Title: **Hybrid breeding skewed the allelic frequencies of molecular variants derived from the *restorer-of-fertility 1* locus for cytoplasmic male sterility in sugar beet (*Beta vulgaris* L.)**

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Abstract

Hybrid breeding of crops may involve the selection of reproductive traits, such as cytoplasmic male sterility (CMS), whose expression is controlled by cytoplasmic and nuclear genes. Intense selection of a single cytoplasm and the consequent lack of cytoplasmic divergence is a potential danger, the so-called genetic vulnerability. However, little is known about the relationship between hybrid breeding and diversity of nuclear genes that suppress the expression of CMS (*Rf*). Despite the multi-allelic nature of *Rf* at the molecular level, a common *Rf* variant was previously found to predominate in sugar beet maintainer lines that were selected for a specific genotype for propagating the CMS line. The question was raised as to the frequency of the common *Rf* variant before the hybrid-breeding era. As the origin of Japanese maintainer lines can be genealogically traced back to seven non-hybrid cultivars, we investigated the allelic diversity of *Rf* in the seven cultivars using molecular markers. Our results indicated that *Rf* diversity differs among the cultivars but exceeds that of the maintainers in total, and the common variant in the maintainers is infrequent in all the cultivars. Therefore, maintainer selection has involved selecting a small number of *Rf* variants in the founder population in Japan.

Introduction

Selection results in the altered allelic frequency of specific gene(s) involved in the selected trait (e.g. Yamasaki et al., 2007). Hence, a change in the crop-breeding method may alter the allelic frequency of genes that were less attractive to breeders before the change. For example, hybrid breeding of maize using cytoplasmic male sterility (CMS) resulted in the increased frequency in commercial cultivars of T-type cytoplasm, one of the four cytoplasms of this crop (Duvick, 1965; Levings, 1993). Having >85% of maize in the production bearing the T-type cytoplasm was ended in 1970 by the southern-corn-leaf-blight epidemic that specifically damaged maize with the T-type cytoplasm (Laughnan and Gabay-Laughnan, 1983). On the other hand, the allelic frequency of a nuclear gene that suppresses CMS (termed *restorer of fertility*, *Rf*) has had less attention. Although the classical genetic model provided only two alleles (i.e. a restoring allele and a nonrestoring allele), the molecular variation of *Rf* has suggested that there are multiple alleles of the *Rf* locus (Wang et al., 2006; Kato et al., 2007). How hybrid breeding impacts the allelic diversity of the *Rf* locus and the possible related consequences are unknown.

Sugar beet is an out-crossing crop that often exhibits genetic heterogeneity even within a breeding line and is known to show a high heterotic response (heterosis) (De Biaggi and Skaracis, 2005). Because of this, almost all of the current commercial sugar beets are hybrid-based cultivars using CMS, which was first described by Owen (Owen, 1942; Bosemark, 2006). Owen proposed that sugar beet CMS is genetically conditioned by a cytoplasmic factor (S) and two recessive genes (*x* and *z*), but he had noticed that expression of sugar beet CMS is sometimes too complex to be explained by this simple genetic model because of the emergence of semi-male sterile phenotypes in the progeny of crossing experiments (Owen, 1945). According to Owen's genetic model, male-sterile sugar beets have an [S]*xxzz* genotype. To propagate CMS lines, maintainer lines that are devoid of the restoring allele but with normal fertile cytoplasm to ensure pollen production (i.e. [N]*xxzz*) have been developed. Identification of the maintainer genotype is the most important process in this hybrid breeding method because the CMS line is

produced by repeated backcrossing of the maintainer line to the CMS progenitor (Skaracis and De Biaggi, 2005). In this crop, the maintainer genotype appears to be rare, making maintainer selection very difficult (Bosemark, 2006). Single-cross hybrids of sugar beet are possible, but because elite CMS lines tend to express inbreeding depression, they are usually unsuitable as final seed parents due to lower seed yield and quality (Skaracis and De Biaggi, 2005). To overcome this shortcoming, three-way hybrids have been developed in which the final seed parent is an F1 plant produced between a CMS line and an unrelated maintainer line, and the pollinators are from a population with a broad genetic base (Skaracis and De Biaggi, 2005).

Molecular cloning of sugar beet *Rf1* (an allele of the *X* locus) revealed a gene cluster consisting of metalloprotease-like genes (Matsuhira et al., 2012). Moritani et al. (2013) investigated the molecular variation of *Rf1* in Japanese sugar beet lines and found that *Rf1* organization varies among restorer lines that often exhibit copy-number variation of the metalloprotease-like genes. The metalloprotease-like gene is not clustered but is present as a single copy in many maintainer lines, in which the same metalloprotease-like variant is shared as a nonrestoring allele, termed *bvORF20L*. Identification of a common variant raised a question about the frequency of *bvORF20L* in the ancestral population of Japanese maintainer lines. This is an important question because the answer would elucidate the dynamics of diversity and frequency of *Rf* alleles during the transition to hybrid breeding.

Japanese maintainer lines received significant genealogical contributions (sometimes ~100%) from seven open-pollinated cultivars (OPCs) that were introduced from the United States or European countries before the 1960s (Taguchi et al., 2006). We hypothesized that the molecular diversity of *Rf1* in these seven OPCs would provide the answer to our question.

In this study, we first improved one of the two *Rf1* markers developed by Moritani et al. (2013) for large-scale genotyping. The seven OPCs were examined for their *Rf1* diversity with special interest on the frequency of *bvORF20L*. We discuss the impact of maintainer selection on genetic diversity.

Materials and methods

Plant materials

Sugar beet (*Beta vulgaris* L.) cultivars Tmm-1 (originated in the United States), Tmm-14 (US), TA-15 (Poland), TA-27 (England), TA-30 (Poland), TA-36 (Germany), and TA-37 (Sweden) are self-incompatible OPCs, introduced by the Japan Sugar Beet Improvement Foundation (succeeded by HARC-NARO) before the 1960s. Plants of TA-36 were different from those used in Moritani et al. (2013). Seeds were sown in plastic pots filled with vermiculite.

Polymerase chain reaction (PCR), nucleotide sequence analysis, and restriction-endonuclease digestion

Total cellular DNA was isolated from seedlings by the modified method of Rogers and Bendich (1985). Nucleotide sequences of PCR primers are 5'-CAATCTGTGGTGCTGACCAA-3' (T1) and 5'-GATTAAAGAGGGCTGCTGAAGCCGAGA-3' (T2). Reactions for s17 PCR contained 0.1 U of Ex-Taq (Takara Bio, Ohtsu, Japan), 0.25 mM of each dGTP, dATP, dTTP and dCTP, 0.2 μM of each T1 primer and T2 primer, and ~10 ng of total cellular DNA in a 20-μL solution. The PCR protocol was 94 ˚C for 5 min, then 35 cycles of 94 ˚C for 1 min, 56 ˚C for 1 min and 72 ˚C for 1 min. PCR for 20L-int was detailed in Moritani et al. (2013). PCR products were subcloned into the pBluescript (SK+) vector and sequenced using an ABI3130 Genetic Analyzer (Applied Biosystems, Foster City, CA) or were directly sequenced. Sequence analysis was conducted using GENETYX (GENETYX CORPORATION, Tokyo, Japan) or Sequencher (Hitachi Software Engineering, Tokyo, Japan). Sequence alignment was modified manually. Nucleotide sequence data were deposited in DDBJ/EMBL/GenBank under the following accession

numbers: AB830620, AB830621, AB830622, AB830623, and AB830624. For cleaved amplified polymorphic sequence (CAPS) detection, PCR products were digested with *Hap*II and *Hin*dIII (Takara Bio) in a 20-μL solution containing 10 μL of PCR-product solution with T buffer (supplied by the manufacturer) at the recommended concentration. The digests were electrophoresed in 1.2%-agarose gels or 5%-polyacrylamide gels.

