Different molecular markers to identify the fertility of sugar beet monogerm germplasm resources

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Research Article

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Abstract

Experiments were conducted to validate different molecular markers associated with sugar beet breeding using available sugar beet monogerm germplasm resources to explore their effectiveness in different sugar beet populations. We used multiple primer pairs to amplify sugar beet monogerm sterile and maintainer lines in order to verify their polymorphism. For the nucleus *Rf1* locus genotype enzyme digestion was also required to verify. The results showed that three pairs of primers, TR1, s17 and 11E8M4S, produced polymorphism when amplifying sugar beet sterile and maintainer lines; primers o7 and AB-18, although polymorphic, did not correlate significantly with sugar beet fertility; primer cpSSR-2 did not produce significant band differences when amplifying sterile and maintainer lines, however, the number of single nucleotide sequence repeats of base A needed to be further verified as a basis for differentiating sugar beet fertility. The polymorphism of 15 pairs of primers related to sugar beet fertility identification was verified and TR1, s17 and 11E8M4S could be used to differentiate sugar beet sterile lines from the maintainer lines and used for subsequent Marker-Assisted Selection (MAS).

Introduction

Cytoplasmic male sterility (CMS) is a trait in sugar beet that results from the interaction of the nuclear and mitochondrial genomes (Arakawa, et al. 2018) and is currently found in over 150 plant species (Chase 2007). A more typical genetic model to explain cytoplasmic male sterility in sugar beet is Owen's CMS genetic expression model, where Owen (Owen 1945) proposed that CMS is composed of a mitochondrial factor 'S' that causes male sterility and two *Rf* genes (*X* and *Z*), where *X* is stronger than *Z* in fertility restoration. In Owen's genetic model, only when S-type sugar beet plants have the non-restoring allele of *Rf*, i.e. S[*rf1rf1rf2rf2*], do the plants exhibit male sterility, while the nuclear fertility restoring gene acts to suppress S (Schnable and Wise 1998). Later researchers found that this genetic model of Owen no longer adequately explained the more complex segregation of fertility, so they tried to include other genetic factors to explain their observations, such as Hogaboam (Hogaboam 1957) who proposed a modified gene, *Sh*, that could enhance the effect of *Rf*. In Hogaboam's genetic model, *Rf* is correlated with the gene (*M*) that controls the number of embryos in the seed. Because the *M* locus was localised on chromosome 4, Arakawa et al. (Arakawa, et al. 2018) then speculated that *Rf* in Hogaboam's genetic model might be *Z*. In sugar beet *Rf*, the *X* gene was localised on chromosome 3 (Pillen, et al. 1993) and one of its alleles was cloned as *Rf1*, whose gene product is a protein similar to the yeast mitochondrial metalloproteinase OMA1 (Matsuhira, et al. 2012).The *Z* gene appears to have a small role in fertility restoration, but a study by Arakawa et al. (Arakawa, et al. 2018) refutes this idea. Several researchers have reported that fertility restoration can be explained by the lack of the *Z* gene (i.e., the *Rf2* gene) (Pillen, et al. 1993).Honma et al. (Honma, et al. 2014) localized the *Rf2* gene to chromosome 4 and suggested that this gene may be the *Z* allele. If the speculation of Honma et al. is followed, then *Rf2* is an important locus.

The main molecular markers used to identify sugar beet cytoplasmic types based on the nucleoplasmic interaction model are the Variable Number of Tandem Repeats (VNTR) molecular marker discovered by

Nishizawa et al. (2000) and The Restriction Fragment Length Polymorphism (RFLP) molecular marker of the chloroplast *petG-psbE* intergenic region identified by Ran et al. (1995). In addition to the molecular markers developed based on nucleoplasmic interactions, Liu (2015) used primers designed by Meng (2014) based on genomic and transcriptomic differences in sugar beet to screen and finally selected seven primers that could be used to identify sterile and maintainer lines; Chen et al. (2017) used isolated populations of sugar beet to screen a Simple Sequence Repeats (SSR) primers for detecting sterile and fertile individuals in sugar beet. Sala et al. (2017) successfully identified sterile and fertile plants in sorghum, wheat, and maize using two chloroplast universal primers, cpSSR-2 and cpSSR-7. Hagihara et al. (2005) successfully distinguished between sterile and fully fertile strains of sugar beet using two STS molecular markers. Although researchers have designed different primers based on the sugar beet nucleoplasmic interaction model or genomic and transcriptomic sequences, the effectiveness of the primers in different sugar beet populations is unknown. Therefore, this experiment is intended to validate some of the available sugar beet monocot germplasm resources in the group to investigate the effectiveness of the primers for sugar beet fertility identification and to pave the way for future marker-assisted selection breeding.

Test Materials

The test materials used in this study were provided by the Crop Genetic Breeding Laboratory, College of Advanced Agriculture and Ecological Environment, Heilongjiang University. The sugar beet seeds were planted in the light culture room, maintained at 25-30 °C with incandescent lighting for 12 h.

DNA molecular markers

The molecular markers used in the assay included VNTR molecular marker for cytoplasmic type identification (Nishizawa, et al. 2000), CAPS molecular marker associated with *Rf1* site (Taguchi, et al. 2014) and DFLP molecular marker associated with *Rf2* site (Arakawa, et al. 2018) as well as molecular markers designed according to genomic and transcriptomic differences (Meng 2014). Table S1. summarizes the DNA molecular markers used in the assay, and the corresponding primers were synthesized by Shanghai Shenggong Biological Engineering.

