We found ultrastructural differences between TA-33B-CMS(G) and TA-33BB-CMS(Owen) anthers. Because the two lines have a nearly identical nuclear background, the difference can be attributed to their mitochondria. The most striking difference was the absence of Ubisch bodies in the Owen CMS lines versus the presence of Ubisch bodies in the G CMS lines. The lack of Ubisch bodies in an Owen CMS line was reported previously (Majewska-Sawka et al. 1993). Ubisch bodies are carriers of sporopollenin, the major component of the pollen cell wall (Ariizumi and Toriyama 2011). Failure in sporopollenin synthesis is known to lead to male sterility (Ariizumi and Toriyama 2011). Therefore, such a failure seems to be associated with the Owen-type CMS plants. Failed sporopollenin production may also account for the poorly developed bacula and tecta in Owen cytoplasm plants. Whereas Ubisch bodies, baculum, and tectum are present in G cytoplasm plants, the microspores were deformed, and the thickness of the pollen cell wall was uneven (Fig. 4). Such a phenotype might be interpreted as a defect in the scaffold for sporopollenin, *i.e.*, primexine, a polymer primarily composed of cellulose and other polysaccharides (Ariizumi and Toriyama 2011; Radja 2020). Further analyses will be necessary to identify which of the pollen production processes is impaired in G cytoplasm expression.

Conclusion

Sugar beet lines with Owen type and G type mitochondria display similar phenotypes. In general, the two CMS lines show a small number of highly electron-dense particles in tapetal cells during the meiosis stage, tapetum vacuolation and hypertrophy at the early microspore stage, and deformed flattened anther locules with cellular residues from tapetal cells in later stages. Mitochondrial abnormalities, such as low electron dense interior and onion-like cristae, are seen during the early tetrad- and later stages in both CMS lines. On the other hand, some observations are specific to either of the two types of CMS. For example, a lack of pro-Ubisch body is observed only in the Owen type CMS. These observations lead us to consider CMS expression as the result of two different processes; one, common to the two CMS, that induces mitochondrial abnormality and one, unique to either of the two CMS, that causes pollen sterility. However, small differences in the mitochondrial abnormalities observed between the tapetal cells of the two CMS lines may

contradict this notion. In both CMS lines, root apical meristems are apparently normal, and the mitochondria are indistinguishable from those of the normal fertile line, suggesting that the CMS mitochondria have the ability to support the development of this high energy requiring tissue. We raise the fundamental question whether these CMS mitochondria are functionally impaired, but it is possible that the energy demand in the tapetum exceeds those in the root apical meristem, and CMS mitochondria can meet the energy requirement of the root apical meristem but fall short for the tapetum.

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Conflict of interest

No conflict of interest is declared.

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Data availability

All data supporting the findings of this study are available within the paper.

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573

574

Figure legends

Fig. 1. Flowers of sugar beets with different cytoplasms. **A, B** Flowers of plants with G- and Owen cytoplasms, respectively. Scale bars of 1 mm are shown. **C, D** Anther contents sampled from sugar beets with G- and Owen cytoplasms, respectively, were stained with Alexander's dye. Scale bars of 20 μm are shown.

Fig. 2. Transverse sections of sugar beet anthers with G cytoplasm. Abbreviations are: en, endothecium; msp, microspore; pmc, pollen mother cell; st, stomium; td, tetrad; and tp, tapetum. Scale bars are 50 μm . **A** Meiosis stage. **B** Early tetrad stage. **C** Late tetrad stage. **D, E, F** Microspore stage. **G, H, I** Later stages.

Fig. 3. Ultrastructure of sugar beet anthers during meiosis stages. The sugar beets differ in their cytoplasms, N- (**A, D, G, J, and M**), G- (**B, E, H, K, and N**), and Owen (**C, F, I, L, and O**). Abbreviations are; e, endoplasmic reticulum; n, nucleus; p, plastid; and v, vacuole. **A, B, C** Pollen mother cells. Scale bars are 2 μm . **D, E, F** Tapetum cells. Scale bars are 2 μm . Arrows indicate lipids. **G, H, I** Mitochondria. Scale bars are 300 nm. **J, K, L** Plastids. Arrows indicate starch granules. Scale bars are 1 μm .

Fig. 4. Ultrastructure of microspores and pollen cell walls of sugar beets with different cytoplasms. Those with N- (**A, D, G, J, M, and P**), G- (**B, E, H, K, N, and Q**), and Owen cytoplasms (**C, F, I, L, O, and R**) are shown. Abbreviations are: ca, callose; el, low electron-dense layer; ne, nexine; pb, probacula; pm, plasma membrane; te, tectum; and tp, tapetum. **A, B, C** Microspores at the early tetrad stage. Scale bars are 20 μm . **D, E, F** Pollen walls at the early tetrad stage. Scale bars are 500 nm. **G, H, I** Microspores at the late tetrad stages. Scale bars are 2 μm . **J, K, L** Pollen walls at the late tetrad stage. Scale bars are 500 nm. **M, N, O** Microspores at the microspore stage. Scale bars are 20 μm . **P, Q, R** Pollen walls at the microspore stage. Scale bars are 500 nm.

Fig. 5. Ultrastructure of mitochondria in the anther tapetum cells of sugar beet with different cytoplasms. Sugar beet with N- (**A, D, and G**), G- (**B, E, and H**), and Owen cytoplasms (**C, F, and I**) are shown. Scale bars are 100 nm. **A, B, C** Early tetrad stage. **D, E, F** Late tetrad stage. **G, H, I** Early microspore stage.

Fig. 6. Ultrastructure of tapetum-microspore interfaces in sugar beet anthers with different cytoplasms. Sugar beet with N- (**A, D, and G**), G- (**B, E, and H**), and Owen cytoplasms (**C, F, and I**) are shown. Abbreviations are; ca, callose; msp, microspore; pu, pro-Ubisch body; tp, tapetum; tw, tapetal cell wall; and u, Ubisch body. Scale bars are 1 μ m. **A, B, C** Early tetrad stage. **D, E, F** Late tetrad stage. **G, H, I** Microspore stage.

Fig. 7. Roots of sugar beets with different cytoplasms. Sugar beets with N- (**A, D, G, and J**), G- (**B, E, H, and K**), and Owen cytoplasms (**C, F, I, and L**) are shown. Abbreviations are: co, cortex; ep, epidermis; n, nucleus; p, plastid; rc, root cap; stl, stele; and v, vacuole. **A, B, C** Vertical sections of young roots. The root apical meristems are enclosed by dotted lines. Scale bars are 50 μ m. **D, E, F** Transverse sections of young roots. Scale bars are 50 μ m. **G, H, I** Cells in the root apical meristems. Scale bars are 2 μ m. **J, K, L** Mitochondria in the root apical meristems. Scale bars are 300 nm.













