MOLECULAR CLONING OF X LOCUS AND MARKER-ASSISTED SELECTION OF NON-RESTORING ALLELE FOR OWEN CYTOPLASMIC MALE STERILITY

Kazunori Taguchi[1*], Mari Moritani[2], Tetsuo Mikami[2] and Tomohiko Kubo[2]
[1]Hokkaido Agricultural Research Center (HARC), Memuro, Hokkaido, JP 082-0052, and
[2]Hokkaido University, N-9, W-9, Kita-ku Sapporo, JP 082-8589

Abstract:

According to Owen (1945), fully male sterile plants have the genotype (S)xxzz, while the remaining eight genotypes usually show varying degree of pollen fertility. To obtain offspring from male-sterile plants, which are themselves male sterile, cytoplasmic male sterility (CMS) plants must be pollinated by so-called maitainer plants (O-type), which carry the same nuclear genes as the male-sterile plants but with normal cytoplasm (N)xxzz. To develop marker-assisted selection (MAS) method, we investigated genetic variation at the *Rf1* locus (Matsuhira *et al*. 2012), one of the two *Rf* loci known in sugar beet. After digestion with HindIII, DNAs from beet plants known to have a restoring *Rf1* allele yielded a range of hybridization patterns on Southern blots, indicating that *Rf1* is a multiallelic locus. However, 22 of 23 maintainer lines showed the same hybridization pattern. The sequences of the *rf1* coding regions of these 22 maintainer lines were found to be identical, confirming that they shared the same *rf1* allele (Moritani *et al*, 2013). Two PCR markers targeting a downstream intergenic sequence and the first intron of *Rf1*, respectively, were developed. The electrophoretic patterns of both markers showed multiple *Rf1* alleles. One of these alleles, named the dd(L) type, was associated with the maintainer genotype.

Introduction:

Hybrid seed production in sugar beet relies on CMS. As time-consuming and laborious test crosses with a CMS tester are necessary to identify maintainer lines, development of a reliable marker-assisted selection (MAS) method for the rf gene (the non-restoring allele of restorer-of-fertility locus) is highly desirable for sugar-beet breeding.

CMS was first discovered and studied in sugar beet by Owen (1945), who found a low frequency of male-sterile plans in the cultivar 'US-1'. Owen proposed that the sterility depends on the interaction between at least two recessive chromosomal genes and a 'sterile cytoplasm' (S). According to Owen, fully male sterile plants have the genotype (S)xxzz, while the remaining eight genotypes usually show varying degree of pollen fertility. To obtain offspring from male-sterile plants, which are themselves male sterile, CMS plants must be pollinated by O-type, which carry the same nuclear genes as the male-sterile plants but in normal cytoplasm (N)xxzz.

Since phenotypic discrimination of O-type plants from the other male fertile plants having another genotypes is impossible, test cross with CMS tester is necessary and currently only method to identify O-type. However, sugar beet breeders have noticed that the frequency of O-type is less than 5% in sugar beet population (Bosemark 2006). This means that current O-type selection is very laborious and costly. One of the solutions may be marker-assisted selection (MAS) of O-type. In other words, it would be beneficial for sugar-beet breeding if we could enrich O-type plants in breeding materials by examining DNA polymorphism.

To meet this, we have cloned sugar beet *Rf1* from chromosome III (Matsuhira *et al*. 2012). A recessive *rf1* allele (bvORF20L) have also been cloned from a Japanese sugar-beet line 'TK-81mm-O'. In this study, we show that *Rf1* is a multi-allelic locus and that the TK-81mm-O-type bvORF20L is predominant in Japanese maintainer lines. We also report the development

and use of molecular markers based on the sequence data from the intronic and flanking regions of bvORF20L. Our analyses indicate that these markers can enrich maintainer genotypes in a broad range of beet germplasms.