DNA extraction

DNA of sugarbeet was extracted by the lysis solution method. Take 2 mm^2 leaves in a 1.5 ml centrifuge tube, add 20 μL of lysis solution to the tube, crush the leaves, then heat at 95 °C for 3 min, centrifuge at 12000 g for 3 min, and then extract the supernatant as DNA template.

Statistical analysis

Statistical analysis was performed on the website (http://aoki2.si.gunma-u.ac.jp/exact/exact.html;

accessed 12 October 2020) and data collation was performed using Microsoft Excel.

Validation of enzymatic cleavage

The amplification product of s17, which belongs to the CAPS molecular marker, requires double digestion with *Hap*II and *Hind*III (TaKaRa), after which five types of band types are generated. The patterns and fragment sizes generated after digestion are shown in Table 1 and the digestion system is shown in Table 2.

Table 1. Patterns and fragment sizes of s17 amplified products after digestion.

Type of pattern after digestion	Fragment size after digestion	Enzyme used for digestion
1	1.0 and 0.8 kbp	*Hap*II and *Hind*III
2	1.2 and 0.5 kbp	
3	1.3 kbp	
4	1.0 and 0.7 kbp	
5	1.7 kbp	

Table 2. Enzymatic system of s17 molecular marker amplification products.

Composition	Dosage
s17 amplification product	5 μL
*Hap*II	0.2 μL
*Hind*III	0.2 μL
10×L Buffer	1 μL
10×Loading Buffer	1 μL
10×M Buffer	1 μL
ddH_2O	1.6 μL
Total	10 μL

PCR amplification and molecular analyses

Identification of cytoplasmic types in sugar beet with TR1 primers, and nucleus-fertility genotype identification with s17 and o7 primers. 10 μL of PCR amplification system contains 5 μL of Mix, 0.4 μL of primers (0.2 μL per pair of primers for dual PCR), 1 μL of genomic DNA of sugar beet, 3.6 μL of ddH_2O. The PCR cycles were: 1 cycle of pre-denaturation 3 min at 95 °C, followed by 28 cycles of 25 s at 94°C, 25 s at 60 °C, 2min at 72°C, and finally 1 cycle of 5 min at 72°C for final extension. The PCR products were separated by 1% agarose gel electrophoresis with Tris, acetic and EDTA (TAE) buffer and stained with 1 μgmL^{-1} red fluorescent nucleic acid dyes and then electrophoresed at 130 V for 30 min. After electrophoresis, the bands were observed by gel imaging.

Results

VNTR, s17 and o7 primers

There are four unlinked VNTR loci (TR1, TR2, TR3 and TR4) in the sugar beet mitochondrial genome, while the highest polymorphism was found in the TR1 tandem repeat sequence, with the number ranging from 2 to 13. Identification of sugar beet cytoplasmic types was performed using primer TR1 acting on the sugar beet mitochondrial VNTR locus (Liu, et al. 2013). The genotype of the *Rf1* locus in the nucleus can be identified by using polymorphisms in the upstream region of *bvORF17* in sugar beet DNA of different nuclear genotypes (Taguchi, et al. 2014). The *Rf2* locus genotype can be identified using molecular markers associated with *Rf2*, another locus associated with sugar beet fertility (Arakawa, et al.

2018). The amplification results are shown in Figs. 1 and 2. The results showed that the amplification of the TR1 primers was 0.5 kbp for both sterile lines and 0.75 kbp for both maintainer lines. (Fisher's exact test, $p < 0.01$). The amplification of s17 molecular markers for sterile lines resulted in both 1.8kbp and 1.8/1.3kbp (both 1.8kbp and 1.3kbp band types), while the amplification of s17 molecular markers for maintainer lines resulted in only 1.8kbp band type, and there was a significant difference between the polymorphism of the amplified bands of primer s17 and sugar beet fertility (Fisher's exact test, $p < 0.05$). Subsequent enzymatic digestion of the s17 amplification product is required to further determine the genotype of the *Rf1* locus in the nucleus. Amplification of sugar beet sterile and maintainer lines with the o7 molecular marker showed three band types of 2.6 kbp, 1.4 kbp and 2.6/1.4 kbp for the maintainer lines and three band types for the sterile lines. There was no significant difference between the polymorphism of the bands amplified by primer o7 and sugar beet fertility (Fisher's exact test, $p = 0.70$).

Enzymatic digestion

For the s17 marker amplification products, enzymatic cleavage using *Hind*III and *Hap*II was required for further genotyping of the *Rf1* locus. The results of the digestion of the beet sterile and maintainer lines are shown in Fig. 4. The digestion verification results showed that the types of digestion were 4/4 and 4/5 for the maintainer lines and 4/4, 4/5 and 3/4 for the sterile lines. In combination with the amplification of TR1, s17 and o7, the statistical results are shown in Table 3. Theoretically, the cytosolic genotypes of both sterile and keeper lines should be purely recessive, while Taguchi et al. (2014) verified the enzymatic cleavage of the *Rf1* locus and found that the enzymatic cleavage type 4/4 was able to increase the percentage of genotypes in the maintainer lines. The primer s17 amplification product enzyme cut type was not significantly different between sugar beet breeding by Fisher's exact test ($p = 0.18$).

