Molecular mapping of *restorer-of-fertility 2* gene identified from a sugar beet (*Beta vulgaris* L. ssp. *vulgaris*) homozygous for the non-restoring *restorer-of-fertility 1* allele

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Abstract

In the hybrid breeding of sugar beet, maintainer-genotype selection is a laborious process because of the dependence on test crossing, despite the very low occurrence of this genotype. Marker assisted selection (MAS) of the maintainer genotype is highly desired by sugar beet breeders. The major *restorer-of-fertility* gene (*Rf*) was identified as *Rf1*, and its non-restoring allele (*rf1*) was discriminated at the DNA level; however, some of the *rf1rf1* selections retained an as yet unidentified *Rf*, another target locus for MAS. The objective of this study was to identify this *Rf*. An *rfrf1* plant was crossed to a cytoplasmic male-sterile (CMS) sugar beet and then backcrossed to obtain progeny segregating the unidentified *Rf*. The progeny exhibited partial male-fertility restoration that was unstable in single plants. The segregation ratio of restored *vs*. non-restored plants suggested the involvement of a single *Rf* in this male-fertility restoration, designated as *Rf2*. We confirmed the feasibility of molecular tagging of *Rf2* by identifying four shared amplified-fragment-length-polymorphic (AFLP) fragments specific to 17 restored plants. Bulked segregant analysis also was performed to screen the *Rf2*-linked AFLP markers, which were subsequently converted into 17 sequence-tagged site markers. All the markers, as well two additional chromosome-IV assigned markers, were linked to each other to form a single linkage map, on which *Rf2* was located. Our data suggested that *Rf2* is likely an allele of *Z*, long known as an elusive *Rf* gene in sugar beet. We also discuss the importance of *Rf2* for sugar beet breeding.

Keywords: cytoplasmic male sterility, hybrid breeding, marker assisted selection, nuclear-mitochondrial interaction, plant mitochondria, pollen

Author contribution

KT, TM and TK designed this study; KT developed all the plant materials; YH, KT, HH and RYK performed the experiments; YH, KT and TK analyzed the data; YH, KT and TK wrote the manuscript.

Key message

By genetically eliminating the major *restorer-of-fertility* gene (*Rf*), a weak *Rf* gene was unveiled. It is an allele of *Z*, long known as an elusive *Rf* gene in sugar beet.

Introduction

Cytoplasmic male sterility (CMS) in plants is a maternally inherited inability to produce functional pollen (Schnable and Wise 1998; Chase 2007). Use of CMS can provide a large number of seed parents without manual or chemical emasculation; therefore, the trait has been employed for hybrid seed production in many crop species (Schnable and Wise 1998; Wise and Pring 2002). Hybrid seed production using CMS involves three lines, namely the CMS line, a maintainer line and a restorer line

(Chen and Liu 2014); however, the latter may be omitted if male fertility is unnecessary for the final yield (Budar et al. 2006). The interactions of cytoplasmic genes and nuclear genes are a prerequisite for these three lines. There needs to be a male sterility inducing cytoplasm (designated as S), a normal cytoplasm (non-male sterility inducing) (N), and alleles of a nuclear fertility-restorer gene (*Rf*) that suppresses the action of S (Schnable and Wise 1998). The genotypes of CMS lines, maintainer lines and restorer lines are designated as [S]*rfrf*, [N]*rfrf*, and [N or S]*RfRf*, respectively (Budar et al. 2006). Note that the maintainer line and restorer line are different in terms of genotype, but are indistinguishable at the phenotypic level because plants of both lines are male fertile. In other words, the presence/absence of *Rf* in a male-fertile plant is difficult to determine without a genetic marker.

Sugar beet breeding owes much to CMS because all current cultivars are hybrids produced using CMS (Bosemark 1993). Sugar beet CMS used for hybrid seed production was first discovered by Owen (Owen 1942, 1945) and this CMS (the so-called Owen-CMS) remains the only practical CMS to date (Panella and Lewellen 2005; Bosemark 2006). Currently, a major problem in the hybrid breeding of sugar beet is the rarity of the maintainer genotype (less than 5% on average) (Bosemark 2006). The maintainer genotype is identified by a test cross using an annual CMS tester, a procedure in which a plant genotype is considered to be maintainer only when all the progeny are fully male sterile (Bosemark 2006); thus, maintainer selection of sugar beet is far from efficient. As a means to increase the efficiency of maintainer selection, marker assisted selection (MAS) appears to be a promising strategy. In this context, the identification and characterization of sugar beet *Rf*s are valuable research objectives for sugar beet breeding.

Identification of sugar beet *Rfs* is rather difficult because fertility restoration tends to be incomplete and the segregation ratio often deviates from those expected from simple genetic models (Owen 1945; Nagao and Kinoshita 1962; Theurer and Ryser 1969; Bliss and Gabelman 1965). Several genetic models explaining fertility restoration of sugar beet CMS have been proposed with different numbers of involved genes and actions. These models could be summarized as follows: there is a principal *Rf* that appears in almost all the investigations (Owen 1945; Hogaboam 1957; Nagao and Kinoshita 1962; Bliss and Gabelman 1965; Pillen et al. 1993). Owen (1945) first described this *Rf* and designated it as *X*. Besides *X*, there may be some minor *Rf*s, one of which was termed *Z* by Owen (Owen 1945).

Progress toward the characterization of *X* has been made recently. The *X* gene is located on chromosome III (Pillen et al. 1993) (we follow Schondelmaier and Jung [1997] for chromosome numbering) and an allele of *X* was cloned as *Rf1*, whose gene product is a protein resembling the yeast mitochondrial metalloprotease OMA1 (Matsuhira et al. 2012). On the other hand, little was known about *Z*, because its small effect has made its genetic study difficult. Additionally, some investigations reported that fertility restoration could be explained in the absence of such a minor gene (Savitsky 1963; Theurer, 1971; Pillen et al. 1993). *Z* was thought to be on chromosome IV on the basis of the observed linkage between fertility restoration and monogerm seed ball, the latter of which is

conditioned by the *M* locus on chromosome IV (Hogaboam 1957; Roundy and Theurer 1974; Schondelmaier and Jung 1997). Hjerdin-Panagopoulos et al. (2002) detected *Z* as two linked quantitative trait loci (QTLs) for fertility restoration that were located on chromosome IV. The precise map position of *Z* is unknown.

Despite a lack of detailed knowledge about *Z*, MAS for the maintainer genotype was attempted on the basis of *Rf1* polymorphism (Moritani et al. 2013). DNA markers linked to one of the non-restoring alleles (i.e. *rf1*) were developed to test the feasibility of MAS for the maintainer genotype (Moritani et al. 2013). As a result, *rf1rf1*-based MAS could enrich the maintainer genotype: up to 83% of the selections had the maintainer genotype in some populations (Moritani et al. 2013). However, none of the selected *rf1rf1* plants from other populations had the maintainer genotype (Moritani et al. 2013). Therefore, an as yet unidentified *Rf* reduced the maintainer-genotype frequency in the *rf1rf1*-selection.

It was not known whether this unidentified *Rf* was an allele of *Z*, whose impact on maintainer-genotype selection has not been elucidated. If this unidentified *Rf* is an allele of *Z*, it will become very clear that *Z* is not a minor *Rf* but an important locus for sugar beet breeding. To this end, we focused our analysis on the sugar beet line that is the most recalcitrant against *rf1rf1*-based MAS, 'TA-36' (Moritani et al. 2013). A plant derived from the *rf1rf1*-selections was used in this study. Here, we report the molecular mapping of the novel *Rf* that reduces the efficiency of maintainer-genotype selection. On the basis of its chromosome-IV localization, this *Rf* is probably an allele of *Z*, which is now becoming a practical target locus for sugar beet breeding.

Materials and Methods

Plant materials

All the sugar beet lines and populations used in this study were developed at the Hokkaido Agricultural Research Center (HARC), National Agricultural and Food Research Organization (NARO), in Japan. 'TA-33BB-CMS' is an annual tester line having Owen-CMS and is devoid of any *Rf*. 'E60' is a selection of 'TA-36', an introduced cultivar from Germany (Moritani et al. 2013). BC_1F_1 and BC_1F_3 were grown in a greenhouse (20°C, 24h day with incandescent light at night).

