Title: **Differences in *restorer-of-fertility 1* haplotype polymorphism are associated with crop history of garden beet and sugar beet (*Beta vulgaris* L.)**

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Key words

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Key message

Although garden beet and sugar beet belong to the same species, the polymorphic pattern of a gene that is important for hybrid breeding differs significantly between these two cultivar groups.

Abstract

Cytoplasmic male sterility (CMS) is used in breeding to facilitate hybrid seed production. *Restorer-of-fertility* (*Rf*), the suppressor of CMS, consists of a gene cluster with multiple haplotypes. Selection of restoring or non-restoring alleles depends on the discrimination of *Rf* haplotypes using DNA markers. The efficacy of this system is decreased if the *Rf* haplotype differs within the population of interest, which can occur during the crop evolution. In sugar beet, the *Rf1* gene has multiple haplotypes that are grouped by a linked polymorphic region (s17), with one group, termed p4, uniquely linked to the recessive *rf1* haplotype. Garden beet is the predecessor cultivar group of the sugar beet group. We questioned whether *Rf1* haplotypes differ between these two cultivar groups to assess the utility of marker-assisted selection. We analyzed 48 garden beet landraces and observed differences in the s17 polymorphism compared to sugar beet, suggesting that the *Rf1* haplotype frequency has changed during evolution of the crop. We next selected non-restoring genotypes from the garden beet landraces through test crosses and identified three recessive *rf1* haplotypes: one is identical to the p4 haplotype and the others are novel haplotypes. The p4 haplotype occurs in a few accessions and its frequency in garden beet is approximately 0.01. We analyzed the s17 polymorphism in modern garden beet hybrids and their constituents. We identified the p4 haplotype and suggest the presence of other recessive *rf1* haplotypes. Selection of p4 haplotype was efficient on the identification of non-restoring genotype in garden beet.

Introduction

Cytoplasmic male sterility (CMS) is used for hybrid seed production in a broad range of crops as it confers male-specific sterility while leaving other organs unaffected (reviewed in Xu et al. 2022). Expression of CMS is genetically conditioned by the presence of male sterility-inducing cytoplasm and the absence of its suppressor gene, known as *restorer-of-fertility* (*Rf*) (reviewed in Kitazaki et al. 2023). Plants with the desired combination of these genetic factors should be efficiently selected in hybrid breeding program, but this remains a practical challenge. DNA marker assisted selection is expected to address this issue, but the rationale for this approach is yet to be established. As our understanding of *Rf* genetics improves, we can elaborate a selection system for *Rf* alleles (see below).

Molecular cloning of *Rf* has facilitated advances in *Rf* genetics. While *Rf* gene products include pentatricopeptide repeat (PPR) proteins and other proteins, it has been observed that some *Rf* loci exhibit similar polymorphic patterns regardless of their gene products (Kubo et al. 2020). In these *Rf* loci, open reading frames (ORFs) that resemble each other form gene clusters, which are polymorphic in terms of ORF copy number and nucleotide sequence (Kato et al. 2007; Arakawa et al. 2020a). In sugar beet (*Beta vulgaris*), citrus (*Citrus* sp.) and rice (*Oryza sativa*), *Rf* gene clusters are considered as haplotypes, and attempts have been made to assign them to *Rf* allelomorphs (Arakawa et al. 2019; Goto et al. 2023; Zhao et al. 2023). The results lead to the discovery of weaker *Rf* alleles (semidominant *Rf*), revealing multiple allelism at some *Rf* loci, a concept that has been previously suggested from the perspective of classical genetics (Duvick 1965; Wise et al. 1996; Lee et al. 2008).

In both sugar beet and rice, variations in the copy number of the ORFs within *Rf* haplotypes have been associated with the differences in allelomorphs (Arakawa et al. 2019; Zhao et al. 2023), suggesting that *Rf* loci are complex loci and involve multiple ORFs in fertility restoration (Arakawa et al. 2020a). This implies that a specific ORF within a haplotype may not be the sole determinant of the allele's function; rather, the haplotype as a whole determines its function. If the *Rf* locus of interest is highly polymorphic and numerous *Rf* haplotypes are found in breeding materials, DNA marker-assisted selection of *Rf* alleles will be challenging without a clear rationale to differentiate between *Rf* haplotypes.

If the molecular basis of fertility restoration is well-established and allows to predict

the function of the haplotype of interest, the nucleotide sequence of the haplotype could help elucidate its functionality (c.f. Yamagishi et al. 2021). Another strategy consists in characterizing haplotype(s) preferred by the breeders in advance, and then selecting them from the breeding materials. This strategy is used in sugar beet breeding. Since the yield of sugar beet comes from the vegetative organ (root), hybrid sugar beet does not need to have male fertility restored, and the breeders' interest is on the recessive *rf* allele. Sugar beet breeders have made tremendous efforts to select genotypes unable to restore male fertility (i.e., lacking the dominant *Rf* allele), as the frequency of such genotypes is generally 3-5% (Bosemark 2006). These selected genotypes are termed “maintainers”. One of the sugar beet *Rf* genes, *Rf1*, has been cloned and encodes a protein similar to OMA1 in budding yeast, which is involved in quality control of mitochondria (Matsuhira et al. 2012). At the *Rf1* locus, ORFs encoding OMA1-like proteins (hereafter referred to as *orf20*-like ORFs) are clustered, with one to four (or possibly more) copies, and a large number of haplotypes have been identified (Moritani et al. 2013). Practically, *Rf1* haplotypes are grouped into five categories based on polymorphisms in a physically linked non-coding region targeted by the DNA marker s17 (Taguchi et al. 2014). Analysis of maintainers from Japan, the United States, and Europe has shown that breeders have selected three haplotypes as the recessive *rf1* (Moritani et al. 2013; Ohgami et al. 2016). One of the three recessive *rf1* haplotypes is uniquely associated with a specific s17 pattern, making DNA marker-assisted selection of maintainers feasible in sugar beet (Moritani et al. 2013; Taguchi et al. 2014).

From the perspective of molecular evolution, beet *Rf1* and PPR-type *Rf* in other plants display a similar evolutionary pattern, generating a large number of haplotypes (Fujii et al. 2011; Arakawa et al. 2020b). Given that crop domestication and subsequent selection were accompanied by a genetic bottleneck, the *Rf* haplotypes observed in a cultivar group represent only a subset of the entire *Rf* haplotypes in the gene pool. This raises the question of whether marker assisted selection of *Rf* (or *rf*) is also effective for other groups in the same species. For example, sugar beet originated from a few fodder beet cultivars at the end of the 18th century (Fischer 1989), while garden beet, the first cultivated root-type beet, appeared in the 16th century (Goldman and Janick 2021). We expected differences in the *Rf1* haplotype between garden beet and sugar beet, both in terms of haplotype repertoire and frequency.

Hybrid garden beet is gaining popularity, but selecting maintainers for this crop

remains very challenging (Goldman and Navazio 2008). The CMS-*Rf* system has been introduced from sugar beet into garden beet to facilitate hybrid breeding in this crop (Bliss and Gabelman 1965). Therefore, some modern garden beet varieties carry the sugar beet *rf1* haplotype, but it remains unknown whether garden beet possesses another *rf1* haplotypes absent in sugar beet. It is also necessary to examine whether, and how, DNA marker-assisted selection of the recessive *rf1* allele can be effectively applied in garden beet hybrid breeding.

In the present study, we demonstrate the differences in the *Rf1* haplotype between garden beet and sugar beet. Despite these differences, the DNA marker-assisted selection adopted for sugar beet has also proven effective for garden beet. Additionally, our study also suggests that *Rf* haplotype polymorphism is not only useful for selection but also provides insights into crop evolution.

Materials and methods

Plant materials

Garden beet and sugar beet accessions used in this study are summarized in Table 1. Accessions prefixed by 'BETA' or 'K' were obtained from The Leibniz Institute of Plant Genetics and Crop Plant Research (IPK), Germany. Garden beet lines W357B and W446B are maintainers developed at the University of Wisconsin (Goldman 1996; https://www.warf.org/technologies/agriculture/plant-varieties/summary/inbred-table-beet-w446a-and-w446b-p01012us.cmsx). Four garden beet hybrids, 'F1 Solo' (Mr. Fothergill's), 'F1 Pablo' (Johnsons Seeds), 'F1 Cardeal' (Johnsons Seeds) and 'F1 Red Titan' (Johnsons Seeds) were purchased online. All sugar beet accessions were obtained from the Hokkaido Agricultural Research Center, National Agriculture and Food Research Organization, Japan. The sugar beet cultivar 'Donyu-2' is a selection derived from the US cultivar 'GW359' and was released in 1954. Sugar beet cultivar 'Hon-iku 192' is derived from the German cultivar 'Kleinwanzlebener' and the French cultivar 'Virmolin White French' and was released in 1935. For the test cross to examine the genotype, we used the sugar beet line TA-33BB-CMS, which possesses a male sterility inducing cytoplasm (Matsuhira et al. 2022). TA-33BB-O line shares the same nuclear genotype as TA-33BB-CMS but differs in having non-sterility inducing cytoplasm. The

procedures for crossing using paper bags is detailed in Ohgami et al. (2016). Plants were grown in a greenhouse from the seedling stage to the young plant stage, after which they were transplanted into the experimental field of the Field Science Center for Northern Biosphere, Hokkaido University. Male fertility was evaluated according to Matsuhira et al. (2022) and Moritani et al. (2013).

Genotyping by DNA markers

Total cellular DNA was isolated according to a procedure described in Matsuhira et al. (2022). The cleaved amplified polymorphic sequence (CAPS) marker s17 is detailed in Taguchi et al. (2014). Oligonucleotide primers for s17 amplification are s17-Fw and s17-Rv (Table S1). PCR products were digested with the restriction endonucleases HapII and HindIII (Takara Bio, Kusatsu, Japan), and electrophoresed in a 1.5% agarose gel. For genotyping using dCAPS-p5, total cellular DNA was PCR amplified with the primers dCAPS-p5-Fw and dCAPS-p5-Rv (Table S1). The PCR products were then digested with HindIII.