Table 3. Amplification results of TR1, s17 and o7 molecular markers and enzymatic cleavage results of s17 amplification products.

Number	Sterile lines				Maintainer lines			
	TR1	s17	Enzymatic digestion	o7	TR1	s17	Enzymatic digestion	o7
1	S	1.8/1.3kbp	4/4	2.6/1.4kbp	N	1.8kbp	4/4	2.6/1.4kbp
2	S	1.8kbp	4/4	2.6/1.4kbp	N	1.8kbp	4/4	1.4kbp
3	S	1.8/1.3kbp	4/5	2.6/1.4kbp	N	1.8kbp	4/5	2.6kbp
4	S	1.8/1.3kbp	4/5	2.6kbp	N	1.8kbp	4/5	2.6/1.4kbp
5	S	1.8kbp	4/5	2.6/1.4kbp	N	1.8kbp	4/5	2.6/1.4kbp
6	S	1.8/1.3kbp	4/4	2.6/1.4kbp	N	1.8kbp	4/4	2.6/1.4kbp
7	S	1.8kbp	3/4	2.6/1.4kbp	N	1.8kbp	4/5	1.4kbp
8	S	1.8kbp	3/4	1.4kbp	N	1.8kbp	4/5	2.6/1.4kbp
9	S	1.8kbp	3/4	2.6kbp	N	1.8kbp	4/5	1.4kbp
10	S	1.8kbp	4/4	1.4kbp	N	1.8kbp	4/4	1.4kbp

cpSSR-2 and cpSSR-7 primers

cpSSR-2 and cpSSR-7 are chloroplast genomic primers. We used these two primer pairs to amplify the genomic DNA of the sugar beet sterile lines and maintainer lines. cpSSR-7 primers were excluded as they did not amplify in either the sterile lines or maintainer lines. cpSSR-2 primers were amplified as shown in Fig 4. amplification results showed that both the maintainer lines and sterile lines were amplified at

around 250 bp. A 272 bp sequence was derived by blast on NCBI (https://www.ncbi.nlm.nih.gov/). To further verify the differences in the amplified sequences of the cpSSR-2 primers between the sterile lines and maintainer lines, the amplified fragment was sequenced and an $(A)_{11}$ sequence was found, as shown in Fig 5. The same result was found in tobacco, but with a 3 base difference in the maintainer lines. To determine if there is a SNP mutation at this locus, sequencing of multiple pairs of maintainer lines and sterile lines will be required at a later date to verify this.

AB-18 and AD-11 primers

In order to test the universality of the AB-18 and AD-11 primers for the verification of sterile lines and fertile lines, we used these two primer pairs to amplify both sterile lines and maintainer lines of sugar beet. The results are shown in Figs 6 and 7. Although there was polymorphism in the AB-18 amplification, it did not correlate significantly with the sterility and fertility of the plants, and there was no polymorphism in the AD-11 primer between the sterile lines and the maintainer lines, so neither AB-18 nor AD-11 was useful for testing the fertility of sugar beet.

Genomic and transcriptomic differential primers

Liu (2015) selected 29 pairs of specific primers based on leaf DNA and bud cDNA polymorphisms of sugar beet sterile lines and maintainer lines designed by Meng (2014) and screened them. However, due to the limitations of the populations tested, the applicability of these screened primers to other sources of sterile lines and maintainer lines needs to be further verified. The seven primer pairs were therefore validated using existing sugar beet sterile lines and maintainer lines. Only primer 11E8M4S was found to be polymorphic between the sterile lines and the maintainer lines and the results are shown in Fig 8. Fig 8 shows the results for the sterile lines, but not for the maintainer lines, which can be used to determine the sugar beet sterile lines and maintainer lines.

BvRE051 primer

In the description by Chen et al. (2017), when the BvRE051 primer was used to amplify sugar beet DNA, the sterile lines produced a band of 172bp and the fertile lines produced three bands of 172bp, 232bp and 274bp (Chen, et al. 2017). When we amplified existing sugar beet germplasm resources with the BvRE051 primers, the sterile lines produced a band of around 170 bp and the maintainer lines produced a band of the same size, and the amplification results are shown in Fig. 9. This situation might be related to the variability between populations.

Discussion

Nucleoplasmic interaction-based studies of sugar beet fertility have provided good insight into the causes of sugar beet failure, which are inextricably linked to the Oma1 gene, a gene encoding a yeast protease (Kaser, et al. 2003) that plays various roles in mitochondrial quality control (Guo, et al. 2020; Migdal, et al. 2017). In sugar beet, a duplicated copy of Oma1 evolved into *Rf1* (Arakawa, et al. 2020). The sugar beet

Rf1 locus is actually a complex locus with multiple Oma1-like genes involved in fertility restoration, but *Rf1* does not necessarily correspond to a single open reading frame (Arakawa, et al. 2020), which adds to the difficulty of testing sugar beet fertility by means of molecular markers. In addition, another *Rf2* gene in sugar beet that is associated with fertility restoration is a locus of uncertain action size.