Statistical analyses

Data were analyzed with Fisher's exact test using the website of Gunma University, Japan (http://aoki2.si.gunma-u.ac.jp/exact/fisher/getpar.html) (accessed on 23rd July, 2013). Confidence intervals were calculated according to the Wald confidence interval (e.g. Agresti and Coul, 1998). When the observed ratio was 0, the upper limit of the probability was given by $1-\alpha^{1/n}$, where α is the significant level and n is the size of the sample. Allelic differentiation was examined by GENEPOP version 4.2 (Raymond and Rousset, 1995) available at the website (http://genepop.curtin.edu.au/index.html) (accessed on 23rd July, 2013) using the default parameters.

Results and Discussion

Development of a novel polymorphic marker targeting the *bvORF17-bvORF20L* intergenic region

bvORF17 and *bvORF20L* are two genes found within the *Rf1* locus of a maintainer line, in which *bvORF17* is located downstream of *bvORF20L* (Matsuhira et al., 2012) (Fig. 1). Marker 17-20L, one of the two DNA markers that tags *bvORF20L* (Moritani et al., 2013), targeted the entire *bvORF17-bvORF20L* intergenic region, which is ~5.5 kbp in length. Difficulty in PCR amplification of a ~5.5-kbp sequence is a shortcoming of marker 17-20L. Therefore, we first improved this marker by confining the polymorphic region in this study.

As detailed in Figure S1, a *bvORF17*-upstream region (~1.8 kbp in length, see Fig. 1) was identified as the polymorphic region, which is the target region of PCR with primers T1 and T2. Our sequence analysis of this PCR-targeted region from several sugar beet plants revealed five different nucleotide sequences (Fig. 1; see Fig. S2), whose differences can be visualized by agarose gel electrophoresis of the PCR products after digestion with *Hap*II and *Hin*dIII (Fig. 2): pattern '1' generated 1.0- and 0.8-kbp fragments; pattern '2' generated 1.2- and 0.5-kbp fragments; pattern '3' generated a 1.3-kbp fragment; pattern '4' generated 1.0- and 0.7-kbp fragments; and pattern '5' generated a 1.7-kbp fragment. Hereafter, the patterns 1 to 5 refer to CAPS (cleaved amplified polymorphic site) marker s17. The target sequence of s17 is single copy in the sugar beet genome. Therefore, s17 is a codominant marker.

Molecular diversity of *Rf1* in cultivars developed in the pre-hybrid breeding era

To analyze *Rf1* diversity, we used two *Rf1* markers, s17 and 20L-int, the latter of which is another *Rf1* marker targeting the first intron of *bvORF20L* and other variants of the metalloprotease-like genes clustered in the *Rf1* locus (Matsuhira et al., 2012; Moritani et al., 2013). Patterns of 20L-int are denoted as S, LS, and L, designations that indicate the presence of a short intron, the presence of both a short intron and a long intron, and the presence of a long intron, respectively (Moritani et al., 2013). Note that types of 20L-int cannot be converted into genotypes because of complex gene clustering (Moritani et al., 2013). Plants homozygous for *bvORF20L* are expected to exhibit pattern-4 homozygous for s17 and type L for 20L-int. Hereafter, we refer to this marker type as 44/L. We genotyped plants that had been inferred to be homozygous for *bvORF20L* by Moritani et al., (2013), and confirmed their marker type as 44/L (Table S1).

A total of 334 plants from the seven OPCs that significantly contributed to Japanese maintainer lines were analyzed (Table 1). The seven OPCs were developed by mass selection, an orthodox breeding method for sugar beet. Of the seven OPCs, seeds of Tmm-14 germinated poorly, perhaps due to inadequate preservation. By analyzing

s17 and 20L-int, we found a total of 16 marker types. Among the seven OPCs, marker types were diversified in TA-30 (14 types) and TA-37 (11 types), whereas the number of marker types was small in Tmm-1 (4 types). The number of marker types in Tmm-14 was also four, but this may be due to the small number of samples.

We compared the number of observed marker-types with those of the 22 Japanese maintainer lines analyzed in Moritani et al. (2013), where the 17-20L marker was used instead of s17. Marker pattern 'a', pattern 'c', and pattern 'd' of 17-20L correspond to pattern 1, pattern 3, and pattern 4 of s17, respectively (see the legend of Fig. S1). As described in the legend of Fig. S1, pattern 'b' of 17-20L includes pattern 2 and pattern 5 of s17. We reanalyzed the plants previously genotyped as having pattern b of 17-20L in Moritani et al. (2013). Using the s17 marker, we found that all the pattern b alleles corresponded to pattern 5 (Table S1). Therefore, the total number of marker types in the 22 Japanese maintainer lines is six (33/S, 34/LS, 35/LS, 44/L, 45/LS and 55/LS; summarized in Table 1). The most diversified line exhibited four marker types (one line), whereas one line had three types, five lines had two types and 14 lines had one type; hence, only one or two marker types occurred in 19 of 22 lines (Table S1), confirming the reduced allelic diversity in the maintainer lines.

In the seven OPCs, the frequency of marker type 44/L is 0 to 0.06 (see Table 1). We tested the null hypothesis that the frequency of 44/L is the same among the seven OPCs. Based on the presence/absence of a short intron in the metalloprotease-like gene of the *Rf1* locus, sugar beet plants can be divided into two classes, those without the short intron (44/L plants) and those with the short intron (the other marker-type plants) (Table S2). Differences in the occurrence of plants without the short intron among the seven OPCs were statistically marginal (Fisher's exact test; p=0.054) (Table S2). Note that frequency of the homozygote for *bvORF20L* in the 22 Japanese maintainer lines was 0.68 on average (see Table 1) and, in 15 out of the 22 lines, reached 0.95–1.00 (Table S1).

Of the seven OPCs, 44/L plants were found only in the TA-36 line; however, it is unlikely that TA-36 is the only 44/L source for Japanese maintainer lines because some maintainer lines that are genealogically independent of TA-36 have the 44/L marker

type. For example, plants of NK-252mm-O and NK-310mm-O exclusively had 44/L (Table S1), but their genealogy indicates no contribution from TA-36; according to Taguchi et al. (2006), NK-252mm-O is 100% Tmm-14-origin, and NK-310mm-O is 100% Tmm-1-origin. We inferred that the *bvORF20L* allele exists at a low frequency in other OPCs, and subpopulations made from these OPCs can generate 44/L plants by random mating. To test this hypothesis, we examined the s17-allelic frequency in the seven OPCs, with particular interest in the frequency of pattern 4, which is linked to *bvORF20L* (Table 2). The seven OPCs tended to exhibit a high frequency of pattern 5 (0.51–0.88, mean=0.67). The pattern-4 frequency varied from 0.00 to 0.17 (mean=0.09), which was not as major as pattern 5. We estimated the 95%-confidence intervals for the frequency of pattern 4 in each OPC as the following: Tmm-1, 0.00–0.03; TA-15, 0.06±0.04; TA-27, 0.08±0.05; TA-30, 0.09±0.05; TA-36, 0.17±0.07; TA-37, 0.13±0.07; and Tmm-14, 0.07±0.13. Therefore, the allelic frequency of *bvORF20L* appeared to remain low in the seven OPCs.