Male-fertility phenotyping

Anthers were visually inspected and evaluated according to Moritani et al. (2013) (Table 1). Five male-fertile classes (N, P, S, G and W, from fully fertile to fully sterile) were indexed from 4 to 0, respectively (Table 1). The mean of the indices calculated from three flowers (on average) on different branches of a plant was used as the male-fertility value of the plant. Male-fertility phenotyping of the

131 B_1F_1 and B_1F_3 was done in the winters of 2010 and 2011, respectively.
132
133 DNA isolation
134
135 The procedure of Doyle and Doyle (1990) was used to isolate total cellular DNA from fresh green
136 leaves.
137
138 Amplified fragment length polymorphism (AFLP) analysis
139
140 AFLP analysis was performed using an AFLP Core Reagent Kit (Invitrogen, Carlsbad, CA, USA).
141 *Eco*RI and *Mse*I were selected as the restriction endonucleases. Adapter-ligated DNA was
142 pre-amplified using Takara *Ex* Taq (Takara Bio, Ohtsu, Japan) using pairs of primers in which one of
143 the four nucleotides at the 3' terminus as a selective nucleotide. The PCR protocol was 20 cycles of
144 94°C for 30 sec, 56°C for 1 min and 72°C for 1 min. For selective amplification, pairs of primers
145 having three selective nucleotides were used. The PCR protocol was 94°C for 5 min; 13 cycles of
146 94°C for 30 sec, 65°C (annealing temperature was decreased by 0.6°C /cycle) for 30 sec and 72°C for
147 1 min; and 13 cycles of 94°C for 30 sec, 56°C for 30 sec and 72°C for 1 min. The amplified products
148 were electrophoresed in the high efficiency genome scanning system (Kawasaki and Murakami 2000;
149 Kikuchi et al. 2003) using a non-denaturing 14% polyacrylamide gel and TBE buffer (89mM Tris,
150 89mM boric acid, 2mM EDTA, pH 8.0). The gel was stained with SYBR Green I nucleic acid gel
151 stain (Takara Bio) and a Typhoon Trio Variable Mode Imager (GE Healthcare, Little Chalfont, UK)
152 scanned the stained gel.
153
154 Unidirectional selective genotyping and bulked segregant analysis (BSA)
155
156 Unidirectional selective genotyping was performed using 17 partially fertile plants with male-fertility
157 values ranging from 2.0 to 3.0. BSA was performed according to the method described by
158 Michelmore et al. (1991). Ten restored individuals and 15 male-sterile individuals from BC_1F_1 were
159 used, and groups of five pre-amplified DNAs were pooled to establish two restored bulks and three
160 male-sterile bulks, respectively.
161
162 Conversion of AFLP markers to sequence-tagged site (STS) markers
163
164 AFLP bands were excised from the gel and the DNA fragments were eluted into TE buffer by
165 repetitive freeze-thaw cycles. DNA fragments were then re-amplified using Green Go Taq Master Mix
166 (Promega, Madison, WI) with its cognate selective primers for AFLP. The PCR products were
167 electrophoresed through a 2% agarose gel and purified using an Ultra Clean 15 DNA Purification Kit

(MoBio Laboratories, Carlsbad, CA, USA). Purified PCR products were cloned into the pBluescript (SK+) vector using Ligation high ver. 2 (Toyobo, Osaka, Japan) and sequenced on an ABI3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). Sequencher (Hitachi Software Engineering, Tokyo, Japan) was used for sequence analysis. STS markers were amplified using Green Go Taq Master Mix (the nucleotide sequences of the primers are shown in Table S1). The PCR protocols were 94°C for 3 min; and 35 cycles of 94°C for 30 sec, 54°C for 30 sec and 72°C for 1 min. For cleaved amplified polymorphic sequence (CAPS) analysis, amplified products were digested with restriction enzymes purchased from Takara Bio or New England Biolabs (Ipswich, MA, USA) and electrophoresed in a 2 % agarose gel or 10% polyacrylamide gel.

Statistical analysis, linkage analysis and quantitative trait locus (QTL) analysis

A chi-square test was done using R version 2.14.0 (R Development Core Team, 2011). Fisher's exact test was performed at the website of Gunma University, Japan (http://aoki2.si.gunma-u.ac.jp/exact/fisher/getpar.html) (accessed on 5 June, 2014). A linkage map was constructed using MAPMAKER/EXP ver3.0 (Lander et al. 1987). The map distances in centimorgans (cM) were calculated from recombination frequencies using the Kosambi function (Kosambi 1944). QTL analysis was performed using the simple interval mapping (SIM) method with MAPMAKER/QTL ver1.1b (Lincoln et al. 1993) and the composite interval mapping (CIM) method with WinQTL Cartographer ver2.5 (Wang et al. 2007), in which the logarithm of odds (LOD) threshold (p=0.05) was generated by 1000 permutation tests. CIM was performed using Model 6 at a walk speed of 1.0 cM and a window size of 10.0 cM. The confidence interval (CI) was defined as the region outside of which the log-likelihood fell by 1.0.

Results

Segregation of male fertility in the progeny of ‘E60’

We crossed ‘E60’ (a selection of ‘TA-36’, *rf1rf1* genotype) with ‘TA-33BB-CMS’ (seed parent) to obtain fertility restored F_1 plants. One of the F_1 plants was used as a pollen parent for backcrossing to ‘TA-33BB-CMS’, and the BC_1F_1 was obtained (115 plants). In the BC_1F_1, we noticed that male fertility often differed between flowers on a single plant. Hence, we first indexed the male-fertility phenotype as shown in Table 1, in which male fertility decreases as the value decreases (from 4 to 0). Subsequently, three flowers (on average) borne on different branches were evaluated for each of the 114 BC_1F_1 plants (one plant died before phenotyping) to calculate the plant's mean index of male fertility (male-fertility value). The obtained male-fertility value distribution is shown in Table 2.

No plant was classified into the 4.0–3.1 class, which can be considered as fully fertile. On the other hand, we never observed any fertility restoration of 'TA-33BB-CMS' plants in our greenhouse. Thus we thought that the observed partial fertility (values 3.0–0.1) was conditioned by the *Rf* encoded in the genome of 'E60'. Assuming a single dominant gene model for this partial fertility, 1:1 segregation of partially fertile plants and fully sterile plants could be expected. A chi-square test supported this genetic model (p=0.349) (Table 2).

One of the BC_1F_1 plants with fairly high male fertility was self-pollinated to generate a BC_1F_2. The number of BC_1F_2 plants was insufficient for genetic analysis; therefore, one of the BC_1F_2 plants was self-pollinated to generate a BC_1F_3 (184 plants). We observed segregation of male fertility in 146 plants of this BC_1F_3 (38 died before phenotyping), and the male-fertility value of each plant was investigated (Table 2). As was the case with BC_1F_1, no fully fertile plant was observed. A single dominant gene model could also explain the occurrence of partially fertile plants (chi-square test for 3:1 segregation; p=0.214) (Table 2). On the basis of these results, we concluded that 'E60' has an *Rf* that restores partial pollen fertility to an Owen-CMS plant. Hereafter, this *Rf* is designated as *Rf2*.

Molecular markers linked to fertility restoration

BSA appeared to be an adequate method to obtain molecular markers tightly linked to *Rf2*; however, varying degrees of male fertility also suggested that this trait may be influenced by other minor gene(s) and/or environmental factors (i.e. it may be a quantitative trait), hence the feasibility of using BSA for this trait was uncertain. Therefore, before BSA, the presence of molecular markers associated with the observed fertility restoration needed to be confirmed.

We used a unidirectional selective genotyping approach (Foolad and Jones 1993; Navabi et al. 2009) in which AFLP fragments shared by 17 restored-BC_1F_1 plants (male-fertility values >2.0), but missing from 'TA-33BB-CMS', were sought. We tested 712 primer combinations that generated approximately 17,000 AFLP fragments, and found four fragments that appeared to be specific to the 17 restored plants (Table 3). The presence or absence of these four fragments was examined in 38 restored and 34 non-restored BC_1F_1 plants (Table 3). The distribution of the four bands was significantly biased toward fertility restored plants (Fisher's exact test; p<0.001), suggesting the feasibility of using BSA.

We conducted BSA using two restored bulks and three non-restored bulks made from the BC_1F_1. A total of 1,836 primer combinations were tested, and the number of AFLP fragments specific to the two restored bulks was 114. The presence or absence of these 114 fragments was examined in each of the bulked plants (Fig. 1). The number of AFLP fragments apparently associated with fertility restoration was 36; however, genetic mapping needs highly reproducible markers (i.e. STS markers) rather than AFLP fragments.

For accurate genetic analysis, we first conducted molecular cloning of the 36 AFLP

fragments, from which we obtained 17 nucleotide sequences. Based on these sequences, we designed 17 pairs of PCR primers. Eleven of the 17 sequences were PCR amplified from restored plants, but not from non-restored plants of the BC_1F_1 [sequenced-characterized amplified region (SCAR) markers] (prefixed by 'sc' in Table S1 and Fig. 2). PCR fragments targeting one sequence exhibited length polymorphism between the restored plants and the non-restored plants [a DNA fragment length polymorphism (DFLP) marker] (prefixed by 'df' in Table S1 and Fig. 2). The remaining five sequences were simultaneously amplified from both restored and non-restored plants. The reason why this occurred may be that the original AFLP between restored and non-restored bulks was generated because of alteration(s) within or close to the *Eco*RI- and/or the *Mse*I restriction endonuclease sites but the nucleotide sequence of the internal AFLP fragment was preserved. Concerning these five sequences, nucleotide sequences of PCR fragments amplified from a restored plant and a 'TA-33BB-CMS' plant were compared to find the sequence alterations in the restriction endonuclease recognition site, and we confirmed polymorphisms in the restriction patterns of the PCR fragments (CAPS markers) (prefixed by 'ca' in Table S1 and Fig. 2). The polymorphisms of these 17 STS markers exactly matched with those of their cognate AFLP fragments in the 25 BC_1F_1 plants that were used for BSA.