There are inherent differences between the cytoplasmic types of sterile lines and fertile strains of sugar beet. In the sugar beet population we validated, there was a significant difference between the polymorphism of the amplified bands of primer TR1 and sugar beet fertility (Fisher's exact test, $p < 0.01$), suggesting that primer TR1 is useful for the identification of sugar beet cytoplasmic types. Since the populations we used were sugar beet sterile lines and maintainer lines, their cytosolic genotypes should theoretically be the same, but the statistical results showed a significant difference between the polymorphism of the amplified bands of primer s17 and sugar beet fertility (Fisher's exact test, $p < 0.05$), which suggests that it is inappropriate to determine the sugar beet *Rf1* locus based on the polymorphism of the amplified product of primer s17 genotype is inappropriate. When we performed enzymatic digestion using the amplification product of primer s17, the type of enzymatic digestion of the primer s17 amplification product was not significantly different between beet breeding (Fisher's exact test, $p = 0.18$), which further suggests that enzymatic validation of the primer s17 amplification product is necessary. When sugar beet sterile lines and maintainer lines were amplified with the o7 molecular marker, there was no significant difference between the polymorphism of the amplified bands of primer o7 and sugar beet fertility (Fisher exact test, $p = 0.70$), but this result does not indicate that primer o7 can be used to genotype the *Rf2* locus in beet nuclei, as we are unable to genotype the amplified bands of primer o7 at this time.

Through the validation of several pairs of primers used to identify sugar beet breeding, some primers that previously showed specificity in other sugar beet populations did not necessarily have specificity in another population. In this trial, primer 11E8M4S was a valid primer for the identification of sterile lines and fertile lines of sugar beet in addition to primers based on nucleoplasmic interaction-related identification, and could be used to assist in the verification of sugar beet breeding phenotypes in subsequent sugar beet breeding determinations. In the experiment of Chen et al. (2017), amplification of sugar beet sterile lines DNA using primer BvRE051 produced only one band of 172 bp, and fertile lines also produced two bands such as 232 bp and 274 bp, but in our experiment, the sterile and fertile lines amplified bands were not polymorphic, therefore, the primer BvRE051 in the validity of primer BvRE051 in different sugar beet populations was negated. Whether the chloroplast-acting primer cpSSR-2 is useful for differentiating sugar beet breeding is a question that needs to be explored subsequently. The primer cpSSR-2 did not show polymorphism between sugar beet sterile lines and maintainer lines in the experiment, but whether the sequence repeats of 11 single bases of A differed between maintainer lines and sterile lines and sugar beet varieties is a matter of concern.

In previous experiments, different researchers have used molecular markers to verify breeding accordingly according to the selected sugar beet populations. Wang et al. (2014) and Liu et al. (2013) successfully distinguished sterile cytoplasm (S) from normal cytoplasm (N) using primers corresponding to the TR1

locus in sugar beet. Chen et al. (2009) used polymorphisms in the chloroplast *petG-psbE* intergenic region and the mitochondrial VNTR locus to study normal and male-sterile cytoplasm in 42 Chinese sugar beet breeding lines, effectively distinguishing between Owen cytoplasm, the maintainer line 'TK-81 mm-O' type cytoplasm (N1 cytoplasm) and the maintainer line 'NK-310 mm-O' type cytoplasm (N2 cytoplasm). Molecular markers designed on the basis of genomic and transcriptomic differences in sugar beet can be used effectively as an aid in breeding sugar beet, helping breeders to select suitable sugar beet genotypes for breeding into desirable varieties. Although the use of molecular markers for sugar beet breeding can effectively distinguish between sterile lines and maintainer lines, and screening can effectively increase the proportion of maintainer genotypes in sugar beet populations (Taguchi, et al. 2014), the precise identification of individual sugar beet plants for breeding can still be determined based on the genetic situation of different populations in combination with molecular markers and field surveys.

Declarations

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Conflicts of interest: The authors declare no conflicts of interest.