Variation in the pattern-4 frequency among the seven OPCs could be explained by something unrelated to *Rf1*, for example, genetic drift or a hitchhiking effect, considering that each OPC was developed by independent mass selection. If so, allelic differentiation of *Rf1* among the seven OPCs would be expected. Allelic differentiation of s17 for each OPC pair was analyzed by GENEPOP (Table 3). The difference between TA-30 and TA-37 was not significant (p=0.12). Also, five out of the six OPC pairs involving Tmm-14 were not significantly different (p=0.08–0.50), but this may be due to the small sample size of Tmm-14. Differences of the other OPC pairs were significant (p=0.00–0.02). Therefore, it is likely that the allelic frequency of *Rf1* had more or less fluctuated before the 1960s.

Conclusion

All the maintainer lines were developed after the launch of hybrid breeding in Japan. The seven OPCs used in this study can be considered as ancestral populations because

of their significant contribution to the genealogy of Japanese maintainer lines (i.e. many Japanese maintainer lines originated from the seven OPCs). The allelic frequency of *bvORF20L* in the seven OPCs remained low but more or less fluctuated during the mass selection for each of the seven OPCs. Marker types and alleles that are missing from the maintainer lines were found in the seven OPCs. In contrast, an increase in the frequency of *bvORF20L* is apparent in the Japanese maintainer lines, and notably, this increase in *bvORF20L* frequency has occurred in at least two genealogically independent maintainer lines (e.g. NK-252mm-O is exclusively derived from Tmm-14, whereas NK-310mm-O is from Tmm-1 [Taguchi et al., 2006]). Therefore, breeders have preferred *bvORF20L* during maintainer selection, and the consequence is predominance of the 44/L-marker type in Japanese maintainer lines.

Three implications are deduced from this conclusion. First, because *bvORF20L* was repeatedly selected from independent populations, the repertoire of nonrestoring alleles of *Rf1* is limited in sugar beet populations. We are currently seeking another nonrestoring alleles from *B. vulgaris* genetic resources. Second, as was seen in the case of *bvORF20L*, the allelic frequency of the nonrestoring allele can fluctuate by mass selection. As such, the nonrestoring allele may be lost from some subpopulations, from which no maintainer line would be expected. This scenario can explain the experience of one of the co-authors in that a population appeared to lack any maintainer genotype (K. Taguchi, unpublished data). Third, the selection intensity for a maintainer genotype is very high because a specific, infrequent allele is selected among the vast majority of multiple alleles. Consequently, the genetic diversity of other loci, especially those linked to *Rf1*, could be reduced after maintainer selection. This concern can be negligible in the commercial varieties if the genetic heterogeneity of the three-way hybrid is warranted by using pollinators with a wide genetic base. However, care should be taken if the pollinator is a maintainer genotype in the case of a single-cross hybrid or three-way hybrids, in which genetic homogeneity around the *X* locus, on which *Rf1* is located, is expected. We speculate that genetic linkage between the *X* locus and several disease resistance genes (e.g. Taguchi et al., 2011) may be a point of concern. It may be important to monitor the genetic diversity of maintainer lines to prevent genetic

vulnerability.

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341

Table 1 Marker types and numbers of plants in seven open-pollinated cultivars (OPCs) and Japanese maintainer lines.

	Type of s17/20L-int																
Name of cultivars	11/S	13/S	13/LS	14/LS	15/S	15/LS	25/S	33/S	33/LS	34/LS	35/S	35/LS	44/L	45/LS	55/S	55/LS	Total
Tmm-1	2				7						12				30		51
TA-15					3						5	1		8	31	24	33
TA-27	2				8		1				2			8	20	8	49
TA-30	3	2	3	2	6	2		2	6	4	7	2		4	8	3	54
TA-36								1	2	2	7	6	3	10	21		52
TA-37	2	4		1	9	1				5	7	1		7	11	1	49
Tmm-14					1						4			1		1	7
Summary of seven OPCs	9	6	3	3	34	3	1	3	8	11	44	10	3	38	121	37	334
Summary of 22 maintainer lines[1]								6		23		5	245	31		52	362

[1]Based on Moritani et al. (2013) and this study. For details, see Table S1.

Table 2 Number of alleles and allelic frequencies (in brackets) of s17 in OPCs

Name of OPC	Alleles of s17					
	1	2	3	4	5	Total
Tmm-1	11 [0.11]	0 [0]	12 [0.12]	0 [0]	79 [0.77]	102 [1]
TA-15	3 [0.02]	0 [0]	6 [0.04]	8 [0.06]	127 [0.88]	144 [1]
TA-27	12 [0.12]	1 [0.01]	2 [0.02]	8 [0.08]	75 [0.77]	98 [1]
TA-30	21 [0.19]	0 [0]	34 [0.31]	10 [0.09]	43 [0.41]	108 [1]
TA-36	0 [0]	0 [0]	21 [0.20]	18 [0.17]	65 [0.63]	104 [1]
TA-37	19 [0.19]	0 [0]	17 [0.17]	13 [0.13]	49 [0.51]	98 [1]
Tmm-14	1 [0.07]	0 [0]	4 [0.29]	1 [0.07]	8 [0.57]	14 [1]
Total	67 [0.10]	1 [0.00]	96 [0.14]	58 [0.09]	446 [0.67]	668 [1]

Table 3 Probabilities of allelic differentiation for each pair of seven OPCs calculated by GENEPOP[1]

	Tmm-1	TA-15	TA-27	TA-30	TA-36	TA-37	Tmm-14
Tmm-1	-	-	-	-	-	-	-
TA-15	0.00[2]	-	-	-	-	-	-
TA-27	0.00[2]	0.02[2]	-	-	-	-	-
TA-30	0.00[2]	0.00[2]	0.00[2]	-	-	-	-
TA-36	0.00[2]	0.00[2]	0.00[2]	0.00[2]	-	-	-
TA-37	0.00[2]	0.00[2]	0.00[2]	0.12	0.00[2]	-	-
Tmm-14	0.09	0.01[2]	0.08	0.60	0.17	0.50	-