Rf2 is located on chromosome IV

We next tested whether the 17 STS markers were linked to each other. Segregation of each of the 17 STS markers in 115 BC_1F_1 plants statistically fit with the expected genetic model (Table S2). These segregation data were analyzed using mapping software. The resultant linkage map contained all the 17 STS markers, covering 28.1cM (Fig. 2a). Chromosomal assignment of this linkage map was investigated using DNA markers developed by Schneider et al. (1999). Chromosome IV, which contains *Z*, was the most likely candidate; therefore, we tested the linkage of two chromosome-IV assigned markers, *nir* and *ant*, to our map. As a result, *nir* and *ant* were found to flank our linkage map (Fig. 2a). Consequently the linkage map was expanded to 46.1cM.

We examined whether *Rf2* could be mapped on our linkage map. Under the dichotomic assumption (i.e. restored *vs*. non-restored), we failed to map *Rf2* on our linkage map. As such, we next assumed that the observed fertility restoration was the quantitative trait involving *Rf2*. The map position of *Rf2* was analyzed by QTL analysis using male-fertility values. In the linkage map shown in Fig. 2a, both SIM and CIM methods detected a QTL for fertility restoration in the region between sc4 and a locus containing ca2, sc3, sc7 and sc10 (LOD=36.15 and 27.68 for SIM and CIM, respectively) (Table 4). The confidence intervals (CIs) identified by SIM and CIM were within the map position of 13.6-15.3 cM in Fig. 2a, the region delimited by sc4 and ca2 (representing four markers). The two methods detected no other consistent QTL for fertility restoration.

We then examined the segregation of the 17 DNA markers, *nir*, and *ant* in the BC_1F_3 (Table

S3). Two markers, sc1 and ca4, could not be mapped because all the plants were homozygous for 'TA-33BB-CMS'-type alleles. Segregation of the other markers fit with the expected genetic model (Table S3), and we analyzed these data using the mapping software. As a result, we obtained a map of 34.5 cM (Fig. 2b). The arrangement of markers is fairly well preserved between the BC_1F_1 and the BC_1F_3 (Fig. 2).

We conducted QTL analysis for fertility restoration in the BC_1F_3 to map *Rf2*. The highest LOD peak for fertility restoration was the region delimited by sc11 and a locus containing sc3 and four other DNA markers, in which map positions of CI were 12.8-16.8 for SIM (LOD=24.93) and 12.0-17.0 for CIM (LOD=32.7) (Table 4 and Fig. 2b). No other QTLs for fertility restoration were consistently detected by the two methods. The map position of the QTL for fertility restoration was very similar between the BC_1F_1 and BC_1F_3 and both of the detected QTLs associated with sc4, sc3, sc7, sc10, and ca2.

The closest markers to *Rf2* appeared to be sc4, sc3, sc7, sc10 and ca2, because the presence or absence of these five markers showed the best association with male-fertility restoration (107/114 in the BC_1F_1 and 132/146 in the BC_1F_3). Therefore, *Rf2* was located in the interval between sc4 and ca2 (one of four markers, see Fig. 2a), ~13.6 to ~15.3 cM away from *nir* toward *ant* (BC_1F_1), or located near the site containing the five markers, ~8.8 cM (BC_1F_3) away from *nir* toward *ant*.

Discussion

Fertility restoration in Owen-CMS is a very complex trait, as pointed out by previous investigations, in which various segregation patterns were described (Owen 1945; Hogaboam 1957; Bliss and Gabelman 1965; Nagao and Kinoshita 1962; Theurer and Ryser 1969; Hjerdin-Panagopoulos et al. 2002). This complexity likely came from the combined action of major and minor *Rf*s, as well as environmental factors (Owen 1945). Hence, genetic dissection of this phenotype is a prerequisite to assessing the action of each *Rf*, a necessary procedure for genetic mapping. Before this study, the major role of *Rf1* in fertility restoration was proposed (e.g. Moritani et al. 2013). We intended to eliminate the action of *Rf1* for the accurate genetic analysis of the minor *Rf*.

Although *Rf2* is a minor *Rf* in terms of genetics, the significance of *Rf2* on sugar beet breeding is another issue. In other words, if *Rf2* is practically important, this locus cannot be ignored by breeders, irrespective of its strength. In the case of *Rf1*, Japanese breeders have been carefully eliminating the restoring *Rf1* allele during maintainer selection, resulting in the selection of a few non-restoring alleles from varieties of *Rf1* alleles (Moritani et al. 2013). This notion was supported by the analysis of the allelic frequency of *Rf1* in the ancestral populations of Japanese sugar beet (Taguchi et al. 2014), suggesting a major impact of *Rf1* on maintainer selection. We speculate that *Rf2* may be another target locus for maintainer genotype selection for the following reason: although the effect of *Rf2* on fertility restoration is small, partially fertile plants in the test-cross progeny will be

316 easily recognized by breeders as a sign of a non-maintainer genotype. In fact, partial male fertility of
317 test-cross progeny derived from 'TA-36' was sufficient to make Moritani et al. (2013) reject all the
318 *rf1rf1* plants as candidates of the maintainer. The question of whether *Rf2* has been one of the target
319 loci for maintainer selection can be tested by examining the molecular polymorphism of *Rf2* in sugar
320 beet lines. Currently, we are working to identify the nucleotide sequence of *Rf2*.
321 The effect of *Rf2* is obviously weak because no fully fertile plants were obtained in this
322 study and the plants are at the most partially fertile. As seen in the male-fertility values, fertility
323 restoration in the BC_1F_1 and the BC_1F_3 appeared to be a quantitative trait. Considering that the BC_1F_1-
324 (and the BC_1F_3-) fertility restoration differed within a plant, it is possible that the penetrance of *Rf2* is
325 influenced by the plant's physiological condition (nutrition, age, etc.). In addition, it is possible that
326 other minor *Rf*(s) and/or environmental factors may be involved. Thus, one of the remaining questions
327 is how the phenotypic expression of *Rf2* changes in relation to other factors. This is an important
328 question, because if the action of *Rf2* can be masked for some reason, the *Rf2* allele might contaminate
329 CMS lines through maintainer lines, potentially decreasing hybrid purity.
330 *Rf2* is situated in the region delimited by *nir* and *ant* on chromosome IV, on which *Z* is the
331 only *Rf* known to be located (Schondelmaier and Jung 1997). Thus *Rf2* is likely an allele of *Z*. In
332 addition, the low restoration effect of *Rf2* is consistent with the postulation of *Z* provided by many
333 sugar beet researchers (Owen, 1945; Hogaboam, 1957; Theurer, 1971).
334 In this context, we think *Rf2* may correspond to one of the two-linked QTLs for fertility
335 restoration reported by Hjerdin-Panagopoulos et al. (2002). The quantitative nature of *Rf2* is
336 consistent with their result. The gene corresponding to the other QTL (i.e. the third *Rf*) was not
337 detected in our study, possibly because the third *Rf* is absent from 'E60'. It is not surprising that the
338 composition of minor *Rf*s differs between sugar beet lines, considering the various segregation
339 patterns of fertility restoration in the previous investigations (see above for references). Unfortunately,
340 because no DNA marker in this study is shared with those of Hjerdin-Panagopoulos et al. (2002),
341 further comparison of the results is impossible. However, the map position of *Rf2* and the markers
342 developed in this study would be useful to compare sugar beet *Rf*s between different populations. The
343 overarching genetic and environmental control of fertility restoration of Owen-CMS should be
344 investigated for the benefit of sugar beet breeding.
345
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352

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434

435 Table 1. Classification of male sterility in this study[1]

	Character of anther			Male fertility
Class	Color	Dehiscence	Pollen production	index
N	Yellow	+	+	4
P	Yellow, sometimes orange	Both + and – are seen	±	3
S	Yellow, sometimes orange	–, rarely +	–	2
G	Light green	–	–	1
W	White or brown	–	–	0

436 [1]Adopted from Moritani et al. (2013) with some modifications.

437

438

439 Table 2. Segregation of observed male fertility

		Male fertility-classes and number of plants						
		Fully fertile	Partially fertile			Fully sterile		
Population	Total number of plants	4.0-3.1[1]	3.0-2.1[1]	2.0-1.1[1]	1.0-0.1[1]	0.0[1]	χ^2 (ratio)	*p*-value
BC1F1	114	0	12 (52)[2]	36	4	62	0.877 (1:1)	0.349
BC1F3	146	0	26 (103)[2]	70	7	43	1.543 (3:1)	0.214

440 [1]Male-fertility value classes.

