Title:

Selection of nuclear genotypes associated with the thermo-sensitivity of Owen-type cytoplasmic male sterility in sugar beet (*Beta vulgaris* L.)

Authors:

Hiroaki Matsuhira [1], Kazuyoshi Kitazaki [2], Katsunori Matsui [2], Keisi Kubota [2], Yosuke Kuroda [1], Tomohiko Kubo [2]

Affiliations:

[1] Hokkaido Agricultural Research Center, National Agriculture and Food Research Organization, Memuro, Hokkaido, Japan

[2] Research Faculty of Agriculture, Hokkaido University, Sapporo, Hokkaido, Japan

Correspondence to: Hiroaki Matsuhira

(ORCID 0000-0002-5940-5964)

ma2hira@affrc.go.jp

or

Kazuyoshi Kitazaki

(ORCID 0000-0003-3012-0604)

kitazaki@abs.agr.hokudai.ac.jp

* Hiroaki Matsuhira and Kazuyoshi Kitazaki contributed equally to this study.

Abstract

The stability of cytoplasmic male sterility expression in several genetic backgrounds was investigated in sugar beet (*Beta vulgaris* L.). Nine genetically heterogenous plants from open-pollinated varieties were crossed with a cytoplasmic male sterile line to obtain 266 F_1 plants. Based on marker analysis using a multiallelic DNA marker linked to *restorer-of-fertility 1* (*Rf1*), we divided the F_1 plants into 15 genotypes. We evaluated the phenotypes of the F_1 plants under two environmental conditions: greenhouse rooms with or without daytime heating during the flowering season. Three phenotypic groups appeared: those consistently expressing male sterility, those consistently having restored pollen fertility, and those expressing male sterility in a thermo-sensitive manner. All plants in the consistently male sterile group inherited a specific *Rf1* marker type named p4. We tested the potential for thermo-sensitive male sterile plants to serve as seed parents for hybrid seed production, and three genotypes were selected. Open pollination by a pollen parental line with a dominant trait of red-pigmented hypocotyls and leaf veins resulted in seed setting on thermo-sensitive male sterile plants, indicating that their female organs were functional. More than 99.9% of the progeny expressed the red pigmentation trait; hence, highly pure hybrids were obtained. We determined the nucleotide sequences of *Rf1* from the three genotypes: one had a novel allele and two had known alleles, of which one was reported to have been selected previously as a nonrestoring allele at a single US breeding station but not at other stations in the U.S., or in Europe or Japan, suggesting environmental sensitivity.

Keywords

Cytoplasmic male sterility, environmental sensitivity, hybrid breeding, *restorer of fertility*, sugar beet

Key message

Cytoplasmic male sterility in sugar beet becomes thermo-sensitive when combined with specific genotypes, potentially offering a means to environmentally control pollination by this trait.

Introduction

Hybrid seed production of some crops relies on heritable male sterility because it converts a hermaphroditic plant into a seed parent that never self-pollinates (Budar et al. 2006). On the other hand, using male sterile plants for hybrid seed production at a commercial scale is costly because the system is complicated, in part, due to problems with how seed parents are propagated. Male sterility can be caused by a nuclear gene (genic male sterility) or by the combination of nuclear and mitochondrial genes (gene-

58 cytoplasmic male sterility or cytoplasmic male sterility, CMS) (Budar et al. 2006). In genic male sterility,
59 the gene responsible for male sterility cannot be fixed, so that male sterile plants should be selected from
60 a population in which male fertile plants co-occur. Practically, the male fertile plants should be removed
61 from the field before the flowers open (Budar et al. 2006). In the case of CMS, cytoplasmic segregation
62 does not occur; however, to propagate the seed parent, pollen must be obtained from a maintainer line
63 that has the identical genotype as the CMS line but be male fertile due to a non-sterility-inducing
64 cytoplasm (Budar et al. 2006). In both cases, such complexity can be reduced if the gene or cytoplasm
65 expresses male sterility in response to an environmental cue such as temperature or photoperiod but
66 otherwise permits pollen production (Chen and Liu 2014). Environmentally inducible male sterility has
67 been reported in rice, wheat, rapeseed, and Chinese cabbage; only the rice and wheat male sterile plants
68 have been used for hybrid seed production (Li et al. 2007; Murai et al. 2008; Sha et al. 2019). The use of
69 environmentally inducible male sterility for the breeding of other crops is rare at present.
70 All commercial varieties of sugar beet (*Beta vulgaris* L.) are hybrids using CMS (McGrath
71 and Panella 2019). Since the discovery of CMS by Owen (1945), breeders have selected maintainer lines
72 with the aim to achieve stable male sterility expression (Kaul 1988; Bosemark 2006). A prerequisite to
73 the maintainer genotype is that two loci, *X* and *Z*, are homozygous recessive (Owen 1945). Whereas little
74 is known about *Z* (Honma et al. 2014), *X* was identified as *restorer-of-fertility 1* (*Rf1*), and its nucleotide
75 sequence was determined (Matsuhira et al. 2012). *Rf1* is a complex locus consisting of a gene cluster
76 whose constituents have sequence similarity with *Oma1*, a gene involved in mitochondrial quality control
77 (Arakawa et al. 2020b; Migdal et al. 2017). The clustered gene copies (hereafter, each copy is referred to
78 as *Rf1-Oma1*) were divided into two classes based on a single property of their encoded protein; namely,
79 those with or without the ability to bind to a CMS-associated sugar beet mitochondrial protein (Arakawa
80 et al. 2020b). This binding activity is crucial for altering the higher-order structure of the CMS-associated
81 mitochondrial protein to restore male fertility (Kitazaki et al. 2015).
82 The *Rf1* locus is highly polymorphic in the number of clustered *Rf1-Oma1* copies and their
83 nucleotide sequences (Arakawa et al. 2020a). The molecular diversity of the *Rf1* locus is associated with
84 the variety of its alleles: dominant, semi-dominant, hypomorphic and recessive alleles with very different
85 types of molecular organization are known. Thus, *Rf1* is considered a multiallelic locus (Arakawa et al.
86 2018, 2019); however, the entire suite of *Rf1* alleles in the sugar beet gene pool is unknown.
87 In our previous study, we genetically analyzed maintainer lines that were developed outside
88 of Japan (Ohgami et al. 2016). We crossed these lines with a CMS line to obtain F_1 plants that should
89 express male sterility if the pollen parent was a maintainer (this experimental procedure is called a test
90 cross). We found a major recessive *rf1* allele with an identical nucleotide sequence. This *rf1* allele