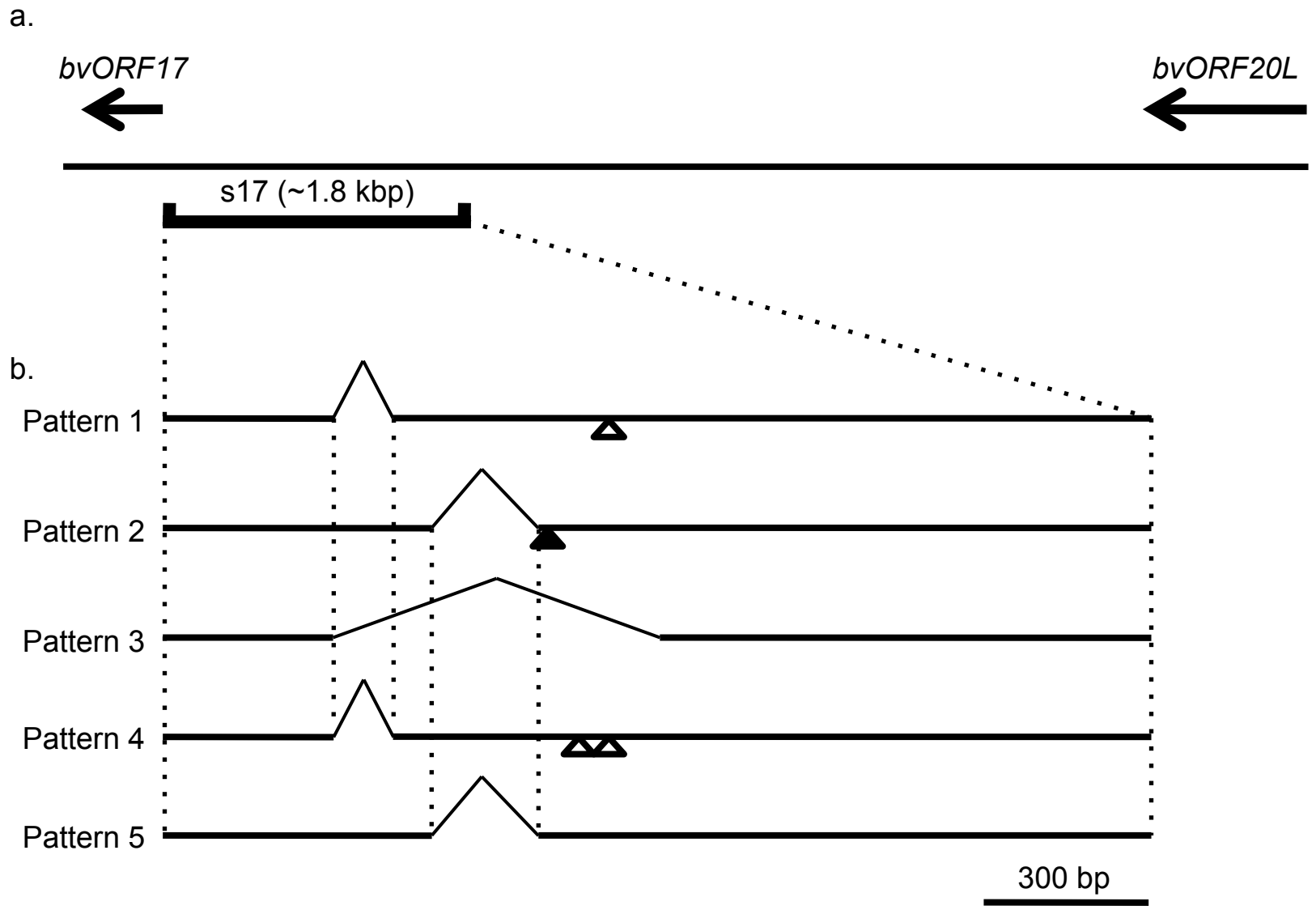
[1]The exact G test was used.

[2]Columns with values less than 0.05 are shaded.

Figure captions

Figure 1 Organization of the *bvORF17-bvORF20L* intergenic region and target region of s17. *Panel a*. Arrangement of *bvORF17* and *bvORF20L* (adopted from Matsuhira et al., 2012). Arrows indicate transcription direction. The target region of s17 is indicated by a bracket. *Panel b*. Organizational comparison of PCR fragments generating patterns 1 to 5 for s17 (for details, see Fig. S2). Wedges indicate deletions. *Hin*dIII restriction site and *Hap*II restriction sites are shown by filled and open triangles, respectively. Scale bar is shown below.

Figure 2 Fragment patterns of s17. PCR products from plants with 1/1, 2/2, 3/3, 4/4, and 5/5 marker types were digested with *Hap*II and *Hin*dIII, and subsequently electrophoresed in a 1.2% agarose gel. M1 and M2 are lambda DNA digested with *Sty*I and pBluescript (SK+) digested with *Hap*II, respectively. Size markers are shown in kbp.


a.
bvORF17
bvORF20L
s17 (~1.8 kbp)
b.
Pattern 1
Pattern 2
Pattern 3
Pattern 4
Pattern 5
300 bp

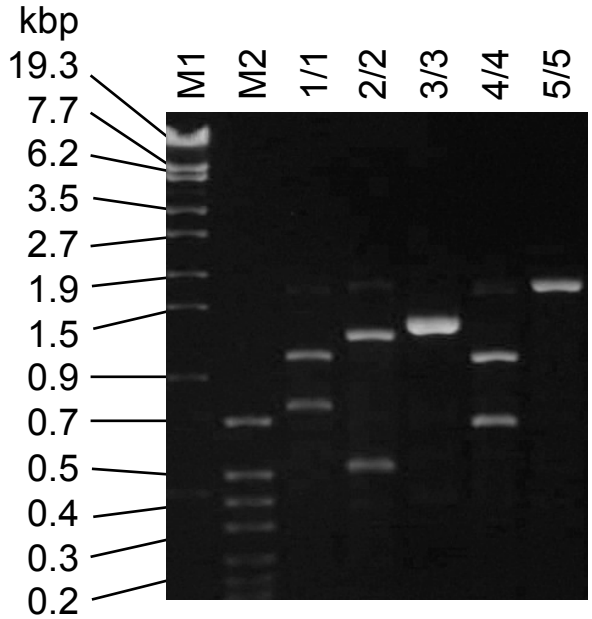

kbp
19.3
7.7
6.2
3.5
2.7
1.9
1.5
0.9
0.7
0.5
0.4
0.3
0.2
M1
M2
1/1
2/2
3/3
4/4
5/5

Table S1 Marker-type coordinates and number of plants[1]

	cc(S)[2]	cd(LS)[2]	bb(LS)[2]		bc(LS)[2]		bd(LS)[2]		dd(L)[2]	
	33/S[3]	34/LS[3]	22/LS[3]	55LS[3]	23/LS[3]	35/LS[3]	24/LS[3]	45/LS[3]	44/L[3]	Total
NK-169mm-O									28	28
NK-172BRmm-O								8	12	20
NK-183BRmm-O									4	4
NK-204mm-O									4	4
NK-205mm-O									4	4
NK-206mm-O									6	6
NK-208mm-O	4								1	5
NK-219mm-O				47						47
NK-222BRmm-O				4		5		9	2	20
NK-226BRmm-O									7	7
NK-252mm-O									7	7
NK-280mm-O									10	10
NK-294mm-O									8	8
NK-296mm-O									20	20
NK-300mm-O								1	19	20
NK-310mm-O									27	27
NK-325mm-O		1							19	20
NK-341mm-O				1				8	11	20
NK-343mm-O								5	15	20
TA-33BB-O									11	11
TK-76mm-O									9	9
TK-81mm-O	2	22							21	45

[1]Based on Moritani et al. (2013) and this study.

[2]Marker type of 17-20L and 20L-int.

[3]Marker type of s17 and 20L-int.

Table S2 Number of plants containing a short intron in the metalloprotease-like gene in the *Rf1* locus.

Name of open pollinated cultivar	With short intron[1]	Without short intron[2]	Total
Tmm-1	51	0	51
TA-15	72	0	72
TA-27	49	0	49
TA-30	54	0	54
TA-36	49	3	52
TA-37	49	0	49
Tmm-14	7	0	7
Total	331	3	334

[1]20L-int types of S and LS.