441 [2]Sum of fertility restored plants.

442

443

444

445 Table 3. Distribution of four AFLP markers in 72 plants

Pair of primers for selective amplification	Size of AFLP marker fragment (bp)	Presence/absence of the marker	Number of plants		Total number of plants	*p*-value (2x2 contingency table, Fisher's exact test)
			Fertility restored	Fully sterile		
E-CCC[1]/M-CCG[1]	290	+	31	0	72	6.46×10^{-14}
		–	7	34		
		N/A[2]	0	0		
E-AAC[1]/M-CCG[1]	150	+	30	1	72	4.25×10^{-12}
		–	7	33		
		N/A[2]	1	0		
E-AAC[1]/M-GAG[1]	120	+	32	0	72	1.05×10^{-14}
		–	6	34		
		N/A[2]	0	0		
E-ACC[1]/M-CTG[1]	170	+	31	1	72	6.98×10^{-13}
		–	6	33		
		N/A[2]	1	0		

446 [1]E- and M- denote *Eco*RI- and *Mse*I primers, respectively. The following three letters indicate selective nucleotides.

447 [2]Not available.

448

449 Table 4. Summary of the QTL analysis

		QTL for fertility restoration							
		SIM				CIM			
Population	Total number of plants	Confidence interval[1]	LOD	R^2	Additive	Confidence interval[1]	LOD	R^2	Additive
BC1F1	115	13.6-15.3	36.15	0.77	1.97	13.6-15.3	27.68	0.51	1.83
BC1F3	184	12.8-16.8	24.93	0.81	0.89	12.0-17.0	32.7	0.59	0.97

450 [1]Map position in Fig. 2.

451

452 **Figure legends**

453

454 **Fig. 1** Images of the AFLP pattern using two restored bulks (A), three non-restored bulks (B), ten
455 fertility restored plants of BC_1F_1 (C), and 15 fully male-sterile plants of BC_1F_1 (D). Selective primers
456 were *Eco*RI-CCC and *Mse*I-GAC. An arrow indicates an AFLP fragment associated with fertility
457 restoration. Size markers are shown on the right (bp)

458

459 **Fig. 2** Linkage map of the chromosomal region around *Rf2* in BC_1F_1 (a) and BC_1F_3 (b) populations.
460 Two chromosome IV-assigned markers are connected by dotted lines. The confidence intervals of
461 QTL for fertility restoration are indicated by vertical bars labeled with '*Rf2*'. Details of the markers
462 are shown in Table S1. Map distances are shown in the left of each maps (cM). In the linkage map
463 shown in b, repulsion of two regions (*nir*–ca2 and sc11-*ant*) was assumed in the linkage analysis

464

Table S1 DNA markers and the nucleotide sequences of primers used in this study

Name of markers	Size (bp)	Forward primer (5'--3')	Reverse primer (5'--3')	Marker type (Restriction endonuclease)	Source
sc1	319	AATACAATCCCTCAAGTATGTCGA	TTAGCAACATGTCAGTGCTTGATG	SCAR	This study
sc2	524	AAGTATTTGACTAAGGCTGGTAG	GCTTAGGGGGTAAGTGTCATA	SCAR	This study
sc3	788	AAGTCGATCTCCTCCACCTT	GGATGTACTGAGTATGAGAGAATA	SCAR	This study
sc4	243	CGCTTGTCTCAATTCACATCA	TTGAGACCTCTATTTCTGCAG	SCAR	This study
sc5	382	GATTGCCCAGGGTTCGAA	GTCCCACGTAGCAGACAT	SCAR	This study
sc6	462	GTTTGGCGGTGAACAGGAC	AGAAACTGGGCTCCAGCAG	SCAR	This study
sc7	377	GTAGTGTGCATATGTATCTCGT	TGAGGAGTGATTGAAGGTGAGT	SCAR	This study
sc8	333	CAGGATGCCACCGTTCTCAAG	TCGTGATACCTCCTCGGTCA	SCAR	This study
sc9	169	GTCCATCTCATTACTTTGGGA	TTTCCAACTCAATAACAAATTCAG	SCAR	This study
sc10	435	GCCTGACCAGCCCAGAAGGCA	TCGGCTCAATGCGGGATCC	SCAR	This study
sc11	319	TCTGCACCTAATTTTTTTCATGCC	AAGATCAAAGCTCTACTCCATAAC	SCAR	This study
df1	390	GAGCGCCAACTCCAATGTC	GCTTTCTTCAGTGTTAGTGACTC	DFLP	This study
ca1	313	TGTTATACCTATGCCACTTGAAG	TCAAGTTCGAACATTGGTGTCGA	CAPS (*Alu*I)	This study
ca2	228	CTTCGCATTCCATCATAATGTTTG	CTCACAAGTCATAACCGCGT	CAPS (*Eco*RI)	This study
ca3	323	AGAGCGTCGTCTTTCTCGG	TCTCTCTCCTCTAATCAGACACT	CAPS (*Taq*I)	This study
ca4	612	GAGAACATGAAATTGCTGCCTG	GTCCACCGGAAGAATGACC	CAPS (*Hin*dIII)	This study
ca5	222	AACCAGAGCATATCTCTCTAGC	GGCATCGGGTCAAATCTCAC	CAPS (*Mbo*I)	This study
nir	1600	GTTAGRCTCAAGTGGCTTGG	GGCATTCTCTTCTCWACCTC	CAPS (*Rsa*I)	Schneider et al. 1999
ant	1000	TGGAGAGGAAACACTGCYAATGT	ATGTTRGCACCAGCWCCCTTGA	CAPS (*Taq*I)	Schneider et al. 1999

Table S2 Segregation of marker genotypes in BC_1F

Name of markers	Plant ID 1_BC1F1	2_BC1F1	3_BC1F1	4_BC1F1	5_BC1F1	6_BC1F1	7_BC1F1	8_BC1F1	9_BC1F1	10_BC1F1	11_BC1F1	12_BC1F1
nir	A[1]	H[2]	H	H	A	H	H	A	A	H	A	H
sc8	A	H	A	H	A	H	H	A	A	H	A	H
sc6	A	H	A	H	A	H	H	A	A	H	A	H
ca5	A	H	A	H	A	H	H	A	A	H	A	H
sc2	A	H	A	H	A	H	H	A	A	A	A	H
sc9	A	H	A	H	A	H	H	A	A	A	A	H
sc5	A	H	A	H	A	H	H	A	A	A	A	H
df1	A	H	A	H	A	H	H	A	A	A	A	H
ca3	A	H	A	H	A	H	H	A	A	A	A	H
ca1	A	H	A	H	A	H	H	A	A	A	A	H
sc4	A	H	A	H	A	H	H	A	A	A	A	H
sc7	A	H	A	H	A	H	H	A	A	A	A	H
ca2	A	H	A	H	A	H	H	A	A	A	A	H
sc10	A	H	A	H	A	H	H	A	A	A	A	H
sc3	A	H	A	H	A	H	H	A	A	A	A	H
sc1	A	H	A	H	A	H	H	A	A	A	A	H
ca4	A	H	A	H	A	A	H	A	A	A	A	H
sc11	A	A	A	H	A	A	H	A	A	A	A	H
ant	A	A	A	H	A	A	H	A	A	A	A	H

[1]TA-33BB-CMS homozygous

[2]Heterozygous

[3]No data

13_BC1F:	14_BC1F:	15_BC1F:	16_BC1F:	17_BC1F:	18_BC1F:	19_BC1F:	20_BC1F:	21_BC1F:	22_BC1F:	23_BC1F:	24_BC1F:	25_BC1F:	26_BC1F:
H	A	H	A	A	A	A	A	A	A	A	A	H	H
H	A	H	A	A	A	A	A	A	A	A	A	H	H
H	A	H	A	A	A	A	A	A	A	A	A	H	H
H	A	H	A	A	A	A	A	A	A	A	A	H	H
H	A	H	A	A	A	A	A	A	A	A	A	H	H
H	A	H	A	A	A	A	A	A	A	A	A	H	H
H	A	H	A	A	A	A	A	A	A	A	A	H	H
H	A	H	A	A	A	A	A	A	A	A	A	H	H
H	A	H	A	A	A	A	A	A	A	A	A	H	H
H	A	H	A	A	A	A	A	A	A	A	A	H	H
H	A	H	A	A	A	A	A	A	A	A	A	H	H
H	A	H	A	A	A	A	A	H	A	A	A	H	H
H	A	H	A	A	A	A	A	H	A	A	A	H	H
H	A	H	A	A	A	A	A	H	A	A	A	H	H
H	A	H	A	A	A	A	A	H	A	A	A	H	H
H	A	H	A	A	A	A	A	H	A	A	A	H	H
H	A	H	A	A	A	H	A	H	A	A	A	H	H
A	A	H	A	A	A	H	A	H	A	A	A	H	A
A	A	H	A	A	A	H	A	H	A	A	A	H	A