Ethics approval: Not applicable

Consent to participate: Not applicable

Consent for publication: Not applicable

Availability of data and material: Not applicable

Code availability: Not applicable

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Figures

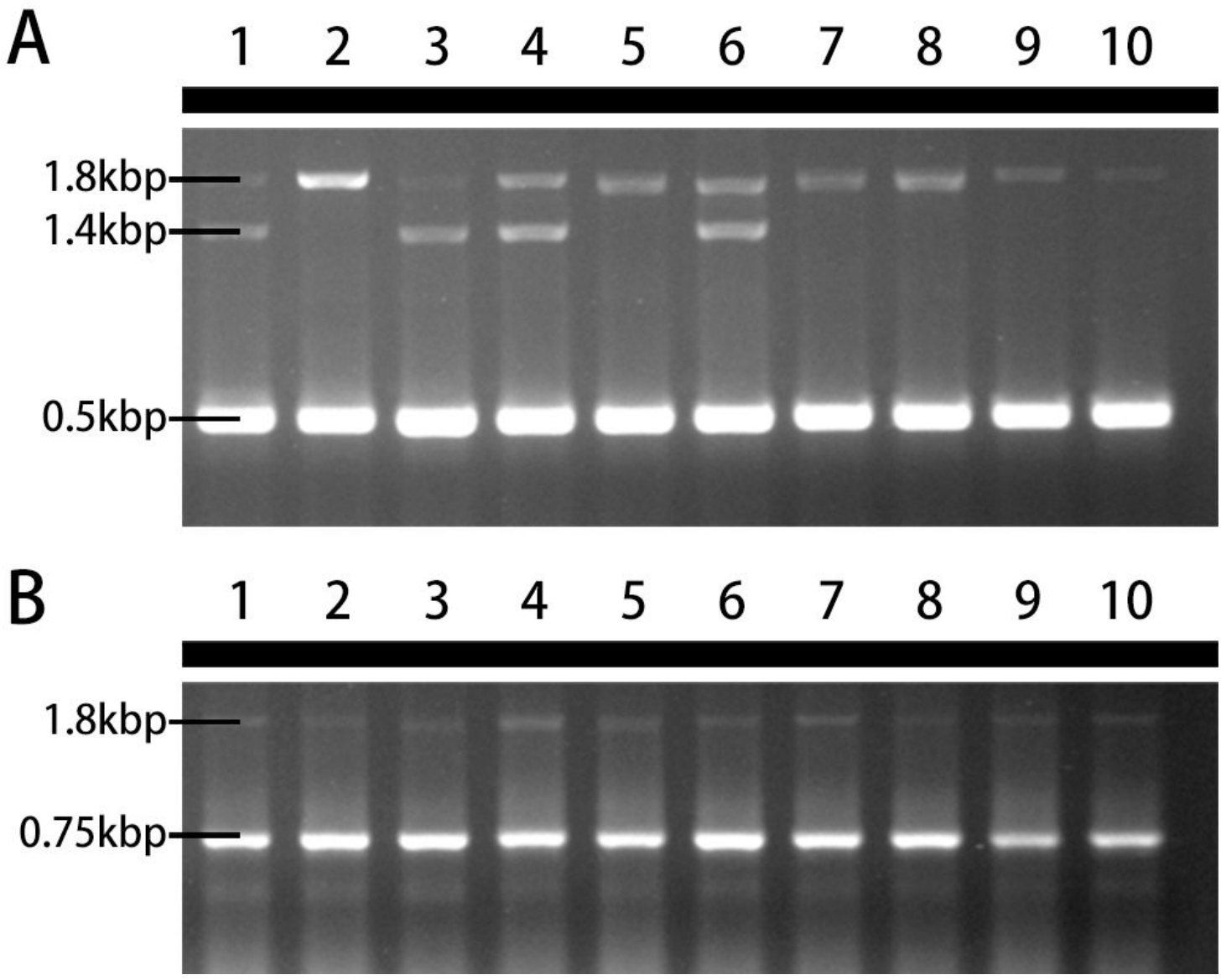

Figure 1

Use of TR1 and s17 molecular markers to characterize the fertility of sugar beet sterile lines and maintainers. A. Dual PCR identification results of molecular markers of sugar beet sterile lines TR1 and s17; B. Dual PCR identification results of molecular markers of sugar beet maintainer lines TR1 and s17.