[2]20L-int types of L.

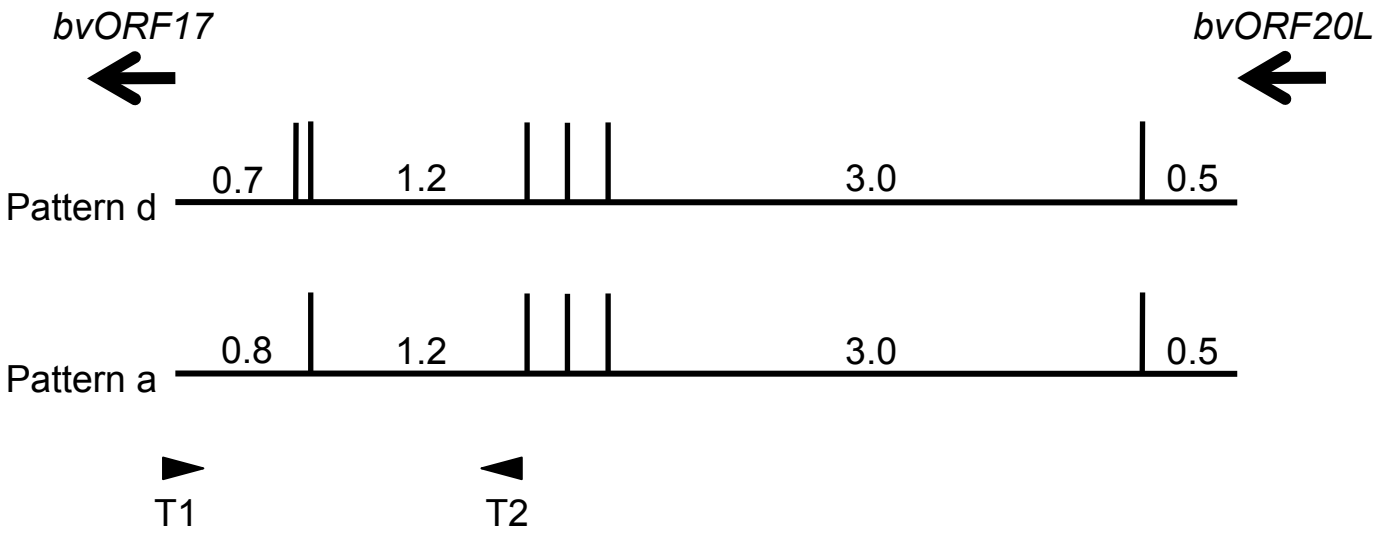


Fig. S1 *Hap*II-restriction map of the *bvORF17-bvORF20L* intergenic region (the target of 17-20L). The map is drawn based on the nucleotide sequences of DDBJ/EMBL/GenBank accession numbers AB646133 and AB646136 that correspond to NK-198 (pattern a) and TK-81mm-O (pattern d), respectively. *Hap*II-restriction sites are shown by vertical lines. Fragment sizes on agarose gel are specified (kbp). Arrows indicate the arrangement of *bvORF17* and *bvORF20L*. Positions of the T1 and T2 primers are shown by arrowheads.

Marker 17-20L is known to generate four patterns, named ‘a’ to ‘d’ (Moritani et al. 2013). We identified two nucleotide sequences of the *bvORF17-bvORF20L*-intergenic region from nucleotide-sequence entries of DDBJ/EMBL/GenBank accession numbers AB646133 and AB646136 that correspond to pattern a and pattern d, respectively. Because the polymorphism of 17-20L can be detected after *Hap*II digestion of the *bvORF17-bvORF20L*-PCR products, we mapped the *Hap*II sites in the *bvORF17-bvORF20L* intergenic region (see above). This analysis revealed that the polymorphic *Hap*II site is located 0.7-kbp upstream of *bvORF17*. In addition, because the other two patterns (pattern ‘b’ and pattern ‘c’) share 3.0- and 0.5-kbp *Hap*II fragments with pattern a and pattern d, the polymorphic region of pattern b and pattern c appeared to be located upstream of *bvORF17* (see above).

We compared the nucleotide sequences of the *bvORF17* upstream regions (~1.8 kbp) that were PCR amplified with primers T1 and T2 from DNA samples of sugar-beet plants known to exhibit patterns a to d (Fig. S2). Since we obtained two different nucleotide sequences from pattern b plants, a total of five different nucleotide sequences were identified that were named patterns ‘1’ to ‘5’ (Fig. S2 and Fig. 1). Patterns 1, 3 and 4 were derived from plants of patterns a, c and d, respectively. Patterns 2 and 5, both were derived from pattern-b plants, differing in single-nucleotide substitutions and indels, one of which generates a polymorphic *Hin*dIII site (Fig. S2 and Fig. 1).

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1_NK-198   GATTAAAGAGGGCTGCTGAAGCCGAGAAAGAAGATAAGAAGTAGAGAATAATATCTTGAT   60
2_NK305    GATTAAAGAGGGCTGCTGAAGCCGAGAAAGAAGACAAGAAGTAGAGAAAAACATTTTGAT   60
3_TK81-O   GATTAAAGAGGGCTGCTGAAGCCGAGAAAGAAGACAAGAAGTAGAGAATAATATTTTGAT   60
4_TA33-O   GATTAAAGAGGGCTGCTGAAGCCGAGAAAGAAGATAAGAAGTAGAGAATAATATCTTGAT   60
5_NK219-O GATTAAAGAGGGCTGCTGAAGCCGAGAAAGAAGACAAGAAGTAGAGAATAATATTTTGAT   60
                                          *             *  *  *

1_NK-198   ACGACCATTTTTTAATTTTTACCTCTTTGTTGATCTTGTGTTTTCTTCAGCTGGTGTTTT  120
2_NK305    ACGACCATTTTTTAATTTTTACCTCTTTGTTGATCT-GTGTTTGCTTCAGCTGGTGTTTT  119
3_TK81-O   ACGACCATTTTTTATTTTTTACCTCTTTGTTGATCTTGTGTTTGCCTCAGCTGGTGTTTT  120
4_TA33-O   ACGACCATTTTTTAATTTTTACCTCTTTGTTGATCTTGTGTTTGCTTCAGCTGGTGTTTT  120
5_NK219-O ACGACCATTTTTTGATTTTTACCTCTTTGTTGATCTTGTGTTTGYTTCAGCTGGTGTTTT  120
                     **                    *      ***

1_NK-198   TCTATACTCTTTTT-ACTGTGAGTTTCATATCCATGATTTCAGAACTGGTATTTATTTTA  179
2_NK305    TTTATACTCTTTTT-ACTGTGAGTTTCATATCCATGATTTCAGAACTGGTATTTATTTTA  178
3_TK81-O   TTAATACTTTTTTTTACTGTGAATTTCATATCCATGATTTCAGAACTGGTATTTATTTTA  180
4_TA33-O   TCTATACTCTTTTT-ACTGTGAGTTTCATATCCATGATTTCAGAACTGGTATTTATTTTA  179
5_NK219-O T-TATACTCTTTTT-ACTGTGAGTTTCATATCCATGATTTCAGAACTGGTATTTATTTTA  178
           **     *     *       *