Materials and methods:

Plant materials used in this study are lines developed by Hokkaido Agricultural Research Center (HARC), National Agriculture and Food Research Organization (NARO), Japan. Individual plants were carefully evaluated for anther color, dehiscence and pollen production during the flowering period. Classification of male-sterile plants is shown in Fig. 1 (modified from Imanishi *et al*. 1970).

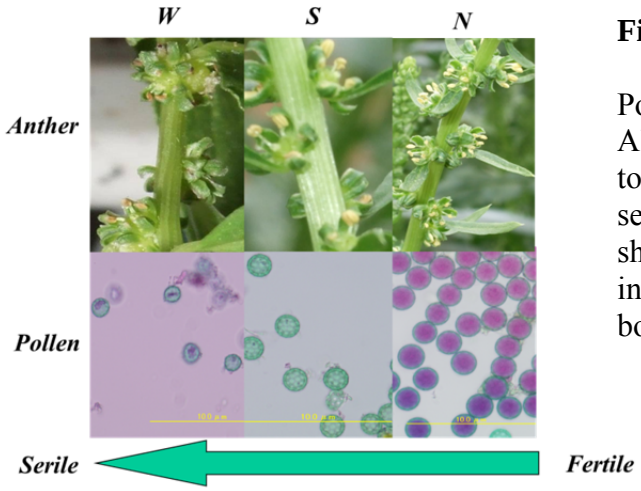

Fig.1 Evaluation of fertility.

Pollen grains were stained with Alexander dye. Starting from the left to the right, complete sterile (W), semi-sterile (S) and fertile (N) are shown, respectively. The top figures indicate anther morphology, and the bottom indicate pollen grains.

Total cellular DNA was isolated using the method of Doyle and Doyle (1990). The sample DNA for gel blot analysis was further purified by centrifugation in CsCl–EtBr continuous density gradients (Sambrook et al. 1989). Polymerase chain reaction (PCR) and cleaved amplified polymorphic sequence (CAPS) detection. The 17-20L sequence was amplified in a 20-µL solution containing 0.4 U of LA-Taq (Takara Bio), 2.5 mM of $MgCl_2$, 0.25 mM of each dNTP, 0.2 ⌈M of each primer, and ~10 ng of genomic DNA with the 1× buffer supplied by the manufacturer. The reaction mixture without $MgCl_2$ was pre-heated in a thermal cycler followed by addition of the $MgCl_2$ solution supplied by the manufacturer. The PCR protocol was 94˚C for 3 min, 35 cycles of 94˚C for 30 s and 68˚C for 5 min plus 10 s per cycle, and 72˚C for 10 min. PCR products were digested with HapII (Takara Bio) in a 20-⌈L solution containing 10 ⌈L of the resultant PCR solution with the manufacturer's recommended buffer concentration. Amplification of the 20L-int sequence was performed in a 10-⌈L solution containing 0.2 ⌈M of each primer, ~5 ng of genomic DNA, and 5 ⌈L of GoTaq (Promega, Madison, WI, USA). The PCR protocol was 35 cycles of 94˚C for 3 s , 58˚C for 1 min and 72˚C for 1 min. The DNA fragment used for preparation of the DNA probe was PCR amplified using the primers shown in Moritani *et al* (2013).

Results and discussion:

Development of markers 17-20L and 20L-int

The bvORF20L copies from the 21 Japanese maintainer lines were PCR amplified with the primers 20L-Fw and 20L-Rv and directly sequenced. The sequences from all the lines were identical and were the same as that in the TK–81mm–O line, indicating that a large number of Japanese maintainer lines have the same rf1 non-restoring allele in common. Therefore, this study has resulted in the development of two agarose gel-based and user-friendly markers (17-20L and 20L-int) for selecting a maintainer genotype for Owen CMS in sugar beet (Fig. 2).