91 predominates among many maintainer lines used by breeders in the U.S., Europe, and Japan (Moritani et
92 al. 2013; Ohgami et al. 2016). In addition, we identified several minor *rf1* alleles that could produce the
93 male sterile phenotype but varied in their molecular organization. These minor *rf1* alleles were favored by
94 a specific breeding station (Ohgami et al. 2016). We also observed that some lines recorded as
95 'maintainers' in a gene bank gave rise to fertility-restored F_1 plants in our experimental growing
96 conditions (Ohgami et al. 2016). The gene organization of *Rf1* alleles of these apparently non-maintainer
97 lines was different from the genetically proven recessive *rf1* alleles at the DNA level (Ohgami et al.
98 2016). We wondered why the molecular organization of *rf1* exhibited differences among the breeding
99 stations and why a maintainer in one breeding station was a non-maintainer in another. *Rf1* alleles with as
100 yet unknown functions possibly exist in the sugar beet gene pool. Theurer and Ryser (1969) suggested
101 that the male fertility of beets with Owen cytoplasm was sensitive to such environmental conditions as
102 moisture stress and temperature. We also observed that the minor *rf1* allele identified in Ohgami et al.
103 (2016) was not selected in another of our studies examining Japanese sugar beets for marker-assisted
104 maintainer selection (Moritani et al. 2013). Our two previous studies differed in how male fertility was
105 evaluated, e.g., the F_1 plants were grown in different fields and seasons. We hypothesized that the
106 environmental sensitivity of Owen CMS expression was influenced by genotype and this cytoplasm-
107 nuclear interaction affected maintainer selection. To support this hypothesis, we needed to show the
108 environmental sensitivity of this CMS expression in various genotypes. Here, we present our data
109 investigating whether environmental conditions during maintainer selection affect the selected *Rf1* allele.
110 As a model experiment for environmental sensitivity, we exposed F_1 plants to normal and high
111 temperatures during flowering. The resultant phenotypes were classified into three categories:
112 consistently male fertile, consistently male sterile, and male fertile at normal temperature but male sterile
113 at high temperature. Our results indicated that sugar beet CMS becomes environmentally sensitive in
114 specific genotypes.
115
116
117 **Materials and methods**
118 *Plant materials*
119 Sugar beet (*Beta vulgaris* L.) open-pollinated varieties were originally developed in Poland, Germany and
120 Sweden and were introduced into Japan by the Japan Sugar Beet Improvement Foundation (JSBIF)
121 (Taguchi et al. 2014). These varieties were preserved and maintained by the Foundation's successor, the
122 Hokkaido Agricultural Research Center, National Agriculture and Food Research Organization (HARC-
123 NARO), Japan, under accession numbers TA-30 (Poland), TA-36 (Germany) and TA-37 (Sweden). Sugar

124 beet line NK-305 was a genetically heterogeneous selection of unknown origin (Arakawa et al. 2019).
125 TA-33BB-CMS is a cytoplasmic male sterile line with Owen cytoplasm, and TA-33BB-O is its
126 maintainer (i.e., the nuclear genotype is the same but with a non-sterility-inducing cytoplasm) (Arakawa
127 et al. 2018). These three lines were developed by the HARC-NARO, Japan. TA-8 is a US inbred line with
128 a non-sterility-inducing cytoplasm and has deep red pigmentation in the hypocotyls and leaf veins. TA-8
129 was introduced from the Great Western Sugar Company by the JSBIF in the 1960s and was maintained
130 by the HARC-NARO. To obtain F_1 seed, TA-33BB-CMS inflorescences and pollen parent inflorescences
131 were first enclosed in paper bags before anthesis. The paper bags were exchanged after anthesis, allowing
132 the pollen grains inside the bags to serve as the pollen donor source. Induced male sterile plants were
133 open-pollinated with TA-8 in a closed room of a greenhouse.
134
135 *Growth conditions*
136 Seeds were sown in paper pots filled with field soil supplemented with chemical fertilizer 'Beet Ikubyou-
137 1' (total nitrogen, 2%; available phosphate (P_2O_5), 18%; soluble potash (K_2O), 1%; total magnesium
138 (MgO), 5%) (Hokuren, Sapporo, Japan) (3 kg/165 L) and soil conditioner 'Kudo-Tankaru' (lime with
139 magnesium) (Hokuren) (300 g/165 L). With occasional watering, seedlings were grown until four to five
140 leaves developed. Plantlets were transplanted to beds in a closed room of a greenhouse in early May in
141 Memuro, Hokkaido, Japan (Fig. 1a). This room was equipped with two kerosene heaters (each had a
142 maximum heating ability of 34890 J/s; MHF 0308 KF, Mitsubishi Heavy Industries, Tokyo, Japan) and
143 six fans to ensure air circulation (Fig. 1a); the room was heated from 10:00 to 16:00 daily, from late June
144 to early September. The maximum temperature was set to 35˚C by three air conditioners (each with a
145 maximum cooling ability of 26283.8 J/s; PA-J280DC-H, Mitsubishi Electric, Tokyo, Japan). Another
146 room was used for the control experiment with no supplemental heating. The temperature of this room
147 was kept in the range of 15 to 25˚C. Temperatures were recorded once every hour in each room by an
148 Ondotori RTR507B data logger (T&D Corporation, Matsumoto, Japan) (Fig. 2).
149
150 *Pollen fertility evaluation*
151 Pollen fertility phenotypes were visually inspected and classified into five categories (N, P, S, G, and W,
152 from fertile to completely sterile) according to the method reported by Moritani et al. (2013). Briefly, N is
153 fully fertile with well-rounded and yellow anthers that can dehisce; P has orange anthers and is less fertile
154 than N; S is semi-sterile and has dull yellow and partially shrunken anthers that cannot dehisce; G is
155 semi-sterile with light green and shrunken anthers; and W is completely sterile with white or brown
156 shrunken anthers. Phenotypes were evaluated twice during a three-week interval (see Fig. 2).