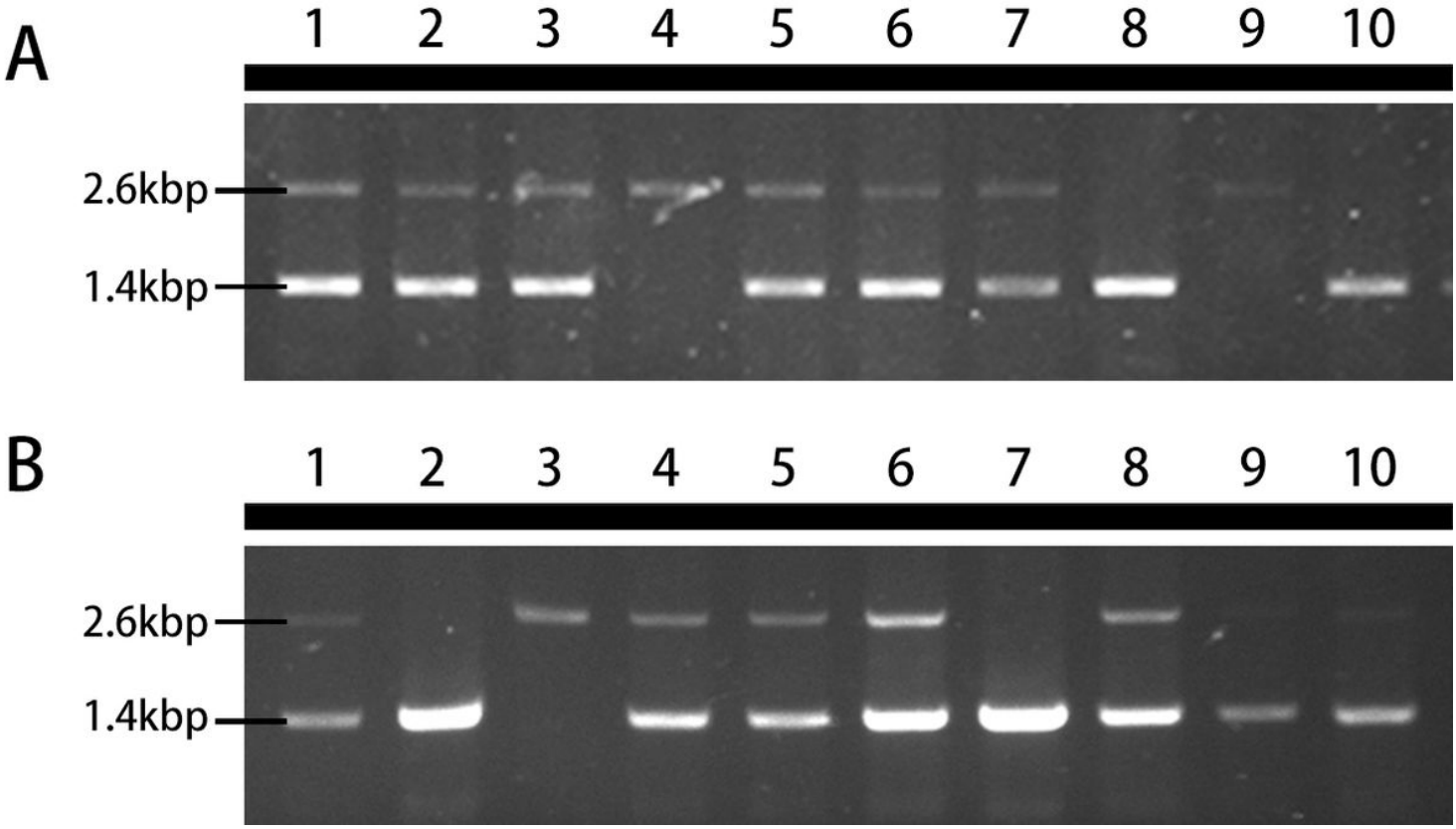

Figure 2

o7 molecular marker identification for sugar beet maintainer lines and sterile lines. A. o7 molecular marker identification for sugar beet maintainer lines; B. o7 molecular marker identification for sugar beet sterile lines.

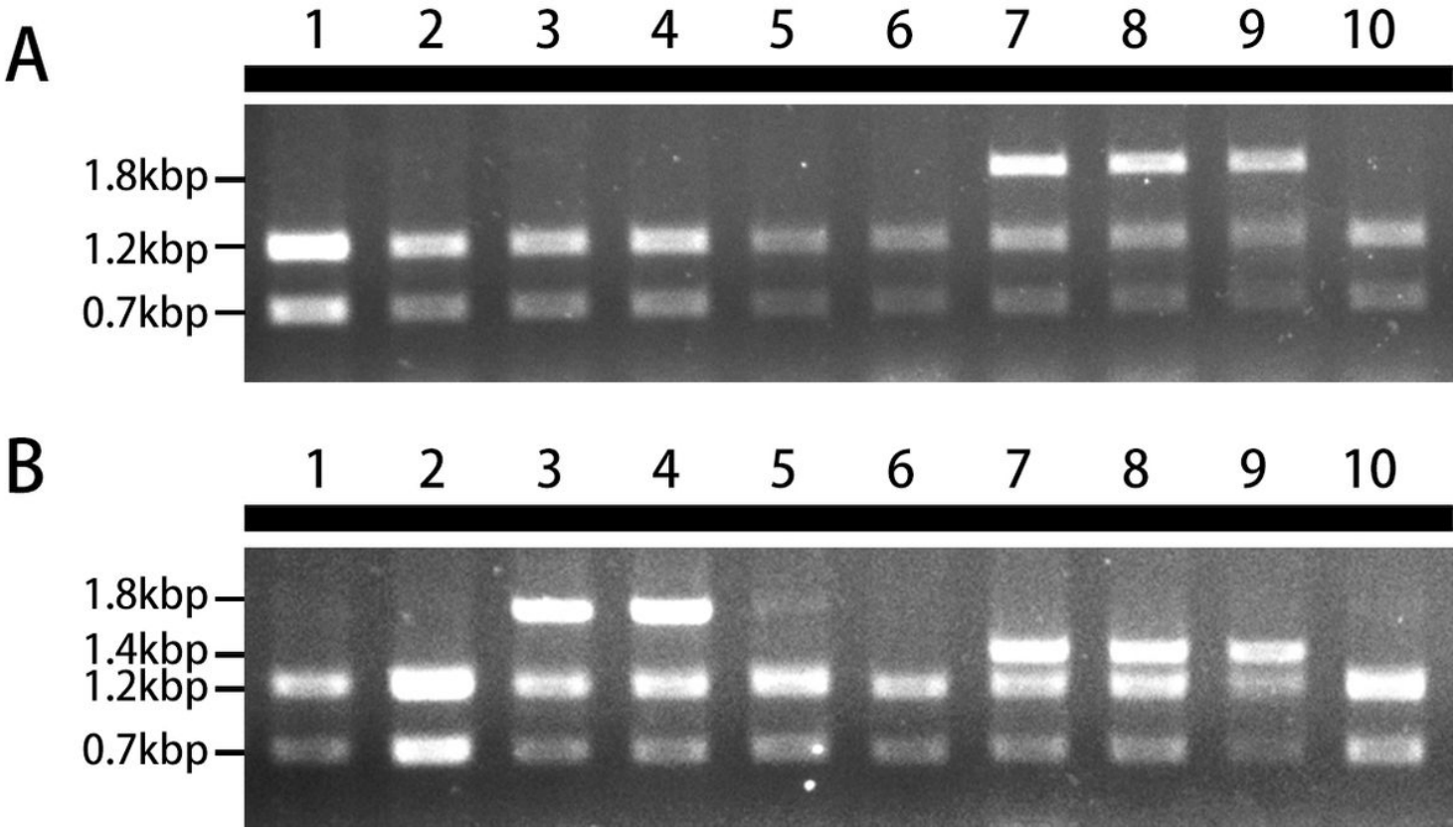

Figure 3

Enzyme digestion results of sugar beet maintainer lines and sterile lines. A. Enzyme digestion results of sugar beet maintainer lines; B. Enzyme digestion results of sugar beet sterile lines.