1_NK-198   TTGGGTGTTTTAATGAAGAATGATTGTCAGGTGCGGATGTGTTCATGTGATGTTGAGCAA  239
2_NK305    TCAGGTGTTTTAATGAATAATGATTGTCAGGTGCGGATGTGTTTATGTGATGTTGAGCAA  238
3_TK81-O   TCGGGTGTTTTAATGAATAATGATTGTCAGGTGCGGATGTGTTTATGTGATGTTGAGCAA  240
4_TA33-O   TCGGGTGTTTTAATGAAGAATGATTGTCAGGTGCGGATGTGTTTATGTGATGTTGAGCAA  239
5_NK219-O TCGGGTGTTTTAATGAATAATGATTGTCAGGTGCGGATGTGTGTATGTGATGTTGAGCAA  238
           **              *                        **

1_NK-198   AAGAAGGTAGAGTTGAATATATTATGAAATTTATCCATTTGTTTTCATTTTGCTGTCTTA  299
2_NK305    AAGAAGGTAGAGTTGAATATATTATGAAATTTATCCATTTGTTTTCATTTTGTTGTCTTA  298
3_TK81-O   AAGAAGGTAGTGTTGAATATATTATGAAATTTATCCATTTGTTTTCATTTTGTTGTCTTA  300
4_TA33-O   AAGAAGGTAGAGTTGAATATATTATGAAATTTATCCATTTGTTTTCATTTTGCTGTCTTA  299
5_NK219-O AAGAAGGTAGAGTTGAATATATTATGAAATTTATCCATTTGTTTTCATTTTGTTGTCTTA  298
                    *                                          *

1_NK-198   CAATTTTTAATACTT-----------------------------------------------  314
2_NK305    CAACTTTTAATGCTTCCTCCATCTCGTTTTAAACGCAACAATTGGAAATTTTATATCTCA  358
3_TK81-O   CAACTTTTAA--------------CGT---------------------------------  313
4_TA33-O   CAATTTTTAATACTT-----------------------------------------------  314
5_NK219-O CAACTTTTAATGCTTCCTCCATCTCGTTTTAAACGCAACAATTGGAAATTTTATATCTCA  358
              *      ************************************************

1_NK-198   ---------------------------------------------------------------  314
2_NK305    CAAATAATACCCTTTTGTTGCATTTGAAACGAAACGGAGGAAGTACTTTGGTAAAATTCT  418
3_TK81-O   ---------------------------------------------------------------  313
4_TA33-O   ---------------------------------------------------------------  314
5_NK219-O CAAATAATACCCTTTTGTTGCATTTGAAACGAAACGGAGGAAGTACTTTGGTAAAATTCT  418
           ************************************************************

1_NK-198   ----CGAATTGGAGCTAGGATTTATAACGCTTTTTCTTTTTGAGGCAAGGATTTAC-TTC  369
2_NK305    TTTACGAATTGGAGCTAGGATTTATATCGTTTTTTCTTTTCGAGGAAAGGATTTATATTG  478
3_TK81-O   ---------------------------------------------------------------  313
4_TA33-O   ----CGAATTGGAGCTAGGATTTATAACGCTTTTTCTTTTTGAGGCAAGGATTTAC-TTC  369
5_NK219-O TTTACGAATTGGAGCTAGGATTTATATCGTTTTTTCTTTTTGAGGAAAGGATTTATATTG  478
           ************************************************************

1_NK-198   ATCC-GTTTCAT-AATACTCGCTACACTTTTCATTATGGGAAGTTTCACATTATTTGCTA  427
2_NK305    ATCTTGATTAATCAAA-----------------------------------------------  494
3_TK81-O   ---------------------------------------------------------------  313
4_TA33-O   ATCC-GTTTCAT-AATATTCGCTACACTTTTCATTATGGGAAGTTTCACATTATTTGCTA  427
5_NK219-O ATTTTGATTTATCGAAA-----------------------------------------------  495
           ************************************************************

1_NK-198   CACTTTTTTTTTGGGCAACAAACTCTCTCTATCTCTTTCCTCTCACATGGGTCCCTTTTC  487
2_NK305    ---------------------------------------------------------------  494
3_TK81-O   ---------------------------------------------------------------  313
4_TA33-O   CACTTCTTTTTTGGGCAACAAACTCTCTCTATCTCTTTCCTCTCACATGGGTCCCTTTTC  489
5_NK219-O ---------------------------------------------------------------  495
           ************************************************************
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1_NK-198   TTTTTACTCTCTATCTCTCTCTTTTCCAAAAATTGCCTAGGACTCTGAATTGAAAAGTAT  547
2_NK305    ------------------------------------------------------------  494
3_TK81-O   ------------------------------------------------------------  313
4_TA33-O   TTTTTACTCTCTATCTCTCTCTTTTCCAAAAATTGCCTAGGAGTCTGAATTGAAAAGTAT  547
5_NK219-O  ------------------------------------------------------------  495
           ************************************************************

1_NK-198   AGCGAATAATATGAAACGGATGAATTTATCAAGTCAAAACTGAAAAAGCATGATGGTATA  607
2_NK305    ---------------------------------GTCAAAACTGAAAAAGCTTGATGGTATA  522
3_TK81-O   ------------------------------------------------------------  313
4_TA33-O   AGCGAATAATATGAAATGGAGGAATTTATCAAGTCAAAACTGAAAAAGCATGATGGTATA  607
5_NK219-O  --------------------------------GTGAAAACTGAAAAAGCATGATGGTATA  523
           ************************************************************

1_NK-198   C-ACTTGCAATTAAAACTCTCAAAACATTATGTGGATTTTGGTGG-GGAATATACTATTG  665
2_NK305    CCACTTGCAATTAAAACTCTCAAAACATTATGTGGATTTTGGCGGGGGAATATACTATTG  582
3_TK81-O   ------------------------------------------------------------  313
4_TA33-O   C-ACTTGCAATTAAAACTCTCAAAACATTATGTGGATTTTGGTGG-GGAATATACTATTG  665
5_NK219-O  CCACTTGCAATTAAAACTCTCAAAACATTATGTGGATTTTGGCGGAGGAATATACTATTG  583
           ************************************************************

1_NK-198   CTGAACTAAACTGGCTTATCCTGTGATGAGTTCTCTATGAAAGTTGACGAATTAAGATGT  725
2_NK305    TTGAACTAAACTGGCTTATCCTGTGATGAGTTCTCTATGAAAGTTGACGAATTAAGATGT  642
3_TK81-O   ------------------------------------------------------------  313
4_TA33-O   TTGAACTAAACTGGCTTATCCGGTGATAAGTTCTCTATGAAAGTTGACGAATTAAGATGT  725
5_NK219-O  TTGAACTAAACTGGCTTATCCTGTGATGAGTTCTCTATGAAAGTTGACGAATTAAGATGT  643
           ************************************************************

1_NK-198   GAAATCAACTAAGAATGAATCTAAGTTGAGGAGTATGCCGGTGATATTAATTAGAATGGA  785
2_NK305    GAAATCAACTAAGAATGAATCTAAGTTGAGGAGTATGCCGATGATATTAATTAGAATGGA  702
3_TK81-O   ------------------------------------------------------------  313
4_TA33-O   GAAATCAACTAAGAATGAATCTAAGTTGAGGAGTATGCCGGTTATATTAATTAGAATGGA  785
5_NK219-O  GAAATCAACTAAGAATGAATCTAAGTTGAGGAGTATGCCGATGATATTAATTAGAATGGA  703
           ************************************************************