Molecular cloning and nucleotide sequencing

For nucleotide sequencing, the s17 region was PCR amplified as three overlapping segments using three pairs of primers (s17-1-Fw and s17-1-Rv, s17-2-Fw and s17-2-Rv, and s17-3-Fw and s17-3-Rv) (Table S1). PCR products for sequencing were treated with Illustra Alkaline Phosphatase (0.1 unit) and Illustra Exonuclease I (1 unit) (GE Healthcare Life Sciences, Marlborough, MA, USA) at 37˚C for 30 min followed by incubation at 80˚C for 15 min. Alternatively, PCR products were electrophoresed in an agarose gel, and the PCR fragment was excised and purified by using the QIAquick Gel Extraction Kit (Qiagen, Tokyo, Japan). *orf20*-like ORFs were PCR amplified from total cellular DNA using the primer pairs orf20-infu-Fw / orf20-infu-Rv or orf20-infu2-Fw / orf20-infu2-Rv (Table S1). PCR products were purified via agarose gel electrophoresis as described above. The purified products were then mixed with a reaction buffer containing In-Fusion Snap Assembly Master Mix and linearized pUC19 plasmid vector DNA (Takara Bio). The mixture was incubated at 50˚C for 15 min, then chilled on ice before being used to transform *E. coli* strain DH5α. Transformants were selected on LB plate containing ampicillin (50 mg/L) and then cultured in liquid LB medium with the same amount of

ampicillin (Sambrook et al. 1989). Plasmid DNA was extracted using the QIAprep Spin Miniprep Kit (Qiagen). Plasmid DNA sequencing was performed using an ABI3130 Genetic Analyzer (Thermo Fisher Scientific, Waltham, MA, USA) with primers M13-Fw, M13-Rv, MPL-intron-Fw, and MPL-intron-Rv (Table S1). PCR amplified *orf20*-like ORF segments were directly sequenced with primers p4-1-Fw, p4-1-Rv, p4-2-Fw, p4-2-Rv, p4-3-Fw and p4-3-Rv (Table S1). Nucleotide sequence analyses were performed using Clustalw (https://www.genome.jp/tools-bin/clustalw) or MEGA 11 (Tamura et al. 2021). Phylogenetic trees were constructed using the neighbor-joining method with default parameters. Nucleotide sequences reported in this study are available under accession numbers AB646133, AB646135, AB646136, LC085626, LC085628, LC385768, LC849784, LC849785, LC849786, LC849787, LC849788, LC849789, LC849790 and LC849791.

Statistical analysis

Fisher's exact test was performed using a webtool (http://aoki2.si.gunma-u.ac.jp/exact/fisher/getpar.html) or the fisher.multcomp function from the R package RVAideMemoire (https://cran.r-project.org). The *P* value adjustment method used was Holm.

Results

Polymorphic patterns of s17 differ between garden beet and sugar beet

To examine the *Rf1* haplotype polymorphism in garden beet, we analyzed the polymorphism of s17, a multiallelic CAPS marker linked to *Rf1* (Taguchi et al. 2014) (Fig. 1). The s17 marker targets a noncoding region located 3.9 kbp away and its polymorphism serves as a useful proxy for estimating *Rf1* polymorphism, as s17 can differentiate *Rf1* haplotypes into several distinct groups (Taguchi et al. 2014). We selected 48 garden beet accessions that are registered as landraces in the genebank of IPK. Although five alleles of s17 are known (p1 to p5), our initial analysis revealed that the frequency was highly skewed towards p5 (>0.9). To determine whether p5 is monomorphic, we sought novel polymorphisms within the p5 allele. We selected one to two plants homozygous for p5 from each accession and determined the nucleotide sequences of their s17 target regions. Comparison of the sequences obtained from 55 plants revealed ten single

nucleotide polymorphic sites (SNPs) which allowed us to group the sequences into three distinct groups (Fig. S1). As two of the three groups are nearly identical, differing by only one nucleotide, we classify p5 into two subtypes: p5A and p5B (Fig. S1). We developed a dCAPS marker (dCAPS-p5) that targets one of the SNPs to differentiate between p5A and p5B (Fig. 1 and detailed in Fig. S1). Plants with p5 were further analyzed using dCAPS-p5.

A total of 485 plants from 48 garden beet accessions were genotyped (Table S2). s17 allelic frequencies of garden beet accessions are shown in Table 1. The frequency is highest in p5A, followed by p5B, p1, p2, p3, and p4, in this order. The combined frequency of p5A and p5B exceeds 0.95. Every garden beet accession used in this study contains either p5A (7 accessions), p5B (8 accessions), or both (33 accessions). We observed a trend where the frequencies of each p1 through p4 are very low (~0.01), with these alleles absent in 31 accessions.

Our study included two sugar beet accessions derived from two old Japanese open-pollinated cultivars (OPCs), 'Donyu-2' and 'Hon-iku 192'. The genotypes and their allelic frequencies are shown in Table S2 and Table 1, respectively. The allelic frequencies in 'Donyu-2' and 'Hon-iku 192' are similar, except for p4, which is reduced in 'Hon-iku 192'. No plants were found to have the p2 allele. Taguchi et al. (2014) analyzed several sugar beet OPCs, though they did not distinguish between p5A and p5B. They reported the allelic frequencies of seven sugar beet OPCs as follows: p1, 0-0.19 (mean 0.10); p2, 0 (0); p3, 0.04-0.31 (0.14); p4, 0-0.17 (0.09); and p5 (which can be considered as the sum of p5A and p5B), 0.41-0.88 (0.67) (Taguchi et al. 2014). Therefore, the data from 'Donyu-2' and 'Hon-iku 192' are consistent with the findings of Taguchi et al. (2014). A comparison of the allelic frequencies of 'Donyu-2' and 'Hon-iku 192' with those of garden beet reveals that p5A is significantly reduced in sugar beet while p4 is increased.

Maintainer selection from garden beet genetic resources: Is p4 selection also effective in garden beet?

To identify maintainers (i.e., genotypes without the restoring *Rf* allele) from garden beet genetic resources, we conducted test crosses. We selected 31 plants from 17 accessions, each one with six s17 alleles, and used them as pollen parent to cross with a sugar beet CMS line TA-33BB-CMS. Since the s17 genotype of TA-33BB-CMS is p4p4, the F1 plants should have one p4 allele. The

F1 plants were grown and flowered in the field and phenotyped for pollen fertility. The results are shown in Table 2.

We examined the male fertility of a total of 317 F1 plants. Of these, 52 were completely sterile, while the remaining were either male-fertile or semi-sterile (Table 2). The relationship between the pollen parental s17 allele and male fertility of the F1 plants is summarized in Figure 2A. Combining male-fertile and semi-sterile phenotypes as “fertility restored”, the ratios of fertility restored to complete sterility varies among the s17 alleles (Fig. 2B). We conducted pairwise comparisons of Fisher's exact test to determine if the occurrence of complete sterility differs among the alleles (Table S3 and Fig. 2B). The results suggest that complete sterility occurs most frequently in p4, followed by p3 and p5B, whereas very few or none in p1, p2 and p5A.

Of the 31 cross combinations, #4, #6 and #24 resulted in F1 plants with complete sterility. In cross combination #6, one of the expected F1 genotype (p4p5) was missing for unknown reason, so we could not definitively conclude that the pollen parent of #6 was a maintainer. Since the number of F1 plants from cross #24 was rather small, we performed additional crosses. One of the F1plants from #24 was used as a seed parent to cross with TA-33BB-O, a maintainer with the same nuclear genotype as TA-33BB-CMS (hence this cross is effectively a backcross). The six BC1 plants obtained were completely sterile in the field.

We focused on the accession BETA 1229 that provided the p4 allele in our test crosses (Table 2). Because p4 is uniquely linked to a recessive *rf1* haplotype preferred by sugar beet breeders (Taguchi et al. 2014), p4 selection has been adopted in sugar beet maintainer selection. Using BETA 1229, we tested whether p4 selection is also effective in garden beet. We genotyped 253 plants of BETA 1229 and identified a p4p4 homozygous plant. This plant was used as the pollen parent for test crosses, and seven F1 plants were obtained. All the seven plants were p4p4 and completely male sterile in the field.

Garden beet recessive rf1 haplotypes are either identical or different from sugar beet

We wanted to know whether the recessive *rf1* haplotypes in garden beet are the same as those in sugar beet. We first examined p4 of BETA 1229. As *rf1* haplotypes are composed of *orf20*-like ORFs (e. g., Arakawa et al. 2020a) (see Fig. 1), we investigated the nucleotide sequence of BETA 1229 p4. The p4 homozygote identified in the previous section was subjected to PCR

amplification of *orf20*-like ORFs. The PCR products yielded a single nucleotide sequence (Fig. S2) which is identical to the one found in the recessive *rf1* haplotype marked by p4 (TK-81mm-O *rf1* in Arakawa et al. 2020a). As no trace of other *orf20*-like ORF was observed, we concluded that this haplotype is identical to TK-81mm-O *rf1*.

We next investigated the haplotype marked by p5B in cross combination #4. One of the F1 plants in #4 was subjected to the molecular cloning of *orf20*-like ORFs. All the *orf20*-like ORFs in this plant were PCR amplified and cloned into a plasmid vector. Multiple recombinant plasmids were analyzed, and five *orf20*-like ORF sequences were obtained, one of which appeared to be derived from the seed parent TA-33BB-CMS. The remaining four are therefore derived from the haplotype marked by p5B. We compared these sequences to the *orf20*-like ORFs identified so far in sugar beet. For this comparison, we chose a total of eleven *orf20*-like ORFs of sugar beet: four from dominant NK-198 *Rf1*, two from semidominant NK-305 *Rf1*, one from recessive TK-81mm-O *rf1*, three from recessive NK-219mm-O *rf1*, and one from recessive PI 615522 *rf1*. No sugar beet copies were found to be identical to the *orf20*-like ORF of p5B of cross #4. We referred the *orf20*-like ORF copies of p5B of #4 to BETA 1229 p5B Copies 1 to 4. Nucleotide identities of coding regions and gene regions (exons and introns) are shown in Tables S3 and S4, respectively.

We analyzed p3 in cross #24. Copies of the *orf20*-like ORF were sequenced using a complete sterile F1 plant from #24. Aside from those derived from TA-33BB-CMS, we obtained one *orf20*-like ORF sequence. As this sequence was not identical to any sugar beet copies identified so far, it was referred to as BETA 223 p3 Copy 1. Nucleotide identities of coding regions and gene regions with other *orf20*-like ORFs are shown in Tables S3 and S4, respectively.