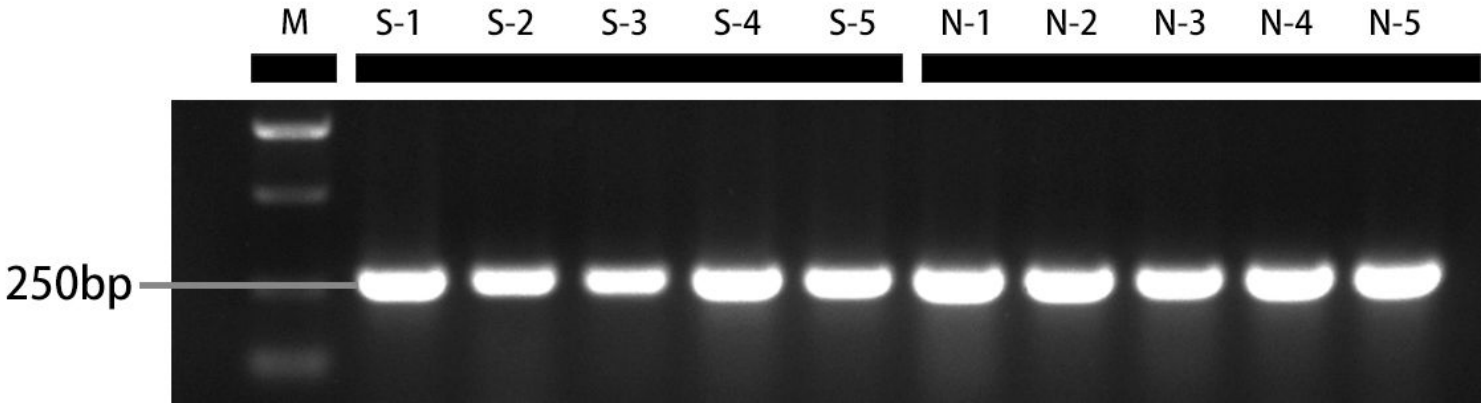

Figure 4

Amplification results of cpSSR-2 primers for sterile and maintainer lines. M: D2000 Maker; S-1~S-5 represent sterile lines, N-1~N-5 represent maintainer lines.

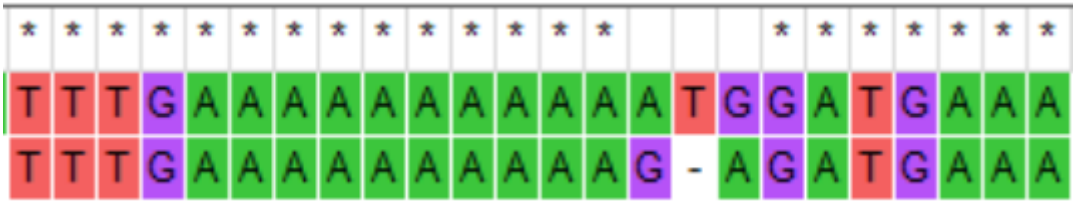

Figure 5

Comparison of partial sequencing results of cpSSR-2 amplification products.

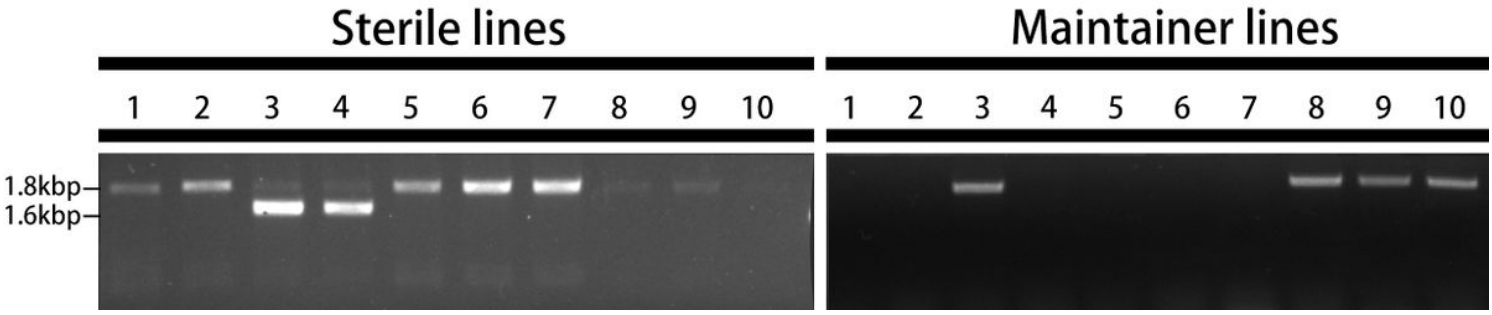

Figure 6

Amplification results of AB-18 primers for sterile lines and maintainer lines.