1_NK-198   ACATATACCAGAATTCAAGTTGGTATCTTAATAATACATGTCAGAATTTCTTCGACGAAG  845
2_NK305    ACCTATACCAGAATTTAAGTTGGTATCTTAATAATACATGTCAGAATTTCCTCGACGAAC  762
3_TK81-O   ---------AG------AGTTGGTATCTTAATAAT----GTCACAATTTCCTCGACGAAG  354
4_TA33-O   ACATCTACCAGAATTTAAGTTGGTATCATAATAATACATGTCAAAATTTCCTCGACGAAG  845
5_NK219-O  ACCTATACCAGAATTTAAGTTGGTATCTTAATAATACATGCCAGAATTTCCTCGACGAAG  763
           *********  ******          *       **** *  *      *        *

1_NK-198   GAAATTTGTAAACAAAATATACTGAAAATCATGTTGTCTGTAACAGCAACATGATCAAAA  905
2_NK305    GAAATTTATAAACAAAATCTACTGAAAATCATGTTGTCTGTAACAGCAACATGATCAAAA  822
3_TK81-O   GAAATTTGTAAACAAAATATACTGGAAATCATGTTG-CTGTTACAGAAACATGATCAAAA  413
4_TA33-O   GAAATTTGCAAACAAAATATACTGAAAATCATGCTGTCTGTAACAGCAACATGATCAAAA  905
5_NK219-O  GAAATTTGTAAACAAAATATACTGAAAATCATGTTGTCTGTAACAGCAACATGATCAAAA  823
                 **         *     *        *  *    *    *

1_NK-198   TGGTTCTTACAACGGAAATCTCGCAATGTATCTACTCTGAAATTGTGATATAGGCGTAGA  965
2_NK305    TGGTTCTTACAACAGAAATCTCGCAATGTATCTACTCTGAAATTGTGATATAGGCGTAGA  882
3_TK81-O   TGGTTCTTACAACAGAAATCTCGCAATGTATCTACTCTGAAATTGTGATATAGGCGTAGA  473
4_TA33-O   TGGTTCTTACAACAGAAATCTCGCAATGTATCTACTCTGAAATTGTGATATAGGCGTAGA  965
5_NK219-O  TGGTTCTTACAGCAGAAATCTCGCAATGTATCTACTCTGAAATTGTGATATAGGCGTAGA  883
                   * *

1_NK-198   GCAGATGAATCAGTCATTAAAGGCTTAGTTCAACTGTTCAACCCACTTTAATTATTAGAG 1025
2_NK305    GCTGATGAATCAGTCATTTAATGCTTA--------GTTCAACCCACTTTATTTATTAGAG  934
3_TK81-O   GCTGATGAATCAGTCATTTAATGCTTA--------CTTCAACCCACTTTATTTACTAGAG  525
4_TA33-O   GCTGATGAATCAGTCATTTAATGCTTA--------GTTCAACCCACTTTATTTATTAGAG 1017
5_NK219-O  GCAGATGAATCAGTCATTAAAGGCTTA--------GTTCAACCCACTTTAATTATTAGAG  935
             *               *  *     *********              *   *

1_NK-198   T-----ACG--T-CAGAATAATTATACTGTTGTTTGATAGAAAATGCAGTTTGATAGTAG 1077
2_NK305    T-----ACG--T-CACAATAATTATACTGTTGTTTGATAGAAAATGCAATTTGATAGTAG  986
3_TK81-O   TGCGTCACAAATTCACAATAATTATACTGTTGTTTGACAGAAAATGCAGTTTGATAGTAG  585
4_TA33-O   T-----ACG--T-CACAATAATTATACTGTTGTTTGATAGAAAATGCAATTTGATAGTAG 1069
5_NK219-O  C-----ACG--T-CAGAATAATTATACTGTTGTTTGATAGAAAATGCAGTTTGRTAGTAG  987
           ******  *** *  *                      *          *    *
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1_NK-198  CAATTGATTAACGTAGTTGTATATATTCTTTATTGATCTTGCTTACATTTGTATGACACA 1137
2_NK305   CAATTGATTAACGTAGTTGTATATATTCTTTATTAATTTTGCTTACATTTGTATGACACA 1046
3_TK81-O  CAATTGATTAACGTAGTTGTATATATTCTTTATTGATCTTGCTTACATTTGTATGACACA  645
4_TA33-O  CAATTGATTAACGTAGTTGTATATATTCTTTATTAATTTTGCTTACATTTGTATGACACA 1129
5_NK219-O CAATTGATTAACGTAGTTGTATATATTCTTTATTGATCTTGCTTACATTTGTATGACACA 1047
                                           *  *

1_NK-198  GAAATAAAAGTACTTGCTTTCAATCATTAGACAATGACATAGCAATTGAAGAATATAGGT 1197
2_NK305   GAAATAGAAGTACTTGCTTGCAATTACCAGACAATGACATAGCAATTGAAGAATATAGGT 1106
3_TK81-O  GAAATAAAAGTACTTGCTTTCAATCATTAGACAATGACATAGCAATTGAAGAATATAGGT  705
4_TA33-O  GAAATAGAAGTACTTGCTTGCAATTACCAGACAATGACATAGCAATTGAAGAATATAGGT 1189
5_NK219-O GAAATAAAAGTACTTGCTTTCAATCATTAGACAATGACATAGCAATTGAAGAATATAGGT 1107
               *            *    * **

1_NK-198  CAAATGTGATTTTAAGTAATACAGGAACACCA-AGTTTTATTACATTGTGTTACTATACT 1256
2_NK305   CAAATGTGATTTTAAGTAATA-AGGAACACTTTAGTTTTATTACATTGTGTTACTATACT 1165
3_TK81-O  CAAATGTGATTTAAAGTAATACAGGAACACTA-AGTTTTATTACATTGTGTTACTATACT  764
4_TA33-O  CAAATGTGATTTTAAGTAATA-AGGAACACTTTAGTTTTATTACATTGTGTTACTATACT 1248
5_NK219-O CAAATGTGATTTTAAGTAATACAGGAACACTA-AGTTTTATTACATTGTGTTACTATACT 1166
                    *        *        ***

1_NK-198  TTTTCTTTGGAATTTGTGTGTTTGAGGATTTTCATCGAAACATAGGCTCCTCATAGCAAC 1316
2_NK305   TTT-CTTTGGAATTTGTGTGTTTGAGGATTTTCATCGAAACATAGGCTCCTCATAGCAAC 1224
3_TK81-O  TTTTCTTTGGAATTTGTGTGTTTGAGGATTTTCATCGAAACATAGGCTCCTCATAGCAAC  824
4_TA33-O  TTTTCTTTGGCATTTGTGTGTTTGAGGATTTTCATCGAAACATAGGCTCCTCATAGCAAC 1308
5_NK219-O TTTTCTTTGGAATTTGTGTGTTTGAGGATTTTCATCGRAACATAGGCTCCTCATAGCAAC 1226
             *      *                          *