We explored the relationship between the novel *orf20*-like ORFs and the sugar beet copies by constructing phylogenetic trees of 16 *orf20*-like ORFs including those from sugar beet and garden beet. The results were consistent across both coding regions (exons) and entire gene regions (exons and introns) (Fig. 3 and Fig. S3). A notable cluster includes *orf20*-like ORFs from NK-198 *Rf1* and NK-305 *Rf1*. However, some of the *orf20*-like ORFs from these *Rf1* alleles are outside of this cluster (i. e., $orf20_{\text{NK-198-2}}$ and $orf20_{\text{NK-305-2}}$). Similarly, not all the *orf20*-like ORFs from recessive *rf1* alleles are clustered. The novel *orf20*-like ORFs identified from garden beet are clustered with the sugar beet copies. For example, BETA 223 p3 Copy 1 is clustered with the

293 copy of PI 615522 *rf1* (Fig. 3). Note that they share features such as being marked by p3 and
294 unable to restore male fertility (Ohgami et al. 2016), suggesting that PI 615522 *rf1* and BETA223
295 p3 haplotypes are close relatives. Two copies of BETA 1229 p5B cluster with those from recessive
296 *rf1* haplotype and dominant *Rf1* haplotype, respectively. The remaining two copies (Copies 2 and
297 4) are relatively isolated.
298
299 *Haplotypes used in hybrid garden beet*
300 We investigated the recessive *rf1* haplotypes currently used in garden beet breeding. First, we
301 genotyped two garden beet maintainer lines W357B and W446B, that were developed in the
302 United States, using s17 and dCAPS-p5 markers. The results are shown in Table S2 (genotypes)
303 and Table 1 (allelic frequencies). We found that W357B is fixed with p4 whereas p4 and p5B
304 were found in W446B. To examine whether p5B in W446B is linked to recessive *rf1*, we selected
305 one of the W446B plants with the p4p5B genotype and crossed it with TA-33BB-CMS. Of the
306 ten F1 plants obtained, six were completely male sterile with a p4p4 genotype and four were male
307 fertile with a p4p5B genotype. Therefore, p5B in W446B is linked to the dominant *Rf1*. To
308 determine the identity of p4 in W357B, we selected one p4p4 homozygote and determined the
309 nucleotide sequence of its *orf20*-like ORF. The sequence obtained was identical to that of TK-
310 81mm-O *rf1*.
311 We next investigated the s17 genotype of four commercial hybrid garden beets
312 developed in the UK. Although we do not have detailed information about their breeding methods
313 (e. g., whether they are developed by single cross or three-way cross, or whether the pollen parent
314 of the hybrid was a maintainer or not), the hybrids are expected to contain the recessive *rf1* allele.
315 We found that p5B is prevalent in 'F1 Solo' and 'F1 Pablo', that four s17 alleles (p3, p4, p5A and
316 p5B) were found in 'F1 Cardeal', and all the examined plants of 'F1 Red Titan' had the p4p5A
317 genotype. Therefore, the recessive *rf1* haplotype linked to p4 is used in current garden beet hybrid
318 breeding. We cannot rule out that other recessive haplotypes are also utilized.
319
320
321 Discussion
322 Hybrid breeding programs of garden beet are prevalent and will continue to develop high

performance varieties (Goldman and Navazio 2003). Therefore, understanding the polymorphism of the garden beet *Rf1* locus is crucial for expediating breeding, particularly when CMS is used for hybrid seed production. In the present study, we evaluated the *Rf1* haplotype polymorphism based on the alleles of s17. Our analysis of garden beet genetic resources suggests that the allelic frequency of s17 is different between garden beet and sugar beet, despite both belonging to *B. vulgaris*. Wu et al. (2024) reported the prevalence of p5 in some garden beet varieties in China, the United States, the United Kingdom and the Netherlands. Note that the accessions used in this study represent a subset of garden beet and sugar beet gene pool, and a more comprehensive study is necessary. The present study provides a good example of how frequency of the *Rf* haplotype can differ between cultivar groups within a crop species. It is possible that the differences observed in this study are associated with the history of the garden beet and sugar beet groups. Garden beet is the first beet cultivar group with swollen roots, from which fodder beet originated (Ford-Lloyd 1995). Sugar beet was selected from fodder beet, but unintentional crosses with other beets, such as leaf beet, is associated with the initial breeding material (Fischer 1989). Such events invoke decrease in population size and the introduction of foreign germplasm, mechanisms that could explain the changes in s17 allelic frequencies. We may focus on p1, p3 and p4 as their frequencies appear to be increased in sugar beet. Although these alleles occur sporadically in European garden beet, they appear to be slightly more frequent in accessions from the East Mediterranean and Black Sea coasts, including regions such as Georgia (p1 and p3), Romania (p1 and p4), Greece (p3) and Turkey (p4). Wascher et al. (2022) analyzed European wild beet (*B. vulgaris* ssp. *maritima*) genomes and found the closest relative of sugar beet in Greece. The role of wild and cultivated beets in this region in the domestication of sugar beet is an interesting question that warrants further investigation.

We conducted test crosses to identify maintainers from the garden beet genetic resources. Although alleles of s17 do not necessarily correspond to a single *Rf1* haplotype but rather to groups of haplotypes, our data reveals certain trends that can assist in selecting maintainers in garden beet. We observed that no or few male sterile F1 plants occurred in certain p17 alleles, such as p1, p2 and p5A, in stark contrast to p4. The alleles of p5A and p3 gave rise to some male sterile plants, but the occurrence frequencies were significantly lower compared to p4. Considering that the majority of garden beets possess p5A or p5B, and that the occurrence of male

sterile F1 with these alleles is 0.06 and 0.24, respectively, we believe that selecting garden beet maintainers from these genetic resources is difficult. This is consistent with Goldman and Navazio (2008) assessment that some garden beet genetic backgrounds are much more recalcitrant sources for maintainers. P4 is a potentially promising s17 allele; unfortunately, its frequency appeared to be very low in the garden beet genetic resources we assessed.

We obtained maintainers from BETA 1229 (Turkey origin) and BETA 223 (Georgia origin) which possess p3, p4 and p5B. We investigated the identity of these recessive *rf1* haplotypes, and to our surprise, BETA 1229 p4 is linked to a recessive *rf1* haplotype identical to that of sugar beet (i.e., TK-81mm-O *rf1*). In sugar beet, *rf1* haplotypes linked to p4 have been sequenced from several different sources and were found to be identical to TK-81mm-O *rf1* at the nucleotide sequence level (Moritani et al. 2013; Ohgami et al. 2016). On the other hand, morphologically, BETA 1229 appears to be a typical garden beet and different from sugar beet (Fig. S4). The TK-81mm-O *rf1* haplotype may be key to understanding the genetic origin of sugar beet.

The *rf1* haplotype linked to BETA 223 p3 is similar to one of the previously identified recessive *rf1* haplotype PI 615522 *rf1*. Although these two alleles are apparently unable to restore male fertility, it is too early to conclude on their usefulness because, for unknown reasons, not all sugar beet breeders have selected PI 615522 *rf1* as maintainer (Ohgami et al. 2016). It is possible that PI 615533 *rf1* and BETA 223 p3 are associated with an undesirable trait. The utility of BETA 1229 p5B should be thoroughly investigated before incorporating it into a breeding program.

Our study revealed the usefulness of p4 (i.e., TK-81mm-O *rf1*) in the current garden beet hybrid breeding. Garden beet maintainers W357B and W446B have complex genealogies involving several lines and cultivars (Goldman 1996). It is possible that a garden beet like BETA 1229 did provide p4 to these maintainer lines. Another possibility is that Warren H. Gabelman, who introduced the CMS system into garden beet (Bliss and Gabelman 1965), may have introduced p4 from sugar beet. The genealogy of W357B includes sugar beet, and US sugar beet maintainers frequently possess the p4 allele (Ohgami et al. 2016). We favor the latter possibility because p4 is rare in garden beet landraces. Altogether, garden beet breeders consider the recessive *rf1* haplotype linked to p4 as a reliable and practical allele. Since no dominant *Rf1* haplotype linked to p4 has been found in garden beet so far, selection of a p4p4 homozygote may

be one of the most effective methods for identifying maintainers in garden beet breeding programs, as demonstrated in the case of BETA 1229, although further study is needed.

It is clear that several recessive *rf1* haplotypes, in addition to p4, are used in garden beet hybrids as some commercial hybrids lack the p4 allele. It has been pointed out that the repertoire of recessive *rf1* haplotypes in sugar beet is limited, and genetic vulnerability is a potential concern (Taguchi et al. 2014). Novel *rf1* haplotypes could widen the choice for sugar beet breeder. However, it should be kept in mind that the seed production methods used in the breeding stations (e.g., field or green house) may impact CMS expression (c. f., Matsuhira et al. (2022)), thus requiring careful evaluation.

Our study focused on *rf1* without considering other factors, such as the presence of other *Rf* genes or environmental conditions (Theurer and Ryser 1969; Matsuhira et al. 2022; Honma et al. 2014; Arakawa et al. 2018). The impact of these factors on garden beet is still unknown. Selection of p4 is recommended as a means to expedite maintainer identification in this crop, but it is necessary to investigate the selected plants in detail.

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Statements and Declarations

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Competing interest

The authors have no relevant financial or non-financial interest to disclose.

Author contributions

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Figure legends

Fig. 1 Schematic illustration of the s17 target region and its alleles. Scale bars are shown below each panel. These panels are based on Figure 1 of Taguchi et al. (2014) with some modifications. **A.** Organization of chromosomal segment containing the *Rf1* locus and the s17 target region. The *Rf1* locus consists of *orf20*-like ORFs, which have slightly differing nucleotide sequences. Each *orf20*-like ORF consists of three exons (open boxes) and two introns (wedges). The copy number of *orf20*-like ORFs varies among the haplotypes (indicated by dashed line). The direction of transcription is indicated by arrows. s17 (shown as a red bold line) is ~3.9 kbp away from the *Rf1* locus. **B.** Organization of six s17 alleles, p1 to p5B. Wedges with dotted lines indicate deletions. Positions of HindIII and HapII recognition sites are shown by filled- and open triangles, respectively. The polymorphic site to distinguish p5A and p5B alleles is shown (A:T or G:C).