27_BC1F:	28_BC1F:	29_BC1F:	30_BC1F:	31_BC1F:	32_BC1F:	33_BC1F:	34_BC1F:	35_BC1F:	36_BC1F:	37_BC1F:	38_BC1F:	39_BC1F:	40_BC1F:
A	A	A	H	H	H	A	A	H	H	A	A	A	H
H	A	H	H	-	H	A	A	H	H	A	A	A	H
H	A	H	H	H	H	A	A	H	H	A	A	A	H
H	A	H	H	H	H	A	A	H	H	A	A	A	H
H	A	H	H	H	H	A	H	H	H	A	A	A	H
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H	A	H	H	H	H	A	H	H	H	A	A	A	H
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H	A	H	H	H	H	A	H	H	H	A	A	A	H
H	A	H	H	H	H	A	H	H	H	A	A	A	H
H	A	H	H	H	H	A	H	H	H	A	A	A	H
H	A	H	H	H	H	A	H	H	H	A	A	A	H
H	H	H	H	H	H	A	H	H	H	H	A	A	H
H	H	H	H	A	H	A	H	H	H	H	A	A	H
H	H	H	H	A	H	A	H	A	H	H	A	A	H

41_BC1F:	42_BC1F:	43_BC1F:	44_BC1F:	45_BC1F:	46_BC1F:	47_BC1F:	48_BC1F:	49_BC1F:	50_BC1F:	51_BC1F:	52_BC1F:	53_BC1F:	54_BC1F:
A	A	A	A	A	H	-[3]	-	A	A	H	H	A	H
A	A	A	A	A	H	H	A	A	A	H	H	A	H
A	A	A	A	A	H	H	A	A	A	H	H	A	H
A	A	A	A	A	H	H	A	A	A	H	H	A	H
A	A	H	A	A	H	H	A	A	A	H	H	A	H
A	A	H	A	A	H	H	A	A	A	H	H	A	H
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A	A	H	A	A	H	H	A	A	A	H	H	A	H
A	A	H	A	A	H	H	A	A	A	H	H	A	H
A	A	H	A	A	H	H	A	A	A	H	H	A	H
A	A	H	A	A	H	H	A	A	A	H	H	A	H
A	A	H	A	A	H	H	A	A	A	H	H	A	H
A	A	H	A	A	H	H	A	A	A	H	H	A	H
A	A	H	A	A	H	H	A	A	A	H	H	A	H
A	A	H	A	A	H	H	A	A	A	H	H	A	H
A	A	H	A	A	H	H	A	A	A	H	H	A	H
A	A	H	A	A	H	H	A	A	A	H	H	A	H
A	H	H	A	A	H	H	A	A	A	H	A	A	H
H	H	A	H	A	A	H	A	A	A	H	A	A	H

55_BC1F:	56_BC1F:	57_BC1F:	58_BC1F:	59_BC1F:	60_BC1F:	61_BC1F:	62_BC1F:	63_BC1F:	64_BC1F:	65_BC1F:	66_BC1F:	67_BC1F:	68_BC1F:
H	A	H	A	A	A	A	H	H	H	A	A	A	H
H	A	H	A	A	A	A	H	H	H	A	A	A	H
H	A	H	A	A	A	A	H	H	H	A	A	A	H
H	A	H	A	A	A	A	H	H	H	A	A	A	H
H	A	H	A	A	A	A	H	H	H	A	A	A	H
H	A	H	A	A	A	A	H	H	H	A	A	A	H
H	A	H	A	A	A	A	H	H	H	A	A	A	H
H	A	H	A	A	A	A	H	H	H	A	A	A	H
H	A	H	A	A	A	A	H	H	H	A	A	A	H
H	A	H	A	A	A	A	H	H	H	A	A	A	H
H	A	H	A	A	A	A	H	H	H	A	A	H	H
H	A	H	A	A	A	A	H	H	H	A	A	H	H
H	A	H	A	A	A	A	H	H	H	A	A	H	H
H	A	H	A	A	A	A	H	H	H	A	A	H	H
H	A	H	A	A	A	A	H	H	H	A	A	H	H
A	A	H	A	A	A	A	H	H	H	A	A	H	H
A	A	H	A	H	A	A	H	H	H	A	A	H	H
A	A	H	A	H	A	A	A	A	H	A	A	H	H
A	A	H	A	H	A	A	A	A	H	A	A	H	H

69_BC1F:	70_BC1F:	71_BC1F:	72_BC1F:	73_BC1F:	74_BC1F:	75_BC1F:	76_BC1F:	77_BC1F:	78_BC1F:	79_BC1F:	80_BC1F:	81_BC1F:	82_BC1F:
A	A	H	A	A	H	A	A	A	A	A	A	H	A
A	A	H	A	A	H	A	A	A	A	A	A	H	A
A	A	H	H	A	H	A	A	A	A	A	A	H	A
A	A	H	H	A	H	A	A	A	A	A	A	H	A
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A	A	H	H	A	H	A	A	A	A	A	A	H	A
A	A	H	H	A	H	A	A	A	A	A	A	A	A
A	A	H	H	A	H	A	A	A	A	A	A	A	A
A	A	H	H	A	H	A	A	A	A	A	A	A	A
A	A	H	H	A	H	A	A	A	A	A	A	A	A
A	A	H	H	A	H	A	A	A	A	A	A	A	A
A	A	H	H	A	H	A	A	A	A	A	A	A	A
A	A	H	H	A	H	A	A	A	A	A	A	A	A
A	A	H	H	A	H	A	A	A	A	A	H	A	A
A	H	H	H	A	H	A	A	A	A	A	H	A	A
A	H	A	H	A	H	A	A	A	A	A	H	A	A
H	H	A	H	A	H	A	H	H	A	A	H	A	A

83_BC1F:	84_BC1F:	85_BC1F:	86_BC1F:	87_BC1F:	88_BC1F:	89_BC1F:	90_BC1F:	91_BC1F:	92_BC1F:	93_BC1F:	94_BC1F:	95_BC1F:	96_BC1F:
A	H	A	A	H	A	H	A	H	A	A	H	A	A
A	H	A	A	H	A	H	A	H	A	A	H	A	A
A	H	A	A	H	A	H	A	H	A	A	H	A	A
A	H	A	A	H	A	H	A	H	A	A	H	A	A
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A	H	A	A	A	A	H	A	H	A	A	H	A	A
A	H	A	A	A	A	H	A	H	A	A	H	A	A
A	H	A	A	A	A	H	A	H	A	A	H	A	A
A	H	A	A	A	A	H	A	H	A	A	H	A	A
A	H	A	A	A	A	H	A	H	A	A	H	A	A
A	H	A	A	A	A	H	A	H	A	A	H	A	H
A	A	A	A	A	A	H	A	A	A	A	H	A	H
A	A	H	A	A	A	A	A	A	A	A	H	H	H

97_BC1F:	98_BC1F:	99_BC1F:	100_BC1I	101_BC1I	102_BC1I	103_BC1I	104_BC1I	105_BC1I	106_BC1I	107_BC1I	108_BC1I	109_BC1I	110_BC1I
A	A	H	A	A	A	-	H	-	-	-	-	A	A
A	H	H	A	H	H	H	H	H	H	H	A	A	A
A	H	H	A	H	H	H	H	H	H	H	A	A	A
A	H	H	A	H	H	H	H	H	H	H	A	A	A
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A	A	H	A	H	H	H	H	H	H	H	A	A	H
A	A	H	A	H	H	H	H	H	H	H	A	A	H
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A	A	H	A	A	H	H	H	H	H	H	A	A	H
A	A	H	A	A	H	H	H	H	H	H	A	A	H
A	A	H	A	A	H	H	H	H	H	H	A	A	H
A	A	H	A	A	H	H	H	H	H	H	A	A	H
A	A	H	A	A	H	H	H	H	H	H	A	A	H
A	A	H	A	A	H	H	H	H	H	H	A	A	H
A	A	H	A	A	H	A	H	H	H	H	A	A	H
A	A	A	A	A	A	A	H	H	A	H	A	A	H