157

158 *Genotyping*

159 Total cellular DNAs were isolated according to the procedure of Doyle and Doyle (1990). Briefly, fresh
160 green leaves were powdered in liquid nitrogen and then poured into a buffer (2% (w/v)
161 hexadecyltrimethylammonium bromide, 1% (w/v) polyvinylpyrrolidone, 100 mM Tris-HCl (pH 8.0), 20
162 mM EDTA, 1.4 M NaCl, and 0.2 mg/ml of RNase A (Nacalai Tesque, Kyoto, Japan)). After incubation
163 (60˚C for 60 min), the mixture was extracted once with an equal volume of chloroform-isoamyl alcohol
164 (24:1 (v/v)). The aqueous phase was collected after centrifugation (15000 x *g* at 20˚C for 5 min), and total
165 cellular DNA was precipitated by adding 2-propanol. DNA markers s17 and 20L-int are cleaved
166 amplified sequence polymorphic- and DNA fragment length polymorphic markers, respectively (Taguchi
167 et al. 2014). GoTaq Green Master Mix (Promega, Madison, WI, U.S.A.) was used according to the
168 manufacturer's instructions. Amplification products were produced on a GeneAmp PCR system 9700
169 thermal cycler (Applied Biosystems, Waltham, MA, U.S.A.) (35 cycles of 94˚C for 30 sec, 60˚C for 30
170 sec and 72˚C for 1 min). DNA fragments were electrophoresed in 2% or 2.5% agarose gels.

171

172 *Nucleotide sequence analysis*

173 *Rf1-Oma1* copies were PCR amplified with primers 5'-
174 CGGTACCCGGGGATCATGGCGTGGTACAGAAATTCAAGGTTTG-3' and 5'-
175 CGACTCTAGAGGATCTTACTTACTGAAGACCTTGAATTGCACGTCC-3' using KOD FX
176 polymerase (Toyobo, Osaka, Japan). PCR products were purified using a QIAquick Gel Extraction Kit
177 (Qiagen, Venlo, The Netherland) and were cloned into plasmid vector pUC19 (In-Fusion HD Cloning
178 Kit; Takara Bio, Kusatsu, Japan). Colonies with the objective DNA fragment were selected by colony
179 PCR and cultured in LB medium (Sambrook et al. 1989). Plasmid DNAs were isolated using a QIAprep
180 Spin Miniprep Kit (Qiagen). Sequencing was accomplished with the primer walking technique using a
181 BigDye Terminator v3.1 Cycle Sequencing Kit and an ABI 3130 Genetic Analyzer (Applied Biosystems,
182 Foster City, CA, USA). Nucleotide sequences were assembled by Sequencher software (Gene Codes,
183 Ann Arbor, MI, USA). Sequences were compared at a website implementing ClustalW
184 (https://clustalw.ddbj.nig.ac.jp/). Nucleotide sequences determined in this study were deposited in
185 DDBJ/EMBL/GenBank under the accession numbers of LC628460, LC628461, LC628462 and
186 LC628463.

187

188

189

190 **Results**

191 *Pollen parent selection from open-pollinated varieties*

192 Pollen parents were selected from three open-pollinated varieties that are genetically heterogeneous. We
193 selected pollen parental genotypes that contain a variety of *Rf1* alleles. The DNA marker s17 that is
194 closely linked to *Rf1*(Taguchi et al. 2014) was used. DNA band patterns of s17 have been linked to the
195 *Rf1* alleles (Arakawa et al. 2018, 2019, 2020b). A total of 169 plants were genotyped using s17 (Table 1),
196 and we observed that these plants had band patterns 1 (hereafter abbreviated as p1), p3, p4, and p5
197 (typical patterns are shown in Fig. S1). We selected seven plants composed of the four s17 band patterns
198 (Table 2). Since p2 was missing from our selections, we added two plants from NK-305, a sugar beet line
199 previously shown to contain p2 (Arakawa et al. 2019) (Table 2). The nine plants were crossed with the
200 CMS plants of TA-33BB-CMS, whose s17 marker type was homozygous at p4 (hereafter p4p4) to obtain
201 F_1 seeds. These F_1 selections were grouped into 15 genotypes according to their parentage and s17 band
202 patterns (Table 2 and see Fig S1).

203

204 *Fertility restoration without supplemental heating*

205 The F_1 plantlets were divided into two groups to grow in two different environmental conditions. One
206 group was planted in a greenhouse room that had no supplemental heat. The pollen fertility of the F_1
207 plants is summarized in Table 2. Out of the 92 plants, eleven were completely male sterile (phenotype
208 class W). The s17 marker type of all eleven plants was p4p4; considering that the s17 marker type of the
209 seed parent TA-33BB-CMS is p4p4, all eleven plants inherited the p4 allele from their pollen parent.

210 The remaining 81 plants had restored pollen fertility (Table 2). Their phenotypes were either
211 N (nine plants) or P (71 plants), both of which are phenotype classes capable of shedding pollen grains.
212 According to their s17 marker types, these fertility-restored plants inherited p1, p2, p3 or p5 from their
213 pollen parent (Table 2).

214

215 *Fertility restoration with supplemental heating*

216 The other F_1 group was planted in a greenhouse room with a heater that operated only during the daytime
217 as supplemental heating. The heating protocol was started just after stalk elongation and ceased at the end
218 of flowering (Fig. 2 and see the Materials and methods section). In the same greenhouse room, plants of
219 TA-33BB-CMS and its maintainer line TA-33BB-O were grown as controls; the phenotypes of twelve
220 TA-33BB-CMS were W, whereas those of TA-33BB-O were N (four plants) and P (seven plants).