1_NK-198  ATGTATGATTAGAGCAGCAGGATGTGCAGGGCCAATCGCAGTCGTTGGAGTCCATGCAGG 1376
2_NK305   ATGTATGATTAGAGCAGCAGGATGTGCAGGGCCAATCGCAGTCGTTGGAGTCCATGCAGG 1284
3_TK81-O  ATGTATGATTAGAGCAGCAGGATGTGCAGGGCCAATCGCAGTCGTTGGAGTCCATGCAGG  884
4_TA33-O  ATGTATGATTAGAGCAGCAGGATGTGCAGGGCCAATCACAGTCGTTGGAGTCCATGCAGG 1368
5_NK219-O ATGTATGATTAGAGCAGCAGGATGTGCAGGGCCAATCGCAGTCGTTGGAGTCCATGCAGG 1286
                                           *

1_NK-198  GGCCATAGC-ACAACCATGGATTTGATACGGCGGGAATTCGTATCCTGGTGGTGGAAGTT 1435
2_NK305   GGCCATAGCCACAACCATGGATTTGATACGGCGGGAATTCGTATCCTGGTGGTGGAAGTT 1344
3_TK81-O  GGCCATAGCCACAACCATGGATTTGATACGGCGGGAATTCGTATCCTGGTGGTGGAAGTT  944
4_TA33-O  GGCCATAGCCACAACCATGGATTTGATATGGCGGGAATTCGTATCCTGGTGGTGGAAGTT 1428
5_NK219-O GGCCATAGCCACAACCATGGATTTGATATGGCGGGAATTCGTATCCTGGTGGTGGAAGTT 1346
                  *                  *

1_NK-198  CCGCAG-CGTTCCTATATCATAAGCAGGTTGTGGTGCAAACATATCATCATTTTCTACAT 1494
2_NK305   CCGCAGGCGTTCCTATATCATAAGCAGGTTGTGGTGCAAACATATCATCATTTTCTACAT 1404
3_TK81-O  CCGCAGGCGTTCCTATATCATAAGCAGGTTGTGGTGCAAACATATCATCATATTCTACAT 1004
4_TA33-O  CTGCAGGCGTTCCTATATCATAAGCAGGTTGTGGTGTAAACATATCATCATTTTCTACAT 1488
5_NK219-O CCGCAGGCGTTCCTATATCATAAGCAGGTTGTGGTGCAAACATATCATTATTTTCTACAT 1406
           *    *                                 *           *  *

1_NK-198  CCTCTGTAGATGGAGGTGGAGATGGTGGAAGAGGTAATAATTTCGGTGGCTCAGAGGGTG 1554
2_NK305   CCTCTGTAGATGGAGGTGGAGATGGTGGAAGAGGTAATAATTTCGGTGGCTCAGAGGGTG 1464
3_TK81-O  CCTCTGTAGATGGAGGTGGAGATGGTGGAAGAGGTAATAATTTCGGTGGCTCAGAGGGTG 1064
4_TA33-O  CCTCTGTAGATGGAGGTGGAGATGGTGGAAGAGGTAATAATTTCGGTGGCTCAGAGGGTG 1548
5_NK219-O CCTCTGTAGATGGAGGTGGAGATGGTGGAAGAGGTAATAATTTCGGTGGCTCAGAGGGTG 1466


1_NK-198  ACATAGGTGGTAGCATGTACTCAGGATCAGCCACATCATAACAGCAGGTATAGTTTGCAC 1614
2_NK305   ACATAGGTGGTAGCATGTACTCAGGATCAGCCACATCATAACAGCAGGTATAGTTTGCAC 1524
3_TK81-O  ACATAGGTGGTAGCATGTACTCAGGATCAGCCACATCATAACAGCAGGTATAGTTTGCAC 1124
4_TA33-O  GCATAGGTGGTAGCATGTACTCGGGATCAGCCACATCATAACAGCAGGTATAGTTTGCAC 1608
5_NK219-O ACATAGGTGGTAGCATGTACTCAGGATCAGCCACATCATAACAGCAGGTATAGTTTGCAC 1526
          *                    *

1_NK-198  AACAGACTGTACAGGGTGCATCACAGTCATTGGAGTCGTTACATGGGTAACCACAACCAT 1674
2_NK305   AACAGACTGTACAGGGTGCATCACAGTCATTGGAGTCGTTACATGGGTAACCACAACCAT 1584
3_TK81-O  AACAGACTGTACAGGGTGCATCACAGTCATTGGAGTCGTTACATGGGTAACCACAACCAT 1184
4_TA33-O  AACAGACTGTACAGGGTGCATCACAGTCATTGGAGTCACTACATGGGTAACCACAACCAT 1668
5_NK219-O AACAGACTGTACAGGGTGCATCACAGTCATTGGAGTCGTTACATGGGTAACCACAACCAT 1586
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1_NK-198  GAATCTGATATGGTGGTGCCGCGATGCCATTACCAGGTACCCATTGTGGAATGGGTGCTT 1734
2_NK305   GAATCTGATATGGTGGTGCCGCGATGCCATTACCAGGTACCCATTGTGGAATGGGTGCTT 1644
3_TK81-O  GAATCTGATATGGTGGTGCCGCGATGCCATTACCAGGTACCCATTGTGGAATGGGTGCTT 1244
4_TA33-O  GAATCTGATATGGTGGTGCCGCGATGCCRTTACCAGGTACCCATTGTGGAATGGGTGCTT 1728
5_NK219-O GAATCTGATATGGTGGTGCCGCGATACCATTACCAGGTACCCATTGTGGAATGGGTGCTT 1646
                                    *  *

1_NK-198  CTGTGAGAGGCAATGGAGCAGGCGAGGGTGATGTGAAGAAGGGCTCATCAAAAGTACAGG 1794
2_NK305   CTGTGGGAGGTAATGGAGCAAGCGAGGGTGATGTAAAGAAGGGCTCATCAAAAGTACAGG 1704
3_TK81-O  CTGTGGGAGGTAATGGAGCAAGCGAGGGTGATGTAAAGAAGGGCTCATCAAAAGTACAGG 1304
4_TA33-O  CTGTGAGAGGTAATGGAGCAAGCGAGGGTGATGTAAAGAAGGGTTCATCAAAAGTACAGG 1788
5_NK219-O CTGTGGGAGGTAATGGAGCAAGCGAGGGTGATGTAAAGAAGGGCTCATCAAAAGTACAGG 1706
               *    *         *             *        *

1_NK-198  TTTGGTCAGCACCACAGATTG 1815
2_NK305   TTTGGTCAGCACCACAGATTG 1725
3_TK81-O  TTTGGTCAGCACCACAGATTG 1325
4_TA33-O  TTTGGTCAGCACCACAGATTG 1809
5_NK219-O TTTGGTCAGCACCACAGATTG 1727
```

Figure S2 Nucleotide-sequence alignment of PCR fragments amplified with primers T1 and T2 from plants of NK-198 (NK-198, s17 pattern is 1/1), NK-305 (NK305, 2/2), TK-81mm-O (TK81-O, 3/3), TA-33BB-O (TA33-O, 4/4), and NK-219mm-O (NK219-O, 5/5). In Moritani et al. (2013), these plants were typed as aa, bb, cc, dd, and bb, respectively, by the 17-20L marker. The numbers of nucleotide residues are shown on the right. Dashes are incorporated for maximum matching. The positions of T1 and T2 primers are indicated by blue and red fonts, respectively. Restriction sites are indicated by single- and double underlines for *Hap*II and *Hin*dIII, respectively. Asterisks denotes positions of nucleotide divergence.