					Chi-square test for segregation			
111_BC1I	112_BC1I	113_BC1I	114_BC1I	115_BC1F1	Genotype segregatio	Not available	$\chi^2(1:1)$	*p* -value
A	H	H	A	H	68 : 40 (A : H)	7	7.259	0.007
A	H	H	A	H	66 : 48 (A : H)	1	2.842	0.092
A	H	H	A	H	65 : 50 (A : H)	0	1.957	0.162
A	H	H	A	H	65 : 50 (A : H)	0	1.957	0.162
A	H	H	A	H	64 : 51 (A : H)	0	1.470	0.225
A	H	H	A	H	64 : 51 (A : H)	0	1.470	0.225
A	H	H	A	H	65 : 50 (A : H)	0	1.957	0.162
A	H	H	A	H	65 : 50 (A : H)	0	1.957	0.162
A	H	H	A	H	66 : 49 (A : H)	0	2.513	0.113
A	H	H	A	H	66 : 49 (A : H)	0	2.513	0.113
A	H	H	A	H	65 : 50 (A : H)	0	1.957	0.162
A	H	H	A	H	65 : 50 (A : H)	0	1.957	0.162
A	H	H	A	H	65 : 50 (A : H)	0	1.957	0.162
A	H	H	A	H	65 : 50 (A : H)	0	1.957	0.162
A	H	H	A	H	65 : 50 (A : H)	0	1.957	0.162
A	H	H	A	H	65 : 50 (A : H)	0	1.957	0.162
A	H	H	A	H	60 : 55 (A : H)	0	0.217	0.641
A	H	H	A	H	70 : 45 (A : H)	0	5.435	0.020
A	H	H	A	H	70 : 45 (A : H)	0	5.435	0.020

Table S3 Segregation of marker genotypes in BC_1F_3

	Plant ID																			
Name of markers	1_BC1F3	2_BC1F3	3_BC1F3	4_BC1F3	5_BC1F3	6_BC1F3	7_BC1F3	8_BC1F3	9_BC1F3	10_BC1F3	11_BC1F3	12_BC1F3	13_BC1F3	14_BC1F3	15_BC1F3	16_BC1F3	17_BC1F3	18_BC1F3	19_BC1F3	20_BC1F3
nir	H[1]	H	H	A[2]	H	A	B[3]	B	H	A	H	A	B	B	H	B	B	A	H	B
sc8	C[4]	C	C	A	C	A	C	C	C	A	C	A	C	C	C	C	C	C	C	C
ca5	H	H	H	A	H	A	B	B	H	A	H	A	B	H	H	B	B	H	H	B
sc6	C	C	C	A	C	A	C	C	C	A	C	A	C	C	C	C	C	C	C	C
sc2	C	C	C	A	C	A	C	C	C	A	C	A	C	C	C	C	C	C	C	C
sc9	C	C	C	A	C	A	C	C	C	A	C	A	C	C	C	C	C	C	C	C
sc5	C	C	C	A	C	A	C	C	C	A	C	A	C	C	C	C	C	C	C	C
df1	H	H	H	A	H	A	B	B	H	A	H	A	B	H	H	B	B	H	H	B
ca1	H	H	H	A	H	A	B	B	H	A	H	A	B	H	H	B	B	H	H	B
ca3	H	H	H	A	H	A	B	B	H	A	H	A	B	H	H	B	B	H	H	B
sc4	C	C	C	A	C	C	C	C	C	A	C	A	C	C	C	C	C	C	C	C
ca2	H	H	H	A	H	H	B	B	H	A	H	A	B	H	H	B	B	H	H	B
sc7	C	C	C	A	C	C	C	C	C	A	C	A	C	C	C	C	C	C	C	C
sc3	C	C	C	A	C	C	C	C	C	A	C	A	C	C	C	C	C	C	C	C
sc10	C	C	C	A	C	C	C	C	C	A	C	A	C	C	C	C	C	C	C	C
sc1	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
ca4	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
sc11[6]	C	C	C	C	A	A	A	A	A	C	C	C	A	C	A	C	A	C	C	A
ant[6]	H	H	H	H	A	A	A	H	A	H	A	B	A	H	H	H	H	H	H	A

[1]Heterozygous.

[2]TA-33BB-CMS homozygous.

[3]E60 homozygous.

[4]E60 homozygous or heterozygous.

[5]No data.

[6]These markers were assumed to be configured in repulsion in the linkage analysis.

21_B(	22_B(	23_B(	24_B(	25_B(	26_B(	27_B(	28_B(	29_B(	30_B(	31_B(	32_B(	33_B(	34_B(	35_B(	36_B(	37_B(	38_B(	39_B(	40_B(	41_B(	42_B(	43_B(
H	H	B	B	B	H	H	H	A	B	A	A	B	A	A	A	B	B	H	B	A	B	H
C	C	C	C	C	C	C	C	A	C	A	A	C	A	A	A	C	C	C	C	A	C	C
H	H	B	B	B	H	H	H	H	H	A	A	H	A	A	A	B	B	H	H	A	H	H
C	C	C	C	C	C	C	C	C	C	A	A	C	A	A	A	C	C	C	C	A	C	C
C	C	C	C	C	C	C	C	C	C	A	A	C	A	A	A	C	C	C	C	A	C	C
C	C	C	C	C	C	C	C	C	C	A	A	C	A	A	A	C	C	C	C	A	C	C
C	C	C	C	C	C	C	C	C	C	A	A	C	A	A	A	C	C	C	C	A	C	C
H	H	H	B	B	B	H	H	H	H	A	A	H	A	A	A	B	B	H	H	A	H	H
H	H	H	B	B	B	H	H	H	H	H	A	H	A	A	A	B	H	H	H	A	H	H
H	H	H	B	B	B	H	H	H	H	H	A	H	A	A	A	B	H	H	H	A	H	H
C	C	C	C	C	C	C	C	C	C	C	A	C	A	A	A	C	C	C	C	A	C	C
H	H	H	B	B	B	H	H	H	H	H	A	H	A	A	A	B	H	H	H	A	H	H
C	C	C	C	C	C	C	C	C	C	C	A	C	A	A	A	C	C	C	C	A	C	C
C	C	C	C	C	C	C	C	C	C	C	A	C	A	A	A	C	C	C	C	A	C	C
C	C	C	C	C	C	C	C	C	C	C	A	C	A	A	A	C	C	C	C	A	C	C
A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
A	C	C	C	C	A	A	C	C	C	C	C	C	C	C	C	A	C	C	C	C	C	C
H	B	H	H	H	A	A	H	H	H	H	B	H	H	H	B	A	H	H	H	H	B	H

44_B(	45_B(	46_B(	47_B(	48_B(	49_B(	50_B(	51_B(	52_B(	53_B(	54_B(	55_B(	56_B(	57_B(	58_B(	59_B(	60_B(	61_B(	62_B(	63_B(	64_B(	65_B(	66_B(
B	B	H	H	A	A	H	B	H	B	H	H	B	H	H	H	A	H	B	H	B	A	B
C	C	C	C	A	A	C	C	C	C	C	C	C	C	A	C	A	C	C	C	C	A	C
B	B	H	B	A	A	H	B	H	B	H	H	B	H	A	H	A	H	B	H	B	A	B
C	C	C	C	A	A	C	C	C	C	C	C	C	C	A	C	A	C	C	C	C	A	C
C	C	C	C	A	A	C	C	C	C	C	C	C	C	A	C	A	C	C	C	C	A	C
C	C	C	C	A	A	C	C	C	C	C	C	C	C	A	C	A	C	C	C	C	A	C
C	C	C	C	A	A	C	C	C	C	C	C	C	C	A	C	A	C	C	C	C	A	C
B	B	B	B	A	A	H	B	H	H	H	H	B	H	A	H	A	H	B	H	B	A	B
B	B	B	B	A	A	H	B	H	H	H	H	B	H	A	H	A	H	B	H	B	A	B
B	B	B	B	A	A	H	B	H	H	H	H	B	H	A	H	A	H	B	H	B	A	B
C	C	C	C	A	A	C	C	C	C	C	C	C	C	A	C	A	C	C	C	C	A	C
B	B	B	B	A	A	H	B	H	B	H	H	B	H	A	H	A	H	B	H	B	A	B
C	C	C	C	A	A	C	C	C	C	C	C	C	C	A	C	A	C	C	C	C	A	C
C	C	C	C	A	A	C	C	C	C	C	C	C	C	A	C	A	C	C	C	C	A	C
C	C	C	C	A	A	C	C	C	C	C	C	C	C	A	C	A	C	C	C	C	A	C
A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
A	C	A	A	C	C	C	A	C	C	C	C	A	C	C	C	C	C	A	C	C	C	C
A	H	A	A	H	B	A	A	H	H	H	H	A	B	H	H	H	H	A	B	H	B	H