221 Of these F_1 plants, the phenotypes of all p4p4 plants were W (Table 2). Unlike the treatments
222 receiving no supplemental heat, the other s17 marker types were associated with W. The phenotypes of

223 all six F_1 plants that inherited p3 from plant #14 of TA-30 (hereafter, this s17 allele is referred to as TA-
224 30-14-p3) were W, and the phenotypes of all twenty-four F_1 plants that inherited TA-37-19-p3 were W
225 (Table 2). W plants occasionally occurred in the F_1 population that had the TA-30-14-p1, NK-305-3-p5,
226 TA-36-1-p3 and TA-36-3-p5 alleles (Table 2). Some other F_1 plants with these four s17 alleles exhibited
227 an indeterminant phenotype in which a plant had flowers with different levels of pollen fertility (denoted
228 as an admixture in Table 2). For example, each of three plants with the TA-30-14-p1 allele had flowers of
229 P, S, and W phenotypes. The admixture phenotype was also observed in plants with other s17 alleles. We
230 identified the admixture phenotype in eight out of the fifteen F_1 populations (Table 2). Note that the
231 admixture phenotype was not present in plants receiving no supplemental heat (Table 2). On the other
232 hand, plants with the TA-30-32-p1 and TA-36-32-p3 alleles had restored pollen fertility when grown in
233 the heated room, as was also seen in plants not receiving supplemental heat, indicating that they were
234 phenotypically stable.
235
236 *Purity of hybrid seed from thermo-sensitive male sterile plants*
237 Because commercial seed production of sugar beet involves hybridization using male sterile F_1 plants as
238 the seed parent (i.e., a three-way cross) (Bosemark 2006), the purity of hybrid seed set on thermo-
239 sensitive F_1 plants under non-permissive conditions was our particular interest. We chose three F_1
240 genotypes with the TA-37-19-p3, TA-30-37-p5 and TA-36-3-p5 alleles as seed parents because these
241 genotypes produced flowers of the W phenotype at high frequency (Table 2). Seedlings with these alleles
242 were selected from F_1 populations by screening with the s17 marker and planted in the greenhouse room
243 with supplemental heat.
244 A total of 69 F_1 plants were grown under supplemental heating. Pollen fertility phenotypes
245 are summarized in Table 3. All 24 F_1 plants with the TA-37-19-p3 allele exhibited the W phenotype,
246 whereas F_1 plants with either the TA-30-37-p5 (22 plants) or TA-36-3-p5 (23 plants) alleles exhibited W
247 and an admixture in which S and G flowers occasionally occurred among the vast majority of W flowers
248 (Table 3). Note that anthers did not dehisce in the S and G flowers. Alongside these F_1 plants, we planted
249 TA-8 as a pollen parent (Fig. 1c); this sugar beet line has a non-sterility-inducing cytoplasm. TA-8 plants
250 shed pollen grains in the same greenhouse room, allowing for open pollination for three-way crosses (i.e.,
251 F_1 x TA-8). The flower phenotypes of the F_1 and TA-8 plants are shown in Fig. 3.
252 The F_1 plants with the TA-37-19-p3, TA-30-37-p5 and TA-36-3-p5 alleles set seeds that were
253 harvested to test whether they were hybrids with TA-8. Deep red pigmentation in the hypocotyls and leaf
254 veins was a unique characteristic of TA-8; this phenotype is governed by a dominant allele of a gene (*R*),
255 as is also documented in garden beet (Hatlestad et al. 2012). We sowed the seeds that set on the F_1 plants

256 to determine the ratio of seedlings with deep red pigmentation. The emerged seedlings exhibited deep red
257 pigmentation that was clearly distinguishable from sugar beet plants with pink hypocotyls and
258 unpigmented veins, phenotypes seen in some sugar beet varieties (Fig. 4). Of 1111 seedlings from the
259 three-way cross between F_1 (TA-33BB-CMS x TA-37-19) and TA-8, 1110 seedlings were intensely
260 pigmented (i.e., 1110/1111=0.999). All 371 seedlings of the three-way cross (TA-33BB-CMS x TA-30-
261 37) x TA-8 and 2316 seedlings of the three-way cross (TA-33BB-CMS x TA-36-3) x TA-8 were highly
262 pigmented (371/371=1.0, and 2316/2316=1.0, respectively).
263 We tested whether the F_1 plants with the TA-37-19-p3, TA-30-37-p5 and TA-36-3-p5 alleles
264 can produce functional pollen under permissive conditions. We grew the three F_1 populations without
265 supplemental heat. These plants flowered and shed apparently functional pollen grains (Fig. 3). The F_1
266 plants were self-pollinated to yield seeds. We sowed these seeds to inspect the hypocotyl- and leaf vein
267 color of the seedlings. None of the seedlings exhibited the deep red pigmentation (0/5, 0/75, and 0/29 of
268 the selfed TA-33BB-CMS x TA-37-19, TA-33BB-CMS x TA-30-37, and TA-33BB-CMS x TA-36-3,
269 respectively).
270
271
272 *Molecular identity of the Rf1 alleles in plants expressing thermo-sensitive male sterility*
273 We investigated whether the TA-37-19-p3, TA-30-37-p5 and TA-36-3-p5 alleles were identical to any of
274 the previously identified *Rf1* alleles and sought to determine the nucleotide sequences of *Rf1-Oma1*
275 copies in these *Rf1* alleles. *Rf1-Oma1* coding regions were PCR amplified from the three F_1 plants that
276 expressed thermo-sensitive male sterility. Because the PCR products were mixtures of the objective DNA
277 fragments and non-objective DNA fragments from the TA-33BB-CMS allele (see Table 3), the PCR
278 products were cloned into plasmid vectors to sort the DNA fragments. To eliminate clones with copies of
279 TA-33BB-CMS *Rf1-Oma1*, we focused on the length of the first intron; TA-30-37, TA-36-3 and TA-37-
280 19 had exclusively short introns, whereas TA-33BB-CMS had a long intron as shown using 20L-int, a
281 DNA marker targeting the intron (Fig. 5). Therefore, we selected and sequenced plasmids with short first
282 introns and compared the nucleotide sequences with those of known *Rf1* alleles.
283 *Rf1-Oma1* derived from TA-37-19-p3 was identical to that found from PI 615522, a U.S.
284 maintainer line (Fig. S2). The s17 marker type of PI 615522 was also reported as p3 (Ohgami et al. 2016).
285 As such, we concluded that the TA-37-19-p3 allele was the same as PI 615522 *rf1*.
286 The *Rf1-Oma1* nucleotide sequence of the TA-30-37-p5 allele was identical to that of the TA-
287 37-19-p3 allele (Fig. S2), but the s17 type of the TA-30-37-p5 allele differed from that of PI 615522 *rf1*.
288 Another U.S. maintainer line, PI 590689, had an *Rf1-Oma1* copy identical to PI 615522 *rf1*, but its s17