17-20L is a CAPS marker and is easy to handle. The informative HapII restriction fragments are relatively long (0.7-1.8 kbp) ensuring that they can be resolved on standard 2% agarose gels. In addition, the presence of several restriction fragments reduces the risk of misidentification. The other marker, 20L-int, originated from the first intron of bvORF20L and can differentiate two intronic size classes (L and S). By combining data for the 17-20L and 20L-int loci, we were able to distinguish 15 marker patterns in a range of cultivated beet accessions. Of these marker patterns, dd(L) particularly attracted our attention because it showed significant association with bvORF20L, an *rf1* variant commonly found in the majority of maintainer lines examined.

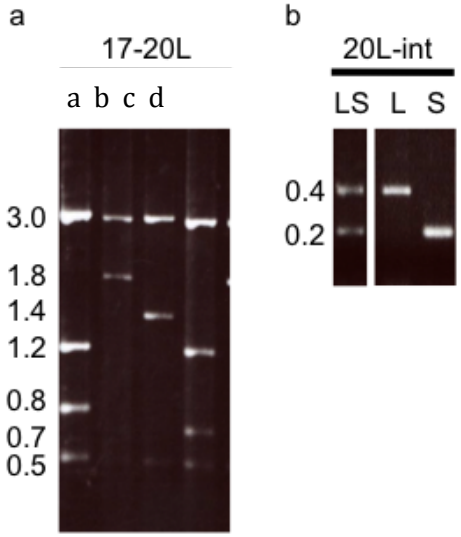

Fig. 2 Agarose gel electrophoresis of 17-20L and 20L-int markers.

Panel A: Four typical band patterns of 17-20 L marker (CAPS). Lanes 1 to 4 correspond to patterns a to d, respectively. Panel B: Three typical band patterns of 20L-int marker.

Testing the 17-20L and 20L-int markers for selection of maintainer genotypes

To test whether selection of dd(L) type plants is indeed effective for identifying maintainer genotypes, the 17-20L and 20L-int markers were used to screen F_4 plants derived from three cross combinations, NK–280mm–O x TA–49, TA–48 x NK–185BRmm–O, and TA–26 x NK–185BRmm–O. The dd(L) type plants, along with several segregants with other marker types, namely, bb(S), bc(S) and bd(S), were selected and used as pollen parents in crosses with the annual Owen-CMS tester line TA–33BB–CMS. TA–33BB–CMS has been maintained by a maintainer line 'TA–33BB–O', scored as dd(L). Our test crosses also included 69 plants of various marker types that were selected from an old cultivar, 'TA–36'. A minimum of 10 progeny from each cross (5038 plants in total) were grown in the greenhouse and examined for male sterility. The numbers of pollen parents that behaved as maintainer (>95% completely male-sterile plants [Class W] in F_1 offspring) or near-maintainer (90 to 95% of completely male-sterile plants [Class W] in F_1 offspring) are summarized in Table 1. No fully male-fertile offspring (Class N) were observed in the test cross progenies of dd(L) plants. We found that the

cross with dd(L) plants from NK–280mm–O x TA–49 had a high rate of perfect male-steriles (between 92% and 100%). Most of the dd(L) plants from TA–26 x NK–185BRmm–O were also classified as either maintainer or near-maintainer, whereas only 4.7% of the dd(L) plants from TA–48 x NK–185BRmm–O were judged to be maintainer. A low proportion of dd(L) plants from TA–36 were maintainer or near-maintainer. By contrast, all plants with marker patterns other than dd(L) were identified as non-maintainer. These plants, when crossed with TA–33BB–MS, yielded fully and partially male-fertile offspring as well as male-sterile offspring. Thus, selection of the dd(L) plants clearly resulted in an increase in the frequency of the maintainer genotype among the breeding materials.