67_B(	68_B(	69_B(	70_B(	71_B(	72_B(	73_B(	74_B(	75_B(	76_B(	77_B(	78_B(	79_B(	80_B(	81_B(	82_B(	83_B(	84_B(	85_B(	86_B(	87_B(	88_B(	89_B(
A	H	H	H	H	H	B	H	H	B	H	H	A	H	B	A	A	H	A	H	H	H	A
A	C	C	C	C	C	C	A	C	C	C	C	A	C	C	A	A	C	A	C	C	C	A
A	H	H	H	H	H	B	A	H	B	H	H	A	H	B	A	A	H	A	H	H	H	A
A	C	C	C	C	C	C	A	C	C	C	C	A	C	C	A	A	C	A	C	C	C	A
A	C	C	C	C	C	C	A	C	C	C	C	A	C	C	A	C	C	A	C	C	A	A
A	C	C	C	C	C	C	A	C	C	C	C	A	C	C	A	C	C	A	C	C	A	A
A	C	C	C	C	C	C	A	C	C	C	C	A	C	C	A	C	C	A	C	C	A	A
A	H	H	H	H	H	B	A	H	B	H	H	A	H	B	A	H	H	A	H	H	A	A
A	H	H	H	H	H	B	A	H	B	H	H	A	H	B	A	H	H	A	H	H	A	A
A	H	H	H	H	H	B	A	H	B	H	H	A	H	B	A	H	H	A	H	H	A	A
A	C	C	C	C	C	C	A	C	C	C	C	A	C	C	A	C	C	A	C	C	A	A
A	H	H	H	H	H	B	A	H	B	H	H	A	H	B	A	H	H	A	H	H	A	A
A	C	C	C	C	C	C	A	C	C	C	C	A	C	C	A	C	C	A	C	C	A	A
A	C	C	C	C	C	C	A	C	C	C	C	A	C	C	A	C	C	A	C	C	A	A
A	C	C	C	C	C	C	A	C	C	C	C	A	C	C	A	C	C	A	C	C	A	A
A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
C	C	C	C	C	C	A	C	C	C	C	C	C	A	A	C	C	C	C	C	C	C	C
B	H	B	H	B	H	A	B	B	B	B	H	B	A	H	H	H	H	B	A	H	B	B

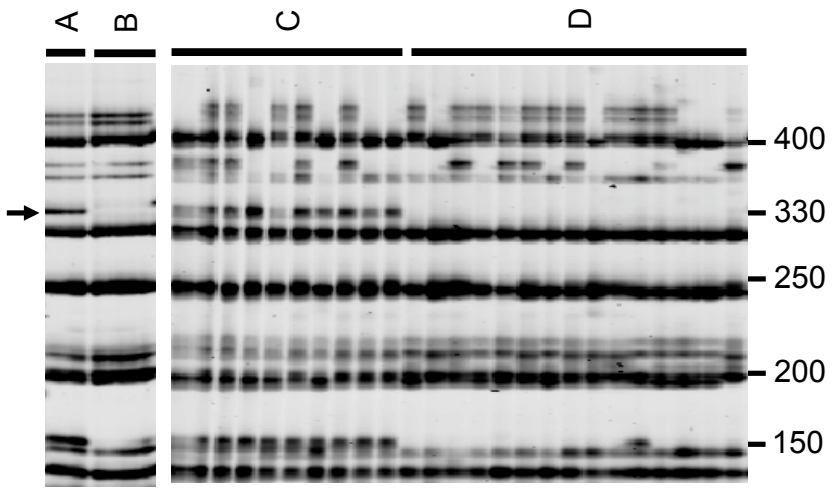
90_B(	91_B(	92_B(	93_B(	94_B(	95_B(	96_B(	97_B(	98_B(	99_B(	100_E	101_E	102_E	103_E	104_E	105_E	106_E	107_E	108_E	109_E	110_E	111_E	112_E
A	H	H	B	H	H	B	A	A	A	H	B	A	H	H	H	A	A	H	A	H	H	B
A	C	C	C	C	C	C	A	A	A	C	C	A	C	C	C	A	C	C	A	C	C	C
A	H	H	B	H	H	B	A	H	A	H	B	A	H	H	H	A	H	H	A	H	H	B
A	C	C	C	C	C	C	A	C	A	C	C	A	C	C	C	A	C	C	A	C	C	C
A	C	C	C	C	C	C	A	C	A	C	C	A	C	C	C	A	C	C	A	C	A	C
A	C	C	C	C	C	C	A	C	A	C	C	A	C	C	C	A	C	C	A	C	A	C
A	C	C	C	C	C	C	A	C	A	C	C	A	C	C	C	A	C	C	A	C	A	C
A	H	H	B	H	H	B	A	H	A	H	B	A	H	H	B	A	H	H	A	H	A	B
A	H	H	B	H	H	B	A	A	A	H	H	A	H	H	B	A	H	H	A	H	A	B
A	H	H	B	H	H	B	A	A	A	H	H	A	H	H	B	A	H	H	A	H	A	B
A	C	C	C	C	C	C	A	A	A	C	C	A	C	C	C	A	C	C	A	C	A	C
A	H	H	B	H	H	B	A	A	A	H	H	A	H	H	B	A	H	H	A	H	A	B
A	C	C	C	C	C	C	A	A	A	C	C	A	C	C	C	A	C	C	A	C	A	C
A	C	C	C	C	C	C	A	A	A	C	C	A	C	C	C	A	C	C	A	C	A	C
A	C	C	C	C	C	C	A	A	A	C	C	A	C	C	C	A	C	C	A	C	A	C
A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
C	C	C	A	C	C	A	C	C	C	C	A	C	C	C	A	C	C	C	C	C	C	A
A	H	H	A	B	H	H	B	H	H	H	A	B	B	H	A	H	H	H	B	H	A	A

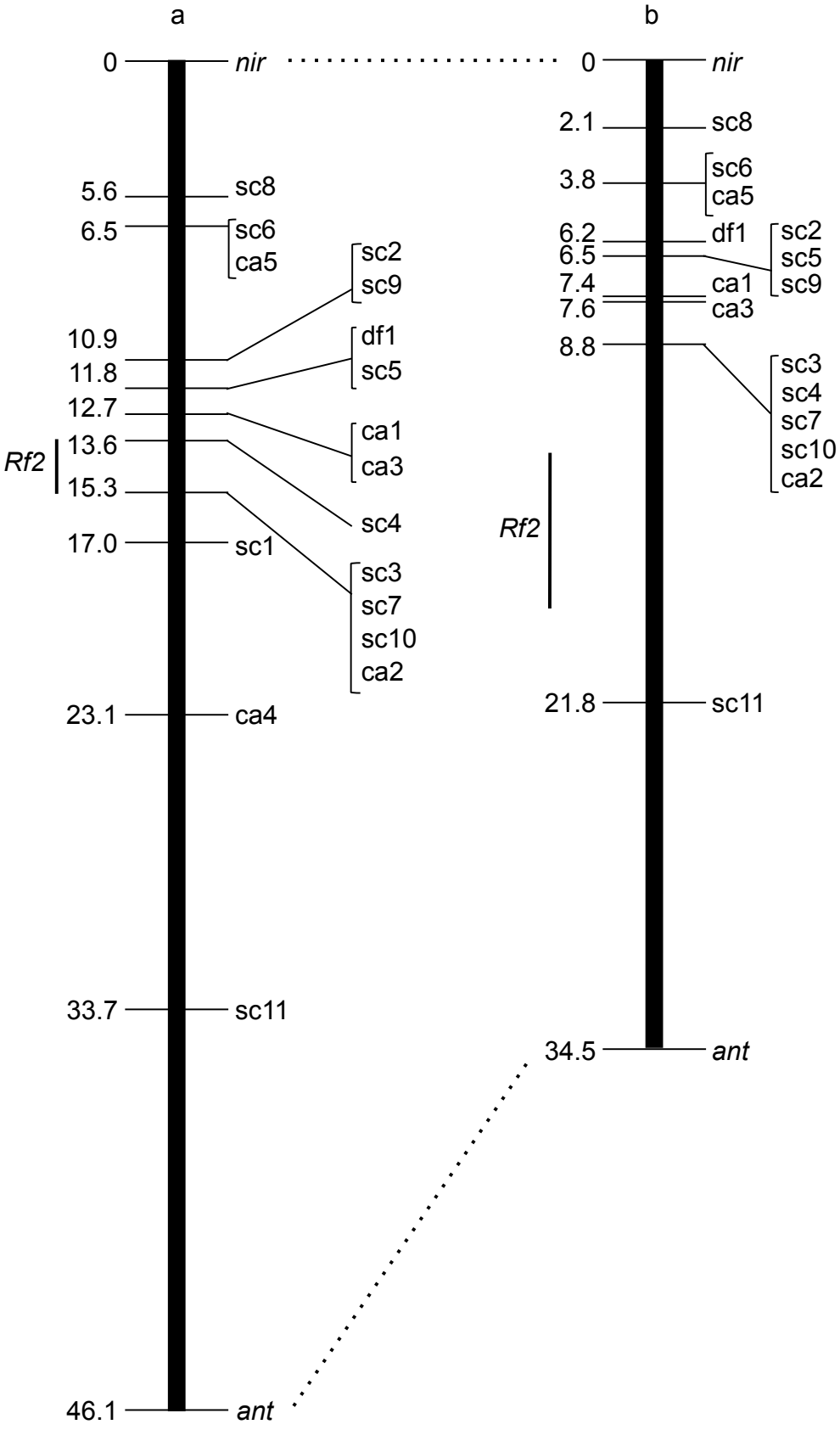
113_E	114_E	115_E	116_E	117_E	118_E	119_E	120_E	121_E	122_E	123_E	124_E	125_E	126_E	127_E	128_E	129_E	130_E	131_E	132_E	133_E	134_E	135_E
B	B	A	B	A	H	A	A	H	A	A	H	H	B	H	H	A	H	H	A	H	H	H
C	C	A	C	A	C	A	A	C	A	A	C	C	C	C	C	C	C	C	A	C	C	C
H	B	H	B	A	A	A	A	H	A	A	H	H	B	H	H	A	H	H	A	H	H	H
C	C	C	C	A	A	A	A	C	A	A	C	C	C	C	C	A	C	C	A	C	C	C
C	C	C	C	A	A	A	A	C	A	A	C	C	C	C	C	A	C	C	A	C	C	C
C	C	C	C	A	A	A	A	C	A	A	C	C	C	C	C	A	C	C	A	C	C	C
C	C	C	C	A	A	A	A	C	A	A	C	C	C	C	C	A	C	C	A	C	C	C
H	B	H	H	A	A	A	A	H	A	A	H	H	B	H	H	A	H	H	A	H	H	H
H	B	H	H	A	A	A	A	H	A	A	H	H	B	H	H	A	H	H	A	B	H	H
H	B	H	H	A	A	A	A	H	A	A	H	H	B	H	H	A	H	H	A	B	H	H
C	C	C	C	A	A	A	A	C	A	A	C	C	C	C	C	A	C	C	A	C	C	C
H	H	H	H	A	A	A	A	H	A	A	H	H	B	H	H	A	H	H	A	B	H	H
C	C	C	C	A	A	A	A	C	A	A	C	C	C	C	C	A	C	C	A	C	C	C
C	C	C	C	A	A	A	A	C	A	A	C	C	C	C	C	A	C	C	A	C	C	C
C	C	C	C	A	A	A	A	C	A	A	C	C	C	C	C	A	C	C	A	C	C	C
A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
C	C	C	C	C	C	C	C	C	C	C	C	C	A	C	A	C	C	C	C	A	C	C
H	B	H	H	B	B	B	B	H	H	H	A	B	A	H	A	B	A	H	B	A	H	A