type was p5 (Ohgami et al. 2016). Therefore, the TA-30-37-p5 allele was likely the same as that of PI 590689 *rf1*.

We obtained two *Rf1-Oma1* sequences from the TA-36-3-p5 allele. This result was consistent with the band pattern of the 20L-int marker in which two short S bands appeared from TA-33BB-CMS x TA-36-3 (Fig. 5). No known *Rf1-Oma1* matched either of the obtained sequences.

Discussion

Sugar beet breeders have preferred stable male sterility in their quest to establish ideal seed parents, and maintainers have been selected to achieve stable male sterility. Unstable male sterility is problematic in breeding if it is uncontrollable, and such genotypes have been discarded in the past without further characterization. In our current study, we observed a novel phenotype of Owen-type CMS in which male sterility is expressed only when the plant experienced overheating, offering a novel means to control male sterility expression. This phenotype can also be interpreted as fertility restoration without supplemental heat but non-restoring with the heat. We propose the existence of two phenotypes for the Owen-type CMS: consistent sterility and thermo-sensitive sterility. These phenotypes were conditioned by the nuclear genotypes, indicating that nuclear-cytoplasmic interaction was involved in these phenotypes. The manifestation of nuclear-cytoplasmic interactions is known to fluctuate depending on environmental conditions, e.g., *Arabidopsis* nuclear substitution lines exhibit differential adaptation to different environmental conditions (Roux et al. 2016). Climate change is a general concern for many human activities, including agriculture (Horton et al. 2021). Our data suggest a potential impact of global warming on the reproductive traits of sugar beets with Owen cytoplasm, hence the influence of climate change on seed production will be a suitable topic for future research.

Rf1 is a principal genetic factor for Owen-type CMS expression, with several different alleles recently reported (Arakawa et al. 2020b). Our previous study identified a molecular variant linked to s17 marker type p4 as the most prevalent form in maintainer lines (Ohgami et al. 2016). Marker-assisted selection of p4 can be used for maintainer line selection (Moritani et al. 2013). The current study showed that plants with the p4 marker expressed male sterility irrespective of supplemental heat (Table 2). This result suggests that breeders have preferred p4 because its ability to condition male sterility is least affected by environmental conditions. This observation, in turn, implies that the other *Rf1* alleles are restoring alleles or unstable male sterility conditioners; however, because not all *Rf1* alleles have been genetically characterized yet, we may expect novel stable male sterility conditioning alleles to be found in the future.

322 We selected three thermo-sensitive male sterile genotypes whose *Rf1* loci were heterozygous
323 for p4 and either of the three *Rf1* alleles. TA-36-3-p5 is, as yet, an uncharacterized *Rf1* allele with two
324 novel *Rf1-Oma1* copies; further genetic and molecular characterization is necessary. The TA-37-19-p3
325 and TA-30-37-p5 alleles were likely PI 615522 *rf1* and PI 59068 *rf1*, respectively, whose *Rf1-Oma1*
326 coding regions were identical but s17 types differed from each other (Ohgami et al. 2016). Arakawa et al.
327 (2020b) investigated the molecular function of PI 615522 *rf1* and concluded that the ability to bind to the
328 CMS-associated sugar beet protein was missing. Therefore, we infer that other genes such as *Z* are
329 associated with thermo-sensitive male sterility. On the other hand, whereas the major *rf1* allele linked to
330 p4 was selected in many breeding stations of the world, including in the US, Europe and Japan, PI
331 615522 *rf1* was preferentially selected by a breeding station in California (Ohgami et al. 2016).
332 Therefore, we cannot exclude the possibility that PI 615522 *rf1* (and PI 59068 *rf1*) are not the maintainer
333 alleles but unstable male sterility conditioners that allow male sterility expression in an environment-
334 specific manner. Detailed genetic analysis with special care for environmental factors will be required.
335 Female fertility of our thermo-sensitive male sterile plants was normal in those receiving the
336 supplemental daytime heat treatment. The purity of hybrid seeds from induced male sterile plants was
337 over 99.9%, indicating that male sterile plants can be seed parents for hybrid seed production. Such a high
338 level of purity cannot be expected when seed parents shed functional pollen grains. Note that our F_1
339 plants can self-pollinate without supplemental heating. Our supplemental heating protocol increased the
340 daytime temperature only and did not alter the nighttime temperature. Therefore, we favor the notion that
341 daytime temperature is critical for male sterility induction. Temperature conditions similar to that in our
342 heated room can be seen in Japan, i.e., >35˚C during the daytime and 15-20˚C at night can occur during
343 the summer season. Investigations of male sterility expression at the field level should be conducted
344 where the environmental conditions are similar to those in this greenhouse study.
345 Many commercial sugar beet seeds are the products of three-way crosses in which male
346 sterile F_1 plants are crossed with a pollinator line (Bosemark 2006). In this breeding scheme, the F_1 plants
347 are derived from the cross between a CMS line and an unrelated maintainer line to ensure their male
348 sterile phenotype (Bosemark 2006). Because the nuclear genotype of a CMS line is equivalent to that of
349 the cognate maintainer line, developing various maintainer lines is a prerequisite to conducting a sugar
350 beet hybrid breeding program. To obtain the desired stability of male sterility, sugar beet breeding must
351 rely on the *rf1* allele linked to the p4, an infrequent occurrence in the sugar beet gene pool (Taguchi et al.
352 2014). The dependency of p4 in sugar beet hybrid breeding could be alleviated by introducing thermo-
353 sensitive male sterility because the male sterile phenotype can be expressed in the F_1 of a CMS x non-
354 maintainer genotype in high temperature. Moreover, it is interesting to consider whether thermo-sensitive