Table 1 Summary of 17-20L and 20L-int marker types and results of test crosses

Plants selected as pollen parent		Number of plants classified as			Total progeny checked
Marker type	Number of plants	Maintainer[a]	Near-maintainer[b]	Others	
From TA–36					
bb(LS)	5	0	0	5	159
bb(S)	36	0	0	36	1138
bc(LS)	1	0	0	1	26
bc(S)	7	0	0	7	234
bd(LS)	2	0	0	2	77
bd(S)	8	0	0	8	267
cc(S)	4	0	0	4	134
cd(LS)	1	0	0	1	43
cd(S)	1	0	0	1	34
dd(L)	4	0	1	3	100
From F4 (NK-280 X TA49)					
bb(S)	8	0	0	8	258
dd(L)	12	10	2	0	434
From F4 (TA48 X NK-185)					
bc(S)	1	0	0	1	33
bd(S)	1	0	0	1	35
dd(L)	21	1	0	20	634
From F4 (TA26 X NK-185)					
bb(S)	2	0	0	2	84
bd(S)	4	0	0	4	171
dd(L)	29	24	2	3	1177

[a] Percentage of completely male-sterile plants (Class W) in the F_1 of the selected pollen parent plant x TA–33–MS: >95%

[b] Percentage of completely male-sterile plants (Class W) in the F_1 of the selected pollen parent plant x TA–33–MS: 90-95%

Possibility of the complete selection for O-type by MAS

A second locus, Z, is known to interact with *Rf1* to fully restore fertility (Owen 1950). Originally, Owen (1945) proposed that the complementary action of the X and Z loci governs male terility or fertility, but he also pointed out that this genetic model did not explain all the

results of the crosses he carried out. Later, he suggested that the Z locus has a minor influence on fertility compared with the X locus (Owen 1950). Recently, we constructed a regional linkage map around the Z locus (provisionally termed *Rf2*) and are currently attempting to develop molecular markers that are diagnostic for genotyping this genomic region (Y. Honma, K. Taguchi, T. Mikami and T. Kubo, unpublished). Combined selection for *Rf1* and *Rf2* loci would enhance the accuracy of marker-assisted selection. It is notable that dd(L) plants are scarce or absent in three Japanese maintainer lines. For example, NK–219mm–O was found to consist exclusively of bb(LS) plants, although the maintainer status of this line was confirmed in test crosses (K. Taguchi, unpublished). This raises the possibility that there might be other as yet uncharacterized allelic forms of *rf1*. Such maintainer lines thus merit further investigation. In NK–219mm–O, other bvORF20-like sequences were found (H. Matsuhira, T. Mikami and T. Kubo, unpublished). It is currently uncertain how many *rf1* alleles are present in the various maintainer lines. The number is likely to be small because of the infrequent occurrence of maintainer genotypes in most sugar beet populations (Bosemark 2006). Currently we are investigating the range of variation in the B. vulgaris gene pool for the architecture of the chromosomal 13 segment corresponding to bvORF20L.

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References:
Bosemark NO (2006) Genetics and Breeding. In: Draycott AP (ed) Sugar Beet. Blackwell Publishing, Oxford, UK, pp 50-88
Doyle JJ, Doyle JL (1990) Isolation of plant DNA from fresh tissue. Focus 12:13-15
Imanishi S, Takeda T, Hosokawa S (1970) Frequency of so-called type O plants as a maintainer of cytoplasmic male sterility in sugar beet populations. Japan J Breeding 20: 40-46
Matsuhira H, Kagami H, Kurata M, Kitazaki K, Matsunaga M, et al. (2012) Unusual and typical features of a novel restorer-of-fertility gene of sugar beet (Beta vulgaris L.). Genetics 192:1347–1358.
Moritani M, Taguchi K, Kitazaki K, Matsuhira H, Katsuyama T, Mikami T, Kubo T (2013) Identification of the predominant nonrestoring allele for Owen-type cytoplasmic male sterility in sugar beet (Beta vulgaris L.): development of molecular markers for the maintainer genotype. Mol Breed, doi: 10.1007/s11032-013-9854-8.
Owen FV (1945) Cytoplasmically inherited male-sterility in sugar beets. J Agr Res 71:423-440
Owen FV (1950) The sugar beet breeder's problem of establishing male-sterile populations for hybridization purposes. Proc Amer Soc Sugar Beet Techn 6: 191-194