136_E	137_E	138_E	139_E	140_E	141_E	142_E	143_E	144_E	145_E	146_E	147_E	148_E	149_E	150_E	151_E	152_E	153_E	154_E	155_E	156_B(	157_E	158_E
B	H	A	A	H	B	B	B	B	H	B	A	H	B	H	H	A	H	H	H	H	A	B
C	C	A	A	C	C	C	C	C	C	C	A	C	C	C	C	A	C	C	C	C	A	C
B	H	A	A	H	B	B	B	B	H	B	A	H	B	H	H	A	H	H	H	H	A	B
C	C	A	A	C	C	C	C	C	C	C	A	C	C	C	C	A	C	C	C	C	A	C
C	C	A	A	C	C	C	C	C	C	C	A	C	C	A	C	A	C	C	C	C	A	C
C	C	A	A	C	C	C	C	C	C	C	A	C	C	A	C	A	C	C	C	C	A	C
C	C	A	A	C	C	C	C	C	C	C	A	C	C	A	C	A	C	C	C	C	A	C
H	H	A	A	H	B	B	B	B	H	B	A	H	B	A	H	A	B	H	H	H	A	B
H	H	A	A	H	B	B	B	B	H	B	A	H	B	A	H	A	B	H	H	H	A	B
H	H	A	A	H	B	B	B	B	H	B	A	H	B	A	H	A	B	H	H	H	A	B
C	C	C	A	C	C	C	C	C	C	C	A	C	C	A	C	A	C	C	C	C	A	C
H	H	H	A	H	B	B	H	B	H	B	A	H	B	A	H	A	B	H	H	H	A	B
C	C	C	A	C	C	C	C	C	C	C	A	C	C	A	C	A	C	C	C	C	A	C
C	C	C	A	C	C	C	C	C	C	C	A	C	C	A	C	A	C	C	C	C	A	C
C	C	C	A	C	C	C	C	C	C	C	A	C	C	A	C	A	C	C	C	C	A	C
A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
C	C	C	C	A	A	C	C	A	C	A	C	C	C	C	C	C	A	C	C	C	C	C
H	H	H	B	A	B	H	H	A	H	H	B	H	H	B	H	B	A	A	H	B	B	H

159_E	160_E	161_E	162_E	163_E	164_E	165_E	166_E	167_E	168_E	169_E	170_E	171_E	172_E	173_E	174_E	175_E	176_E	177_E	178_E	179_E	180_E	181_E
B	B	B	H	B	H	B	H	H	H	A	H	B	H	H	A	H	H	H	A	A	A	H
C	C	C	C	C	C	C	C	C	C	A	C	C	C	C	A	C	C	C	A	A	A	C
H	B	B	H	B	H	B	H	H	H	A	H	H	H	H	A	H	H	H	A	A	A	H
C	C	C	C	C	C	C	C	C	C	A	C	C	C	C	A	C	C	C	A	A	A	C
C	C	C	C	C	C	C	C	C	C	A	C	C	C	C	A	C	C	C	A	A	A	C
C	C	C	C	C	C	C	C	C	C	A	C	C	C	C	A	C	C	C	A	A	A	C
C	C	C	C	C	C	C	C	C	C	A	C	C	C	C	A	C	C	C	A	A	A	C
H	B	B	H	B	H	B	H	H	H	A	A	H	H	H	A	H	H	H	A	A	A	H
H	B	B	H	B	H	B	H	H	H	A	H	H	H	H	A	H	H	H	A	A	A	H
H	B	B	H	B	H	B	H	H	H	A	H	H	H	H	A	H	H	A	A	A	A	H
C	C	C	C	C	C	C	C	C	C	A	C	C	C	C	A	C	C	A	A	A	C	C
H	B	B	H	B	H	B	H	H	H	A	H	H	H	H	A	H	H	A	A	A	H	H
C	C	C	C	C	C	C	C	C	C	A	C	C	C	C	A	C	C	A	A	A	C	C
C	C	C	C	C	C	C	C	C	C	A	C	C	C	C	A	C	C	A	A	A	C	C
C	C	C	C	C	C	C	C	C	C	A	C	C	C	C	A	C	C	A	A	A	C	C
A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A	A
C	A	A	C	A	C	A	C	C	C	A	C	C	C	C	C	C	C	C	C	C	C	C
B	A	A	H	A	B	A	B	B	H	A	B	H	H	H	H	H	B	B	H	B	H	A

			Chi-square test for segregation			
182_E	183_E	184_BC1F3	Genotype segregatior	Not available	χ^2	*p* -value
B	H	H	48 : 86 : 50 (A : H : B)	0	0.826 (1 : 2 : 1)	0.662
C	A	C	48 : 136 (A : C)	0	0.116 (1 : 3)	0.734
B	A	H	47 : 94 : 43 (A : H : B)	0	0.261 (1 : 2 : 1)	0.878
C	A	C	47 : 137 (A : C)	0	0.029 (1 : 3)	0.865
C	A	C	49 : 135 (A : C)	0	0.261 (1 : 3)	0.610
C	A	C	49 : 135 (A : C)	0	0.261 (1 : 3)	0.610
C	A	C	49 : 135 (A : C)	0	0.261 (1 : 3)	0.610
B	A	H	50 : 91 : 43 (A : H : B)	0	0.554 (1 : 2 : 1)	0.758
B	H	-[5]	48 : 93 : 42 (A : H : B)	1	0.443 (1 : 2 : 1)	0.802
B	H	H	49 : 93 : 42 (A : H : B)	0	0.554 (1 : 2 : 1)	0.758
C	C	C	46 : 138 (A : C)	0	0 (1 : 3)	1
B	H	B	46 : 96 : 42 (A : H : B)	0	0.522 (1 : 2 : 1)	0.770
C	C	C	46 : 138 (A : C)	0	0 (1 : 3)	1
C	C	C	46 : 138 (A : C)	0	0 (1 : 3)	1
C	C	C	46 : 138 (A : C)	0	0 (1 : 3)	1
A	A	A	-	0	-	-
A	A	A	-	0	-	-
A	C	A	42 : 142 (A: C)	0	0.464 (1 : 3)	0.496
A	H	A	44 : 92 : 48 (A : H : B)	0	0.174 (1 : 2 : 1)	0.917


A
B
C
D
400
330
250
200
150


a
b
0
nir
5.6
sc8
6.5
sc6
ca5
sc2
sc9
10.9
df1
11.8
sc5
12.7
ca1
13.6
ca3
Rf2
15.3
sc4
17.0
sc1
sc3
sc7
sc10
ca2
23.1
ca4
33.7
sc11
46.1
ant
0
nir
2.1
sc8
3.8
sc6
ca5
6.2
df1
6.5
sc2
sc5
7.4
ca1
sc9
7.6
ca3
8.8
sc3
sc4
sc7
sc10
ca2
Rf2
21.8
sc11
34.5
ant