male sterile lines can be developed, as is the case for rice (Li et al. 2007). Combining Owen cytoplasm and an appropriate genotype, it may be possible to produce a line that is male sterile in high temperatures and male fertile in moderate temperatures. Such a line would be propagated by selfing at a moderate temperature but serve as a seed parental line at a high temperature, making a maintainer line unnecessary. A practical test of this possibility is ongoing.

Declarations

Funding

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Conflict of interest

The authors have no conflicts of interest to declare that are relevant to the content of this article.

Author contribution statement

H. Matsuhira, K. Kitazaki, and T. Kubo conceptualized and designed the study. H. Matsuhira, K. Kitazaki, K. Matsui, K. Kubota, and Y. Kuroda prepared materials and collected and analyzed the data. The draft was written by H. Matsuhira and K. Kitazaki and finalized by T. Kubo. All authors read and approved the final manuscript.

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445 Table 1 Number of plants with the DNA marker type in three open-pollinated varieties

Variety	s17 marker types								Total
	p1p1	p1p3	p1p5	p3p3	p3p4	p3p5	p4p5	p5p5	
TA-30	2	5	27	2		8	1	18	63
TA-36		2	1	1	7	15	13	26	65
TA-37		2	5	5		11	1	17	41
Total	2	9	33	8	7	34	15	61	169

446

447 Table 2 Pollen parental plants and the results of test crosses

Pollen Parent			F_1									
Variety	Plant #	s17 type	Group #	s17 type	Supplemental Heating	Pollen fertility phenotype [*1]						Total
						N	P	S	G	W	Admixtures[*2]	
TA-30	32	p1p4	1	p1p4	No		6					6
					Yes		5					5
			2	p4p4	No					5		5
					Yes					6		6
TA-30	14	p1p3	3	p3p4	No		6					6
					Yes					6		6
			4	p1p4	No		5					5
					Yes					3	3 (P, S and W)	6
TA-30	37	p1p5	5	p1p4	No	1	7					8
					Yes		17				1 (P, S, G, and W)	18
			6	p4p5	No		4					4
					Yes					6		6
NK-305	1	p2p2	7	p2p4	No	4	5					9
					Yes		23				1 (P, S, G, and W)	24
NK-305	3	p2p5	8	p2p4	No		5					5
					Yes		10				1 (P, S, G, and W)	12

											1 (S, G, and W)	
			9	p4p5	No		6					6
					Yes					3	9 (P, S, G, and W)	12
TA-37	19	p3p3	10	p3p4	No		7					7
					Yes					24		24
TA-36	32	p3p4	11	p3p4	No		5					5
					Yes		6					6
			12	p4p4	No					6		6
					Yes					6		6
TA-36	1	p3p5	13	p3p4	No		7					7
					Yes		3			1	7 (P, S, G, and W)	11
			14	p4p5	No	5						5
					Yes		12				1 (P, S, G, and W)	13
TA-36	3	p5p5	15	p4p5	No		8					8
					Yes					8	3 (P, S, G, and W) 1 (S, G, and W)	12

*1 Pollen fertility is higher in N, P, S, and G, in this order, and W is completely pollen sterile (for details, see the Materials and methods section)

*2 Pollen fertility phenotypes found in the admixtures are shown in parentheses.

450 Table 3 Phenotypes of F_1 plants used as seed parents in supplemental heat treatment

Cross combinations	Selected s17 type	Phenotype						Total
		N	P	S	G	W	Admixtures*	
TA-33BB-CMS x TA-37-19	p3p4					24		24
TA-33BB-CMS x TA-30-37	p4p5					14	8 (S, G and W)	22
TA-33BB-CMS x TA-36-3	p4p5					15	8 (S, G and W)	23

451 * Pollen fertility phenotypes found in the admixtures are shown in parentheses.

452

453

454 Figure legends

455 Fig. 1 Greenhouse room used in this study. **a.** Drawing and dimensions of the greenhouse room used in
456 this study. Positions of the kerosene heaters, air conditioners, and fans are shown by red-, blue-, and pale
457 blue boxes, respectively. Two doorways are indicated by arcs. **b.** An overview of the sugar beet growing
458 room. Red and blue boxes, respectively, indicate a kerosene heater and two air conditioners. Pale-blue
459 arrows indicate fans. **c.** F_1 plants as seed parents and TA-8 plants as pollen parents were planted in
460 alternate rows for the hybrid seed production experiment

461

462 Fig. 2 Temperature fluctuations in closed greenhouse rooms (see Fig. 1). Vertical and horizontal axes
463 depict the temperature in degrees Celsius and the date, respectively. The red line indicates the greenhouse
464 room temperature with supplemental heating, and the blue line indicates the greenhouse room
465 temperature without supplemental heating. Dates for transplanting, bolting started, two pollen fertility
466 evaluations, and seed harvest are indicated by black arrows

467

468 Fig. 3 Flowers of three F_1 populations grown with and without supplemental heat. DNA marker types
469 for the s17 marker are shown in parentheses. The plus and minus signs indicate with or without
470 supplemental heat, respectively. **a. and e.** Flowers of TA-33BB-CMS x TA-37-19. **b and f.** TA-33BB-
471 CMS x TA-30-37. **c and g.** TA-33BB-CMS x TA-36-3. **d.** TA-8