Fig. 2 Inherited s17 allele from pollen parent and its impact on male fertility phenotype in the F1 progeny. **A.** Number of plants with male fertile (MF), semi-sterility (SS), fertility restored, and complete sterility (CS) phenotypes. The F1 plants with unknown p5 identity are omitted. **B.** Comparison of ratios of fertility restored and complete sterile plants among s17 alleles. ns, nonsignificant pair as determined by Fisher's exact test (Table S3).

Fig. 3 Neighbor-Joining tree of *orf20*-like ORFs constructed using the nucleotide sequence of amino acid coding regions. Labels are as follows (see also Arakawa et al. 2020a): NK-198-1, *orf20* $_{\text{NK198-1}}$; NK-198-2, *orf20* $_{\text{NK198-2}}$; NK-198-3, *orf20* $_{\text{NK198-3}}$; NK-198-4, *orf20* $_{\text{NK198-4}}$; NK-219-1, *orf20* $_{\text{NK219-1}}$; NK-219-2, *orf20* $_{\text{NK219-2}}$; NK-219-3, *orf20* $_{\text{NK219-3}}$; NK-305-1, *orf20* $_{\text{NK305-1}}$; NK-305-2, *orf20* $_{\text{NK305-2}}$; PI 615522, *orf20* $_{\text{PI 615522}}$; and TK-81, *orf129* $_{\text{TK-81}}$. Copies from dominant- and semidominant *Rf1* haplotypes are highlighted in red. Copies from recessive *rf1* haplotypes are highlighted in blue. The following labels denote copies identified in this study: BETA 1229 Copy 1, BETA 1229 p5B Copy 1; BETA 1229 Copy 2, BETA 1229 p5B Copy 2; BETA 1229 Copy 3, BETA 1229 p5B Copy 3; BETA 1229 Copy 4, BETA 1229 p5B Copy 4; and BETA 223 Copy 1, BETA 223 p3 Copy1.

545 Table 1 Allelic frequency of s17 in garden beet genetic resources, sugar beet, garden beet
546 maintainer lines, and garden beet commercial hybrids.

Accession	Country of origin	Number of s17 allele						Total number of alleles
		p1	p2	p3	p4	p5A	p5B	
Garden beet genetic resources								
BETA 1032	TURKEY	0	0	0	0	16	6	22
BETA 1037	GEORGIA	0	0	0	0	25	13	38
BETA 1058	GERMANY	0	0	0	2	37	3	42
BETA 1065	IRAN	0	9	0	0	11	6	26
BETA 1159	GREECE	0	0	1	0	19	8	28
BETA 1165	GREECE	0	0	0	0	9	15	24
BETA 1229	TURKEY	0	0	0	6	29	3	38
BETA 1257	UZBEKISTAN	0	0	0	0	4	2	6
BETA 1285	AZERBAIJAN	0	0	0	0	12	12	24
BETA 1306	GREECE	0	0	0	0	14	0	14
BETA 1343	PAKISTAN	2	0	0	0	2	24	28
BETA 1388	SOVIET	0	0	0	0	27	3	30
BETA 1463	GREECE	0	0	0	0	14	0	14
BETA 1468	GREECE	0	0	0	0	10	4	14
BETA 1478	GREECE	0	0	0	0	13	23	36
BETA 155	RUSSIA	0	0	0	0	0	18	18
BETA 1594	TURKEY	0	0	0	0	15	7	22
BETA 1618	UNKNOWN	0	0	0	0	19	25	44
BETA 1681	GREECE	0	0	0	0	8	0	8
BETA 1744	GREECE	0	0	0	0	0	14	14
BETA 1774	GERMANY	0	0	0	0	0	2	2
BETA 1777	GERMANY	0	0	1	0	5	6	12
BETA 179	CHINA	0	0	0	0	4	10	14

BETA 1795	NETHERLAND	0	0	0	0	4	0	4
BETA 184	SLOVAKIA	0	0	0	0	8	12	20
BETA 187	GEORGIA	5	0	1	0	1	11	18
BETA 1901	FRANCE	0	0	0	0	0	44	44
BETA 2040	NETHERLAND	0	0	0	0	24	2	26
BETA 2056	FRANCE	0	0	0	0	0	20	20
BETA 2071	ITALY	0	0	1	0	35	2	38
BETA 2129	USA	0	0	0	0	12	12	24
BETA 222	POLAND	0	0	0	0	6	0	6
BETA 223	GEORGIA	0	5	3	0	1	7	16
BETA 245	IRAQ	0	0	0	0	17	7	24
BETA 248	SPAIN	1	0	0	0	0	7	8
BETA 273	TAJIKISTAN	0	0	0	0	25	3	28
BETA 33	CANADA	0	0	0	0	12	2	14
BETA 334	ROUMANIA	0	0	0	1	1	0	2
BETA 336	SLOVAKIA	3	0	0	0	1	2	6
BETA 340	ROUMANIA	1	0	0	0	14	1	16
BETA 355	GEORGIA	0	0	0	0	5	1	6
BETA 3818	CUBA	0	0	0	0	10	4	14
BETA 3861	GEORGIA	0	0	0	0	6	2	8
BETA 3879	GEORGIA	4	0	0	0	0	18	22
BETA 3881	GEORGIA	0	0	0	0	22	0	22
BETA 965	UNKNOWN	3	0	0	0	16	25	44
BETA1723	ITALY	0	0	0	0	0	2	2
K 7136	GEORGIA	0	0	3	0	12	5	20
Garden beet genetic resources total		19	14	10	9	525	393	970
(Ratio)		(0.02)	(0.01)	(0.01)	(0.01)	(0.54)	(0.41)	(1)

Sugar beet

Donyu-2	JAPAN	4	0	4	19	2	35	64
Hon-iku 192	JAPAN	2	0	2	2	2	32	40
Suga beet total		6	0	6	21	4	67	104
(Ratio)		(0.06)	(0)	(0.06)	(0.20)	(0.04)	(0.64)	(1)

Garden beet maintainer

W357B	USA	0	0	0	28	0	0	28
W446B	USA	0	0	0	12	0	6	18
Garden beet maintainer total		0	0	0	40	0	6	46
(Ratio)		(0)	(0)	(0)	(0.87)	(0)	(0.13)	(1)

Garden beet commercial hybrid

F1 Solo	UK	0	0	0	1	0	19	20
F1 Pablo	UK	0	0	0	0	0	20	20
F1 Cardeal	UK	0	0	6	11	2	1	20
F1 Red Titan	UK	0	0	0	10	10	0	20
Garden beet hybrid total		0	0	6	22	12	40	80
(Ratio)		(0)	(0)	(0.075)	(0.275)	(0.15)	(0.5)	(1)

547

548

549

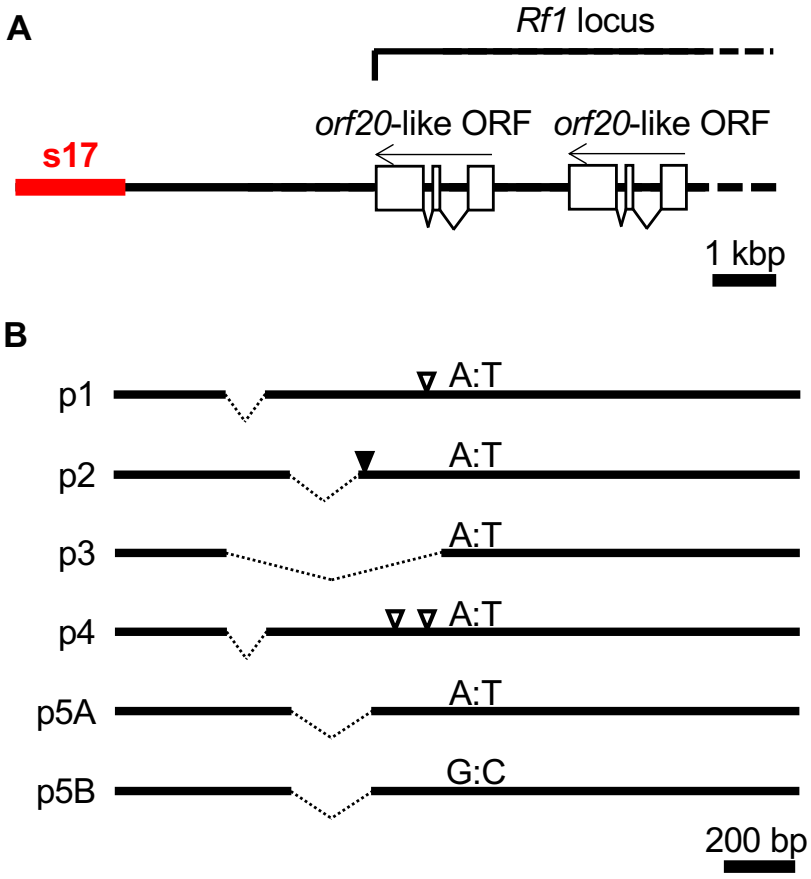
550 Table 2 Cross combinations, s17 genotype of pollen parent and F1 plants, and phenotype of F1
551 plants.