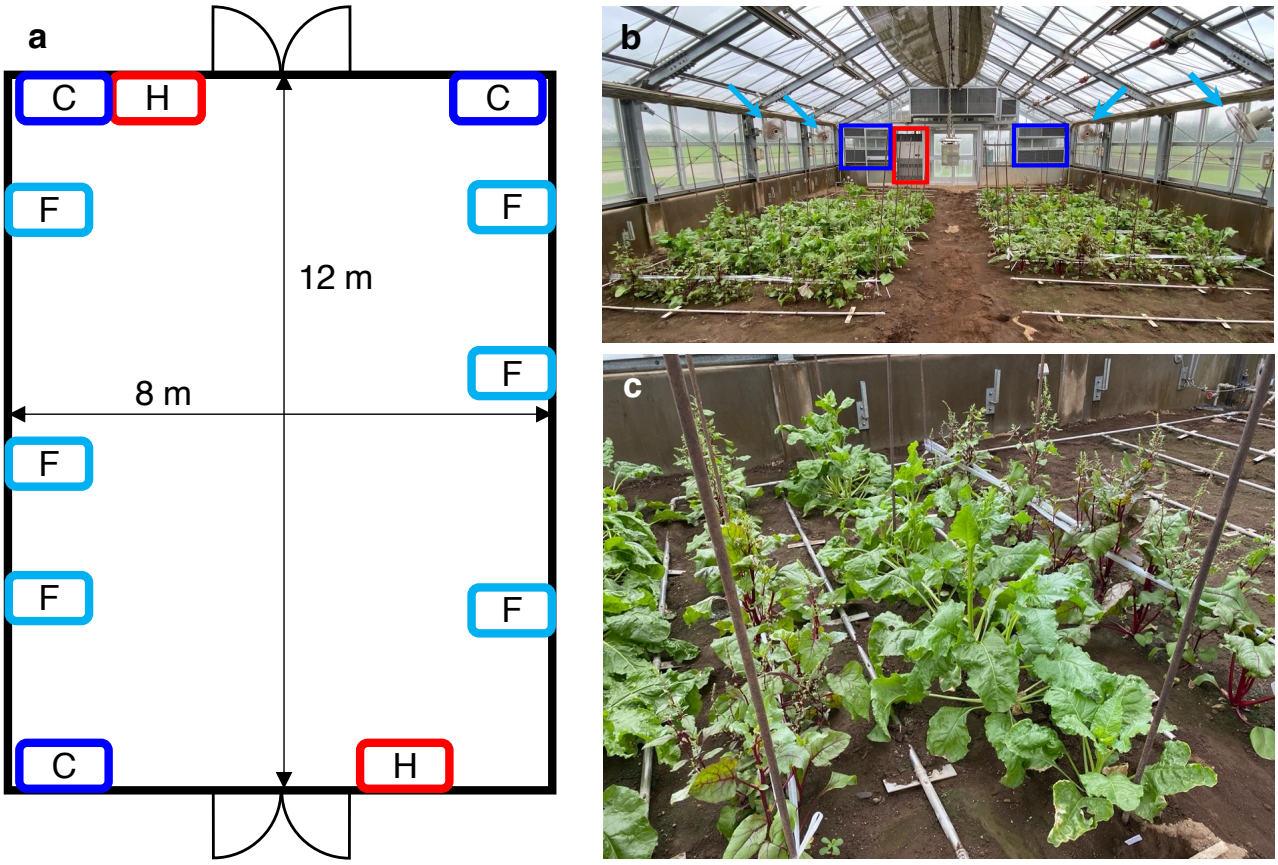
472

473 Fig. 4 Phenotypes of sugar beet seedlings. **a.** Seedlings from a three-way cross emerged from the seeds
474 set on thermo-sensitive male sterile F_1 plants (TA-33BB-CMS x TA-37-19). The pollen parent was TA-8.
475 **b.** Seedlings of a sugar beet cultivar that segregated for the pink hypocotyl phenotype (TA-30). **c.**
476 Seedlings of selfed F_1 (TA-33BB-CMS x TA-30-37)

477

478 Fig. 5 Gel electrophoresis of PCR fragments targeting the first intron of *Rf1-Oma1*. Plant IDs and cross
479 combinations are shown above. Size markers are shown on the left (bp). Positions of the short intron (S)
480 and long intron (L) are shown on the right

481


a
C
H
C
F
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12 m
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8 m
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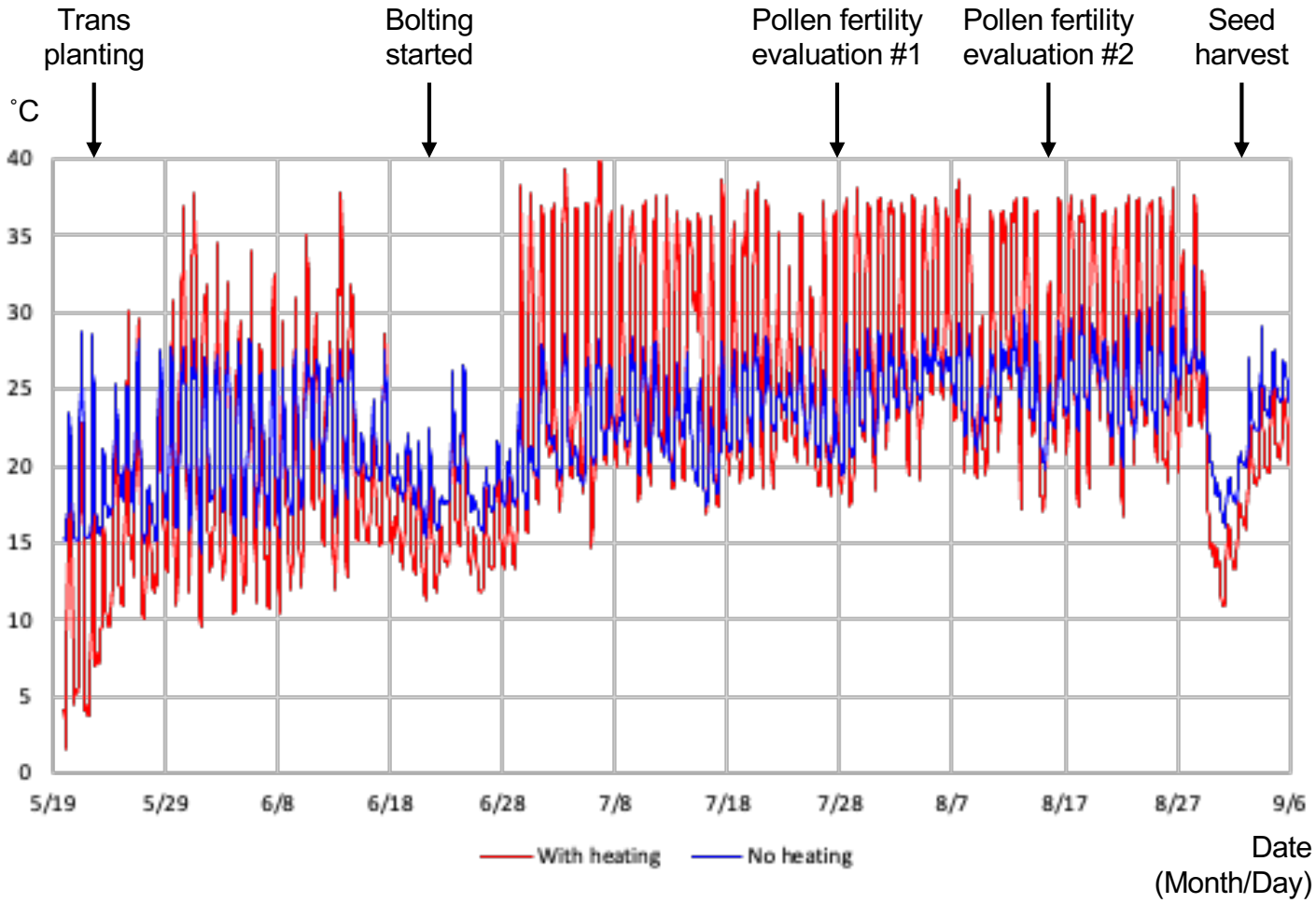
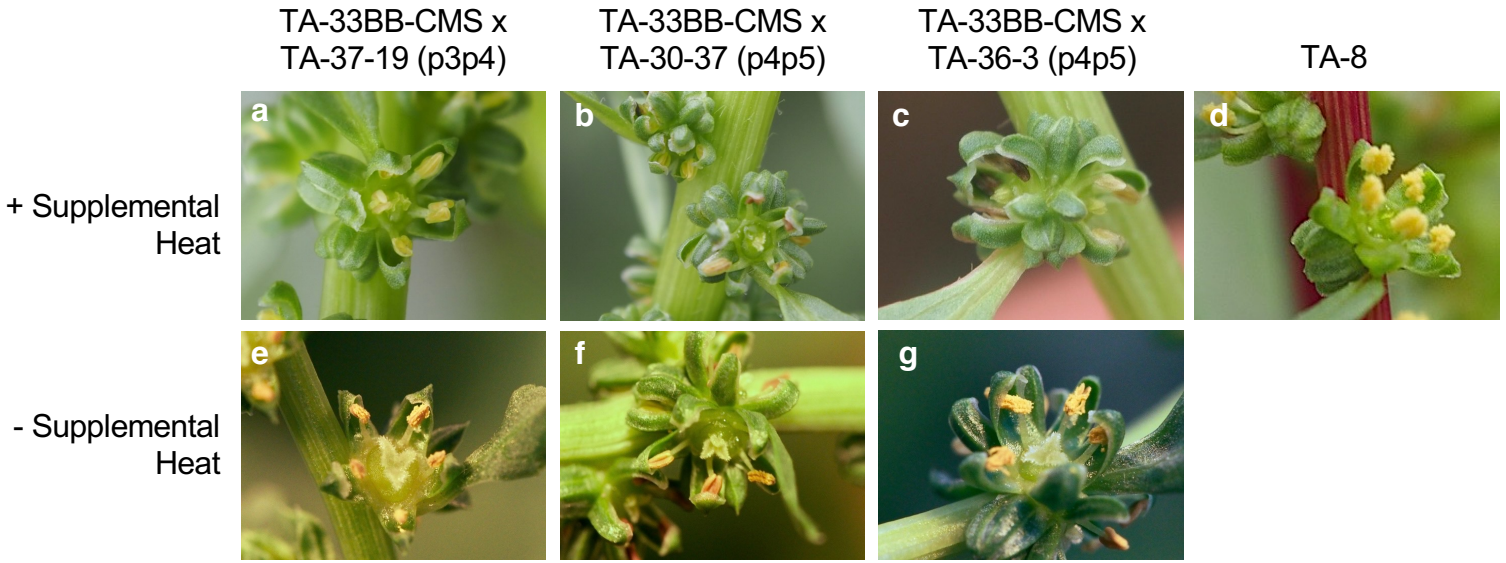
F
F
C
H
b
c


Trans planting
Bolting started
Pollen fertility evaluation #1
Pollen fertility evaluation #2
Seed harvest
˚C
40
35
30
25
20
15
10
5
0
5/19
5/29
6/8
6/18
6/28
7/8
7/18
7/28
8/7
8/17
8/27
9/6
With heating
No heating
Date (Month/Day)


TA-33BB-CMS x
TA-37-19 (p3p4)
TA-33BB-CMS x
TA-30-37 (p4p5)
TA-33BB-CMS x
TA-36-3 (p4p5)
TA-8
+ Supplemental
Heat
- Supplemental
Heat
a
b
c
d
e
f
g


a
b
c

TA-30-37
TA-36-3
TA-37-19
TA-33BB-CMS
TA-33BB-CMS x TA-30-37
TA-33BB-CMS x TA-36-3
TA-33BB-CMS x TA-37-19
No template DNA
489
404
328
242
190
L
S