Cross combination #	Pollen parent		F1				
				Pollen fertility phenotype and number of plants [2]			
	Accession	s17 genotype	s17 genotype [1]	MF	SS	CS	Total
1	BETA 1058	p5Ap5A	p4p5	8	1		9
2	BETA 1065	p2p5A	p2p4	12			12
			p4p5	3			3
3	BETA 1066	p2p5B	p2p4	4	5	1	10
			p4p5		3		3
4	BETA 1229	p4p5B	p4p4			8	8
			p4p5			8	8
5	BETA 1229	p5Ap5A	p4p5	1	3	2	6
6	BETA 1229	p4p5A	p4p4			2	2
7	BETA 1229	p5Ap5A	p4p5	6		1	7
8	BETA 1343	p5Bp5B	p4p5	8	2		10
9	BETA 1343	p5Ap5B	p4p5	2	10	1	13
10	BETA 1478	p5Ap5B	p4p5	16			16
11	BETA 1478	p5Bp5B	p4p5	6	8		14
12	BETA 187	p1p5B	p1p4	3	3		6
			p4p5	2	6		8
13	BETA 187	p5Bp5B	p4p5	10	1	4	15
14	BETA 2040	p5Ap5A	p4p5	6	2	1	9
15	BETA 2040	p5Ap5B	p4p5	3	1	2	6
16	BETA 2040	p5Ap5A	p4p5	8			8
17	BETA 2040	p5p5 [1]	p4p5	4			4
18	BETA 2056	p5Bp5B	p4p5	13	2		15
19	BETA 2058	p5Ap5A	p4p5	4			4

20	BETA 2071	p5Ap5A	p4p5	7	3		10
21	BETA 2071	p5Ap5A	p4p5	3	1		4
22	BETA 223	p3p5B	p3p4	4	6		10
			p4p5		2	5	7
23	BETA 223	p2p5B	p2p4		1		1
24	BETA 223	p3p3	p3p4			2	2
25	BETA 245	p5Ap5B	p4p5	3	6	5	14
26	BETA 340	p1p5A	p1p4	4	6		10
			p4p5	4			4
27	BETA 3879	p5Bp5B	p4p5	1	9	6	16
28	BETA 3879	p1p5B	p1p4	2	3		5
29	BETA 965	p5Ap5B	p4p5	14	2		16
30	BETA 965	p1p5A	p1p4	7	1		8
			p4p5	6			6
31	K 7136	p3p3	p3p4		4	4	8
Total				174	91	52	317

[1] Type of dCAPS-p5 is not determined.

[2] Abbreviations are: MF, male-fertile; SS, semi-sterile; and CS, complete sterility.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p2
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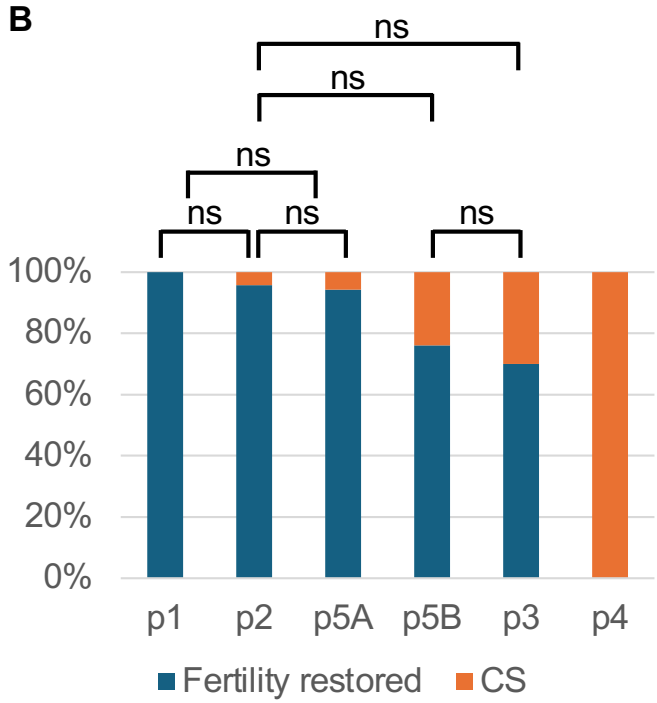
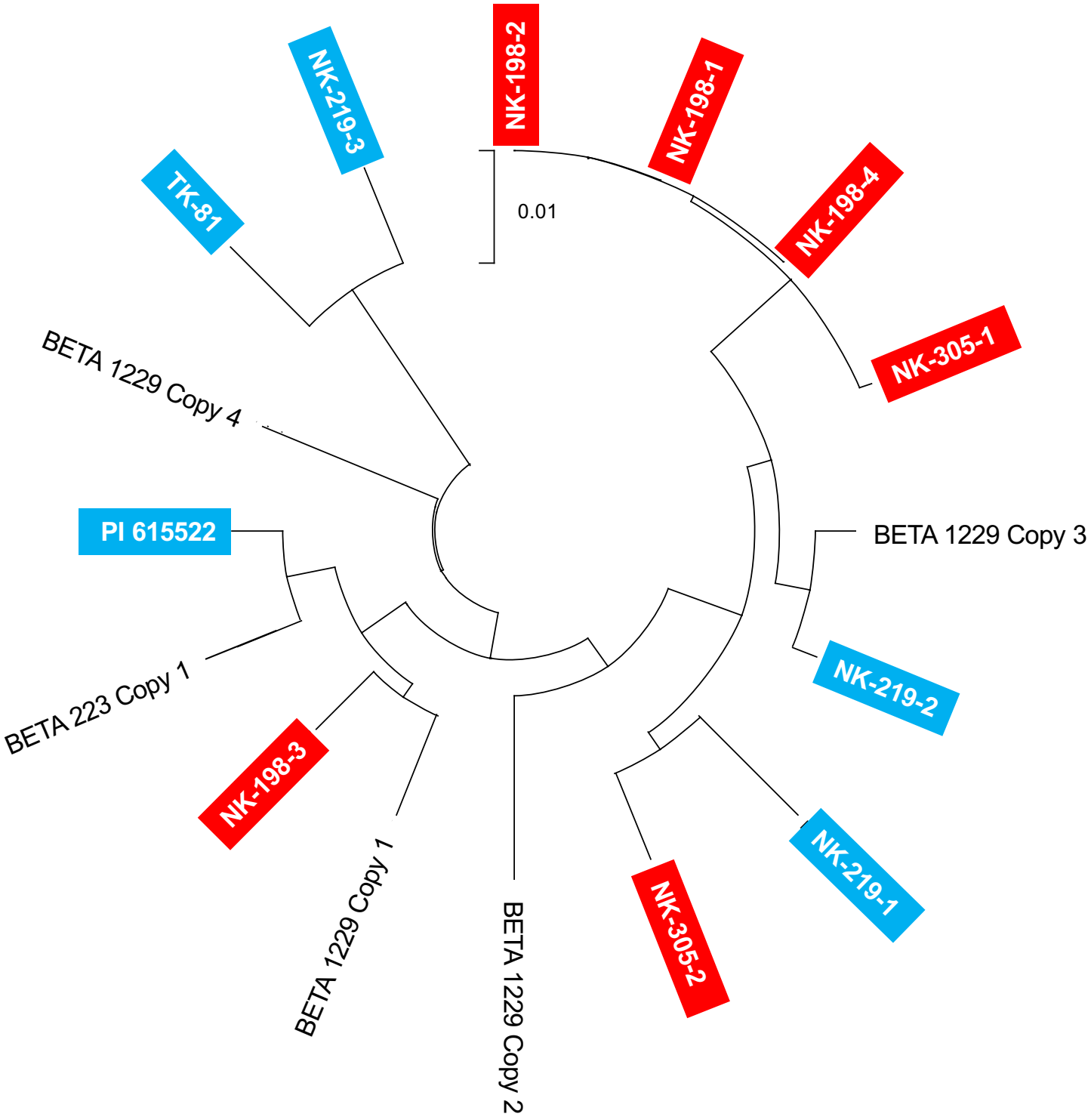
p4
A:T
p5A
A:T
p5B
G:C
200 bp

A

s17 allele from pollen parent	Pollen fertility phenotype and number of plants			
	MF	SS	CS	Total
	Fertility restored			
p1	16	13	0	29
	29			
p2	16	6	1	23
	22			
p3	4	10	6	20
	14			
p4	0	0	10	10
	0			
p5A	56	10	4	70
	66			
p5B	40	33	23	96
	73			
Total	132	72	44	248
	204			

B

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BETA 1229 Copy 1
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BETA 223 Copy 1
PI 615522
BETA 1229 Copy 4
TK-81
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P5_3        CTTTTGATGAACCCTTCTTTACATCACCCTCGCTTGCTCCATTACCTCTCACAGAAGCAC    60

P5_1        CCATTCCACAATGGGTACCTGGTAATGGTATCGCGGCACCACCATATCAGATTCATGGTT    120
P5_2        CCATTCCACAATGGGTACCTGGTAATGGTATCGCGGCACCACCATATCAGATTCATGGTT    120
P5_3        CCATTCCACAATGGGTACCTGGTAATGGTATCGCGGCACCACCATATCAGATTCATGGTT    120

P5_1        GTGGTTACCCATGTAACGACTCCAATGACTGTGATGCACCCTGTACAGTCTGTTGTGCAA    180
P5_2        GTGGTTACCCATGTAACGACTCCAATGACTGTGATGCACCCTGTACAGTCTGTTGTGCAA    180
P5_3        GTGGTTACCCATGTAACGACTCCAATGACTGTGATGCACCCTGTACAGTCTGTTGTGCAA    180

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P5_2        ACTATACCTGCTGTTATGATGTGGCTGATCCTGAGTACATGCTACCACCTATGTCACCCT    240
P5_3        ACTATACCTGCTGTTATGATGTGGCTGATCCTGAGTACATGCTACCACCTATGTCACCCT    240

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P5_2        CTGAGCCACCGAAATTATTACCTCTTCCACCATCTCCACCTCCATCTACAGAGGATGTAG    300
P5_3        CTGAGCCACCGAAATTATTACCTCTTCCACCATCTCCACCTCCATCTACAGAGGATGTAG    300

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P5_2        AAAATGATGATATGTTTGCACCACAACCTGCTTATGATATAGGAACGCCTGCGGAACTTC    360
P5_3        AAAATGATGATATGTTTGCACCACAACCTGCTTATGATATAGGAACGCCTGCGGAACTTC    360

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P5_2        CACCACCAGGATACGAATTCCCGCCGTATCAAATCCATGGTTGTGGCTATGGCCCCTGCA    420
P5_3        CACCACCAGGATACGAATTCCCGCCGTATCAAATCCATGGTTGTGGCTATGGCCCCTGCA    420

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P5_3        TGGACTCCAACGACTGCGATTGGCCCTGCACATCCTGCTGCTCTAATCATACATGTTGCT    480

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P5_2        ATGAGGAGCCTATGTTTCGATGAAAATCCTCAAACACACAAATTCCAAAGAAAAAGTATA    540
P5_3        ATGAGGAGCCTATGTTTCGATGAAAATCCTCAAACACACAAATTCCAAAGAAAAAGTATA    540

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P5_3        GTAACACAATGTAATAAAACTTAGTGTTCCTGTATTACTTAAAATCACATTTGACCTATA    600

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P5_3        TACAAATGTAAGCAAGATCAATAAAGAATATATACAACTACGTTAATCAATTGCTACTAT    720

P5_1        CAAACTGCATTTTCTATCAAACAACAGTATAATTATTCTGACGTGCTCTAATAATTAAAG    780
P5_2        TAAACTGCATTTTCTATCAAACAACAGTATAATTATTCTGACGTACTCTAATAATTAAAG    780
P5_3        TAAACTGCATTTTCTATCAAACAACAGTATAATTATTCTGACGTACTCTAATAATTAAAG    780

P5_1        TGGGTTGAACTAAGCCTTTAATGACTGATTCATCTGCTCTACGCCTATATCACAATTTCA    840
P5_2        TGGGTTGAACTAAGCCTTTAATGACTGATTCATCTGCTCTACGCCTATATCACAATTTCA    840
P5_3        TGGGTTGAACTAAGCCTTTAATGACTGATTCATCTGCTCTACGCCTATATCACAATTTCA    840

P5_1        GAGTAGATACATTGCGAGATTTCTGCTGTAAGAACCATTTTGATCATGTTGCTGTTACAG    900
P5_2        GAGTAGATACATTGCGAGATTTCTGTTGTAAGAACCATTTTGATCATGTTGCTGTTACAG    900
P5_3        GAGTAGATACATTGCGAGATTTCTGTTGTAAGAACCATTTTGATCATGTTGCTGTTACAG    900

                                          934
P5_1        ACAACATGATTTTCAGTATATTTTGTTTACAAATTTCCTTCGTCGAGGAAATTCTGGCAT    960
P5_2        ACAACGTGATTTTCAGTATATTTTGTTTACAAACTTCCTTCGTCGAGGAAATTCTGGCAT    960
P5_3        ACAACGTGATTTTCAGTATATTTTGTTTACAAACTTCCTTCGTCGAGGAAATTCTGGCAT    960
                ---------->
                dCAPS-Fw

P5_1        GTATTATTAAGATACCAACTTAAATTCTGGTATAGGTTCCATTCTAATTAATATCATCGG    1020
P5_2        GTATTATTAAGATACCAACTTAAATTCTGGTATAGGTTCCATTCTAATTAATATCATCGG    1020
P5_3        GTATTATTAAGATACCAACTTAAATTCTGGTATAGGTTCCATTCTAATAAATATCATCGG    1020

P5_1        CATACTCCTCAACTTAGATTCATTCTTAGTTGATTTCACATCTTAATTCGTCAACTTTCA    1080
P5_2        CATACTCCTCAACTTAGATTCATTCTTAGTTGATTTCACATCTTAATTCGTCAACTTTCA    1080
P5_3        CATACTCCTCAACTTAGATTCATTCTTAGTTGATTTCACATCTTAATTCGTCAACTTTCA    1080

P5_1        TAGAGAACTCATCACAGGATAAGCCAGTTTAGTTCAACAATAGTATATTCCTCCGCCAAA    1140
P5_2        TAGAGAACTCATCACAGGATAAGCCAGTTTAGTTCAACAATAGTATATTCCTCCGCCAAA    1140
P5_3        TAGAGAACTCATCACAGGATAAGCCAGTTTAGTTCAACAATAGTATATTCCTCCGCCAAA    1140
                                                  <----------
                                                  dCAPS-Rv

P5_1        ATCCACATAATGTTTTGAGAGTTTTAATTGCAAGTGGTATACCATCATGCTTTTTCAGTT    1200
P5_2        ATCCACATAATGTTTTGAGAGTTTTAATTGCAAGTGGTATACCATCATGCTTTTTCAGTT    1200
P5_3        ATCCACATAATGTTTTGAGAGTTTTAATTGCAAGTGGTATACCATCATGCTTTTTCAGTT    1200
            ----------

P5_1        TTCACTTTCGATAAATCAAAATCAATATAAATCCTTTCCTCAAAAAGAAAAAACGATATA    1260
P5_2        TTCACTTTCGATAAATCAAAATCAATATAAATCCTTTCCTCAAAAAGAAAAAACGATATA    1260
P5_3        TTCACTTTCGATAAATCAAAATCAATATAAATCCTTTCCTCAAAAAGAAAAAACGATATA    1260

P5_1        AATCCTAGCTCCAATTCGTAAAAGAATTTTACCAAAGTACTTCCTCCGTTTCGTTTCAAA    1320
P5_2        AATCCTAGCTCCAATTCGTAAAAGAATTTTACCAAAGTACTTCCTCCGTTTCGTTTCAAA    1320
P5_3        AATCCTAGCTCCAATTCGTAAAAGAATTTTACCAAAGTACTTCCTCCGTTTCGTTTCAAA    1320
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P5_1    TGCAACAAAAGGGTATTATTTGTGAGATATAAAATTTCCAATTGTTGCGTTTAAAACGAG   1380
P5_2    TGCAACAAAAGGGTATTATTTGTGAGATATAAAATTTCCAATTGTTGCGTTTAAAACGAG   1380
P5_3    TGCAACAAAAGGGTATTATTTGTGAGATATAAAATTTCCAATTGTTGCGTTTAAAACGAG   1380

P5_1    ATGGAGGAAGCATTAAAAGTTGTAAGACAACAAAATGAAAACAAATGGATAAATTTCATA   1440
P5_2    ATGGAGGAAGCATTAAAAGTTGTAAGACAACAAAATGAAAACAAATGGATAAATTTCATA   1440
P5_3    ATGGAGGAAGCATTAAAAGTTGTAAGACAACAAAATGAAAACAAATGGATAAATTTCATA   1440

P5_1    ATATATTCAACTCTACCTTCTTTTGCTCAACATCACATACACACATCCGCACCTGACAAT   1500
P5_2    ATATATTCAACTCTACCTTCTTTTGCTCAACATCACATACACACATCCGCACCTGACAAT   1500
P5_3    ATATATTCAACTCTACCTTCTTTTGCTCAACATCACATACACACATCCGCACCTGACAAT   1500

P5_1    CATTATTCATTAAAACACCCGATAAAATAAATACCAGTTCTGAAATCATGGATATGAAAC   1560
P5_2    CATTATTCATTAAAACACCCGATAAAATAAATACCAGTTCTGAAATCATGGATATGAAAC   1560
P5_3    CATTATTCATTAAAACACCCGATAAAATAAATACCAGTTCTGAAATCATGGATATGAAAC   1560

P5_1    TCACAGTAAAAAGAGTATAAAAAACACCAGCTGAAGCAAACACAAGATCAACAAAGAGGT   1620
P5_2    TCACAGTAAAAAGAGTATAAAAAACACCAGCTGAAGCAAACACAAGATCAACAAAGAGGT   1620
P5_3    TCACAGTAAAAAGAGTATAAAAAACACCAGCTGAAGCAAACACAAGATCAACAAAGAGGT   1620

P5_1    AAAAATCAAAAAATGGTCGTATCAAAATATTATTCTCTACT    1661
P5_2    AAAAATCAAAAAATGGTCGTATCAAAATATTATTCTCTACT    1661
P5_3    AAAAATCAAAAAATGGTCGTATCAAAATATTATTCTCTACT    1661
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Fig. S1 Nucleotide sequences of s17 target region of garden beet genetic resources showing p5. Polymorphic sites are highlighted in green. The positions of dCAPS-Fw and dCAPS-Rv primers are indicated by arrows. PCR products obtained by using these primers have a HindIII site when they are amplified from P5_2 or P5_3 but not from P5_1. We refer P5_1 as p5A, and P5_2 and P5_3 as p5B.

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[*] Corresponding author (kitazaki@agr.hokudai.ac.jp)

```
TK-81mm-0_p4        ATGGCGTGGTACAGAAATTCAAGGTTTGTCTACAATGCTTTAAAACTCAA      50
BETA1229_orf20      ATGGCGTGGTACAGAAATTCAAGGTTTGTCTACAATGCTTTAAAACTCAA      50
                    **************************************************
                    ↳ Exon 1

TK-81mm-0_p4        CTTGCGTTCCAAAACATTTGGTACTATTCCAACTCCAAGAGTTCATTCGA      100
BETA1229_orf20      CTTGCGTTCCAAAACATTTGGTACTATTCCAACTCCAAGAGTTCATTCGA      100
                    **************************************************

TK-81mm-0_p4        ATTCCTCATCTTTGTTTTACAATCAATCTACTAAGTGTAGTGGGTTATTT      150
BETA1229_orf20      ATTCCTCATCTTTGTTTTACAATCAATCTACTAAGTGTAGTGGGTTATTT      150
                    **************************************************

TK-81mm-0_p4        GGGTCTGCAAAATCTGGGTATTTTAATGGGTTTAAACATCATCAAGAGAT      200
BETA1229_orf20      GGGTCTGCAAAATCTGGGTATTTTAATGGGTTTAAACATCATCAAGAGAT      200
                    **************************************************

TK-81mm-0_p4        TAGCTCTTTCTCTGGTTTTGCAAGGAGAAACTATCATGGTGTTAAAACCG      250
BETA1229_o          TAGCTCTTTCTCTGGTTTTGCAAGGAGAAACTATCATGGTGTTAAAACCG      250
                    **************************************************

TK-81mm-0_p4        AAGTAAGTGTTGAATTTCGGGTGGAAAAATTACTTCTTGGAATTGCACTA      300
BETA1229_orf20      AAGTAAGTGTTGAATTTCGGGTGGAAAAATTACTTCTTGGAATTGCACTA      300
                    **************************************************

TK-81mm-0_p4        ATAATCTCGCATTCTGGTATGATTGCTTTCTTTTATTTGCACCCAGTAGT      350
BETA1229_orf20      ATAATCTCGCATTCTGGTATGATTGCTTTCTTTTATTTGCACCCAGTAGT      350
                    **************************************************

TK-81mm-0_p4        TGTGCCATATACAGGAAGGAAGCATTATGTGATTTTGTCAACAACTCATG      400
BETA1229_orf20      TGTGCCATATACAGGAAGGAAGCATTATGTGATTTTGTCAACAACTCATG      400
                    **************************************************

TK-81mm-0_p4        AGAATGAAAATGGAGAATTTGAGAAGCGGAAAATACAACCTGCTACACAC      450
BETA1229_orf20      AGAATGAAAATGGAGAATTTGAGAAGCGGAAAATACAACCTGCTACACAC      450
                    **************************************************

TK-81mm-0_p4        CCTGATACTGAGAGGGTTAGGTCTATATTCCAACACATTCTTGAATCACT      500
BETA1229_orf20      CCTGATACTGAGAGGGTTAGGTCTATATTCCAACACATTCTTGAATCACT      500
                    **************************************************

TK-81mm-0_p4        GGAAAGAGAGATTAATCACCATGAACTCGAACTCGAACTCGAACTCGAAA      550
BETA1229_orf20      GGAAAGAGAGATTAATCACCATGAACTCGAACTCGAACTCGAACTCGAAA      550
                    **************************************************
```

```
TK-81mm-0_p4        GAGATGAAACTTTCAAGGAGAAAACCATTTGGAAGGAGGAGACAGATCAT    600
BETA1229_orf20      GAGATGAAACTTTCAAGGAGAAAACCATTTGGAAGGAGGAGACAGATCAT    600
                    **************************************************

TK-81mm-0_p4        GATAAAGATAGTAGGAAGAAGCATAGTGGGGCTAAGATAACTACTAACCA    650
BETA1229_orf20      GATAAAGATAGTAGGAAGAAGCATAGTGGGGCTAAGATAACTACTAACCA    650
                    **************************************************

TK-81mm-0_p4        TGAAGGGATGAATTGGGAAATTTTCGTTGTCGATAAACCGTGGGTTGAGT    700
BETA1229_orf20      TGAAGGGATGAATTGGGAAATTTTCGTTGTCGATAAACCGTGGGTTGAGT    700
                    **************************************************

TK-81mm-0_p4        CCAGTTGTATATTTGGTGGGAAGATTGTTGTTTACACTGGATTGCTCAAC    750
BETA1229_orf20      CCAGTTGTATATTTGGTGGGAAGATTGTTGTTTACACTGGATTGCTCAAC    750
                    **************************************************

TK-81mm-0_p4        CATTGCATCTCTGATGCTGAATTGGCTACAATTATCGCGCATCAGGTATA    800
BETA1229_orf20      CATTGCATCTCTGATGCTGAATTGGCTACAATTATCGCGCATCAGGTATA    800
                    **************************************************
                                                    Exon 1 →

TK-81mm-0_p4        TAAAACTATTCGTGGGACTCCAACCATCGGCTTAAGCTCATGGTTGATAG    850
BETA1229_orf20      TAAAACTATTCGTGGGACTCCAACCATCGGCTTAAGCTCATGGTTGATAG    850
                    **************************************************

TK-81mm-0_p4        AATATAAAATTATTTCTGGGACTCCAACCAAAAGCTCAGGAATAGCTTTA    900
BETA1229_orf20      AATATAAAATTATTTCTGGGACTCCAACCAAAAGCTCAGGAATAGCTTTA    900
                    **************************************************

TK-81mm-0_p4        TATTCTATCAGTAGCTTGTAGTAGTTCTTTACTAGACTGCAGGAATAGAT    950
BETA1229_orf20      TATTCTATCAGTAGCTTGTAGTAGTTCTTTACTAGACTGCAGGAATAGAT    950
                    **************************************************

TK-81mm-0_p4        AATATTTGGTTATCAATATACATGGGTGATTCCGGTTCATATTTAGGCGT   1000
BETA1229_orf20      AATATTTGGTTATCAATATACATGGGTGATTCCGGTTCATATTTAGGCGT   1000
                    **************************************************

TK-81mm-0_p4        ATCAAATGATTTAAGATATTGTATGAAGTTATGAACTATACAAAAAACAT   1050
BETA1229_orf20      ATCAAATGATTTAAGATATTGTATGAAGTTATGAACTATACAAAAAACAT   1050
                    **************************************************

TK-81mm-0_p4        GAAAGGAATTATATGAAAAAAAAAACTGATGAATTTTAGGTTATTATATT   1100
BETA1229_orf20      GAAAGGAATTATATGAAAAAAAAAACTGATGAATTTTAGGTTATTATATT   1100
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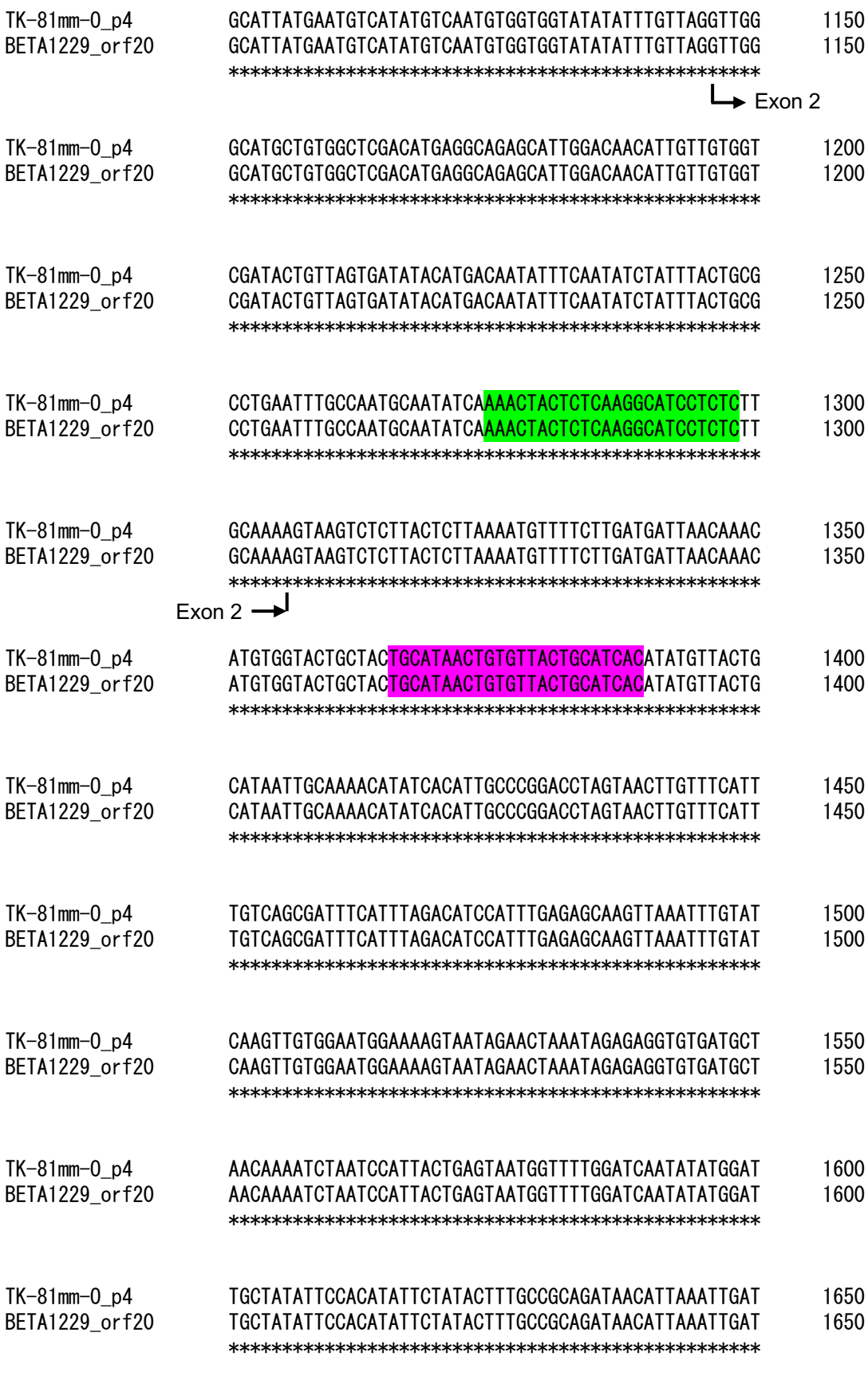
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BETA1229_orf20 GCATTATGAATGTCATATGTCAATGTGGTGGTATATATTTGTTAGGTTGG 1150
Exon 2
TK-81mm-0_p4 GCATGCTGTGGCTCGACATGAGGCAGAGCATTGGACAACATTGTTGTGGT 1200
BETA1229_orf20 GCATGCTGTGGCTCGACATGAGGCAGAGCATTGGACAACATTGTTGTGGT 1200
TK-81mm-0_p4 CGATACTGTTAGTGATATACATGACAATATTTCAATATCTATTTACTGCG 1250
BETA1229_orf20 CGATACTGTTAGTGATATACATGACAATATTTCAATATCTATTTACTGCG 1250
TK-81mm-0_p4 CCTGAATTTGCCAATGCAATATCAAAACTACTCTCAAGGCATCCTCTCTT 1300
BETA1229_orf20 CCTGAATTTGCCAATGCAATATCAAAACTACTCTCAAGGCATCCTCTCTT 1300
TK-81mm-0_p4 GCAAAAGTAAGTCTCTTACTCTTAAAATGTTTTCTTGATGATTAACAAAC 1350
BETA1229_orf20 GCAAAAGTAAGTCTCTTACTCTTAAAATGTTTTCTTGATGATTAACAAAC 1350
Exon 2
TK-81mm-0_p4 ATGTGGTACTGCTACTGCATAACTGTGTTACTGCATCACATATGTTACTG 1400
BETA1229_orf20 ATGTGGTACTGCTACTGCATAACTGTGTTACTGCATCACATATGTTACTG 1400
TK-81mm-0_p4 CATAATTGCAAAACATATCACATTGCCCGGACCTAGTAACTTGTTTCATT 1450
BETA1229_orf20 CATAATTGCAAAACATATCACATTGCCCGGACCTAGTAACTTGTTTCATT 1450
TK-81mm-0_p4 TGTCAGCGATTTCATTTAGACATCCATTTGAGAGCAAGTTAAATTTGTAT 1500
BETA1229_orf20 TGTCAGCGATTTCATTTAGACATCCATTTGAGAGCAAGTTAAATTTGTAT 1500
TK-81mm-0_p4 CAAGTTGTGGAATGGAAAAGTAATAGAACTAAATAGAGAGGTGTGATGCT 1550
BETA1229_orf20 CAAGTTGTGGAATGGAAAAGTAATAGAACTAAATAGAGAGGTGTGATGCT 1550
TK-81mm-0_p4 AACAAAATCTAATCCATTACTGAGTAATGGTTTTGGATCAATATATGGAT 1600
BETA1229_orf20 AACAAAATCTAATCCATTACTGAGTAATGGTTTTGGATCAATATATGGAT 1600
TK-81mm-0_p4 TGCTATATTCCACATATTCTATACTTTGCCGCAGATAACATTAAATTGAT 1650
BETA1229_orf20 TGCTATATTCCACATATTCTATACTTTGCCGCAGATAACATTAAATTGAT 1650

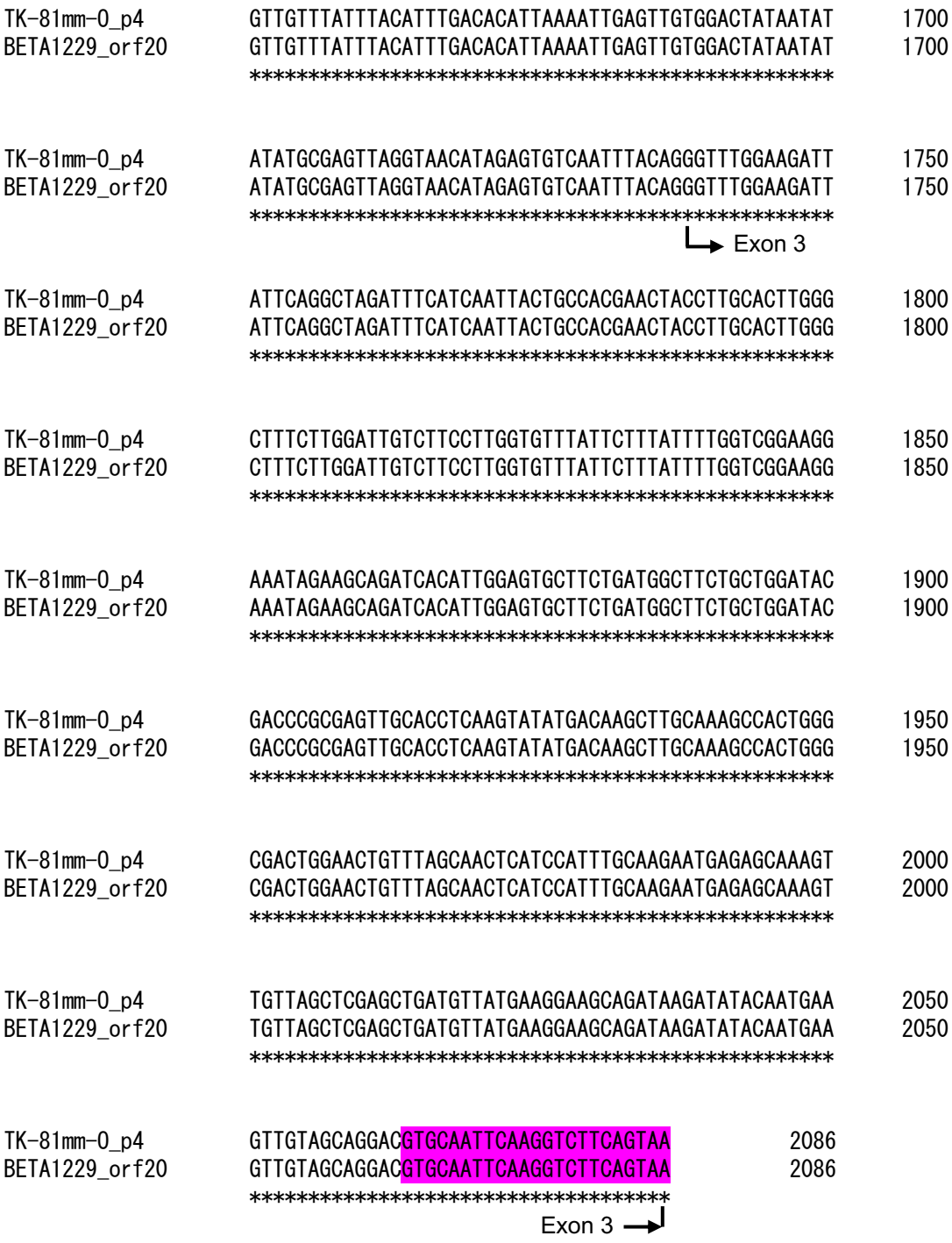

Fig. S2 Nucleotide sequences of exons and introns of orf20-like genes from TK-81mm-O (DDBJ accession number AB646136) and BETA 1229 (this study) associated with the p4 allele of s17. Primers used for sequence analysis are highlighted in green (forward) and purple (reverse). The extents of exons are indicated by arrows. Nucleotides residues are numbered from the translation initiation codon.

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[*] Corresponding author (kitazaki@agr.hokudai.ac.jp)

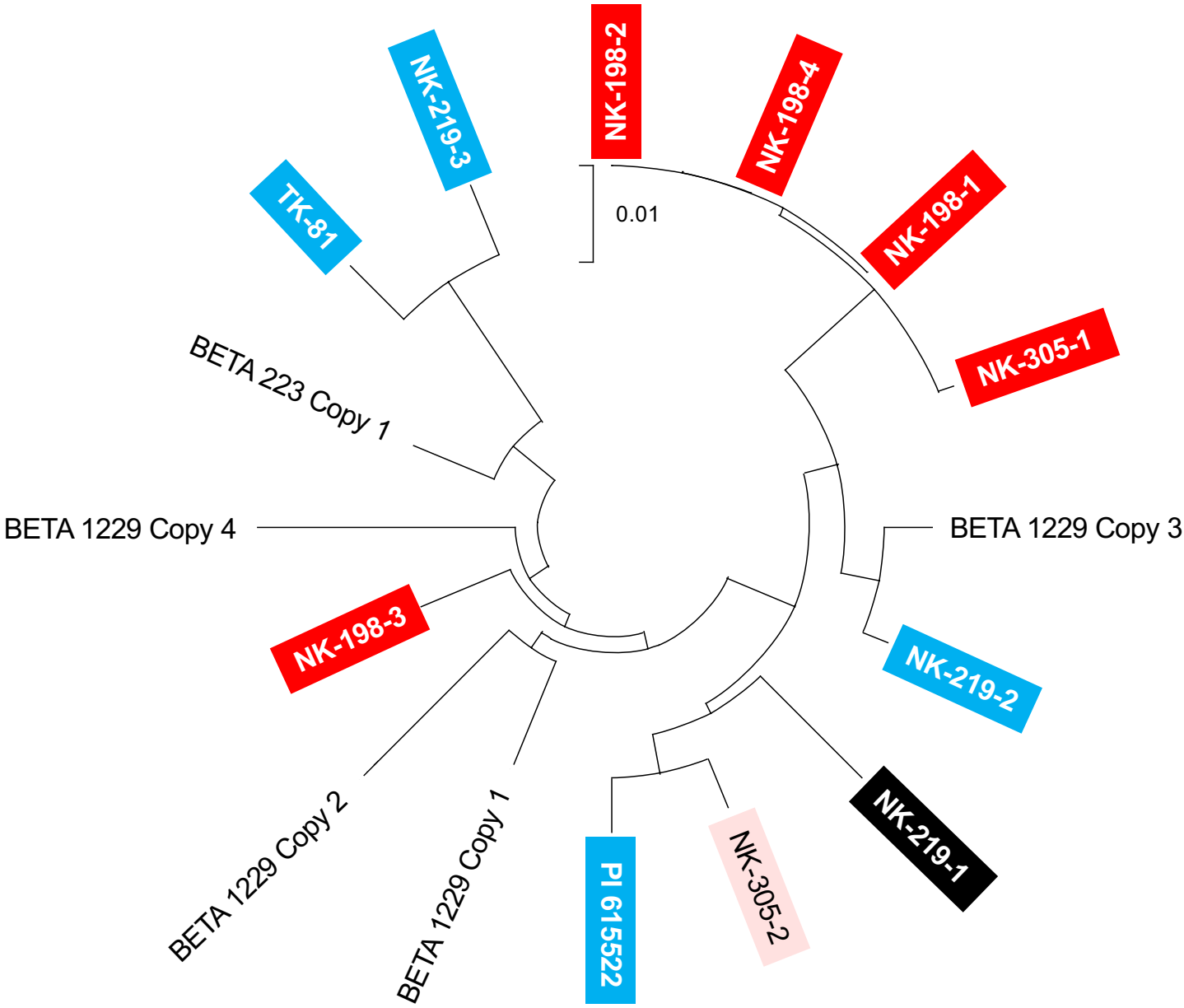

Fig. S3 Neighbor-Joining tree of orf20-like ORFs constructed by using the nucleotide sequence of amino acid coding regions and introns. Labels are as follows (see also Arakawa et al. 2020a): NK-198-1, *orf20* $_{NK198\text{-}1}$; NK-198-2, *orf20* $_{NK198\text{-}2}$; NK-198-3, *orf20* $_{NK198\text{-}3}$; NK-198-4, *orf20* $_{NK198\text{-}4}$; NK-219-1, *orf20* $_{NK219\text{-}1}$; NK-219-2, *orf20* $_{NK219\text{-}2}$; NK-219-3, *orf20* $_{NK219\text{-}3}$; NK-305-1, *orf20* $_{NK305\text{-}1}$; NK-305-2, *orf20* $_{NK305\text{-}2}$; PI 615522, *orf20* $_{PI\ 615522}$; and TK-81, *orf20* $_{TK\text{-}81}$. Copies from dominant- and semidominant Rf1 haplotypes are highlighted in red. Those from recessive rf1 haplotypes are highlighted in blue. Identified copies in this study are: BETA 1229 Copy 1, BETA 1229 p5B Copy 1; BETA 1229 Copy 2, BETA 1229 p5B Copy 2; BETA 1229 Copy 3, BETA 1229 p5B Copy 3; BETA 1229 Copy 4, BETA 1229 p5B Copy 4; and BETA 223 Copy 1, BETA 223 p3 Copy1.

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[2] Hokkaido Agricultural Research Center, National Agriculture and Food Research Organization, Memuro, Japan
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[*] Corresponding author (kitazaki@agr.hokudai.ac.jp)

Fig. S4. Taproot morphology of BETA 1229. Side view (left) and transverse section (right) are shown.

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