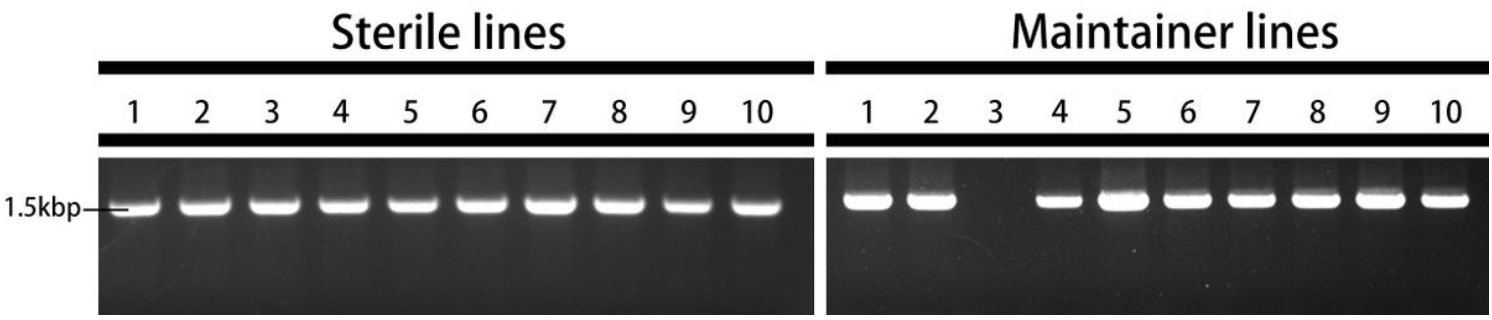

Figure 7

Amplification results of AD-11 primers for sterile lines and maintainer lines.

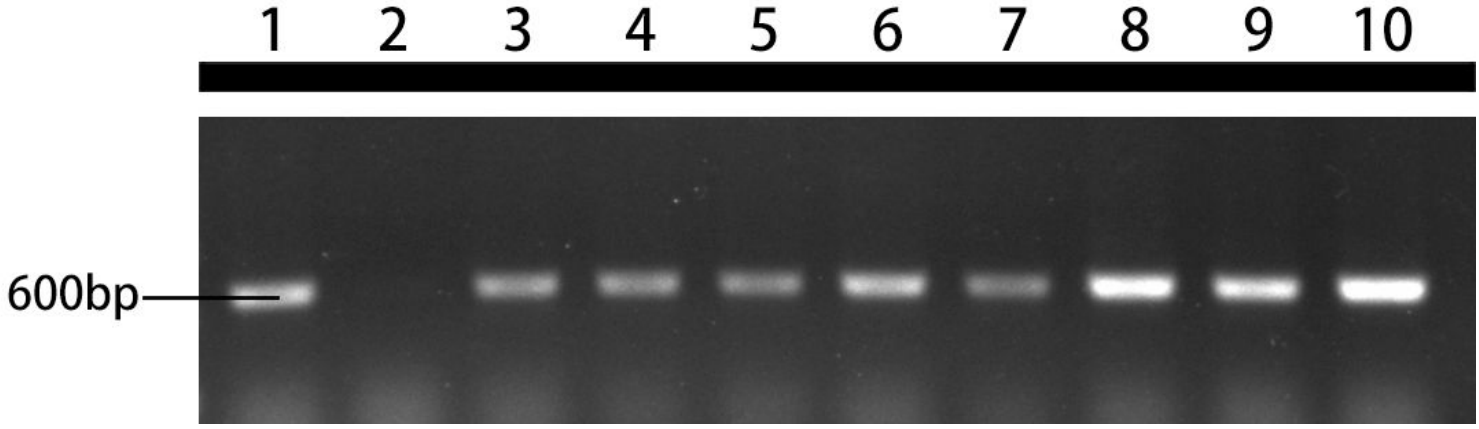

Figure 8

Amplification results of 11E8M4S primers for sterile lines.

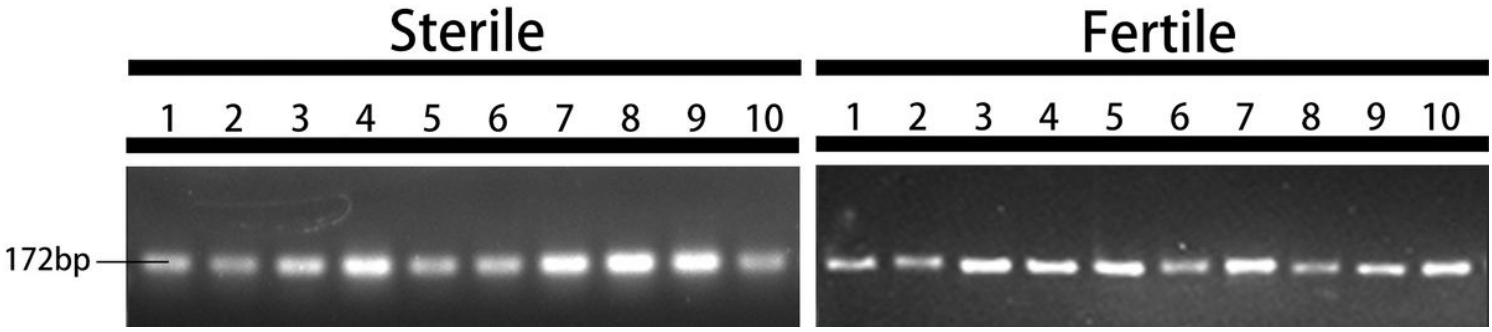

Figure 9

Amplification results of BvRE051 primers for sugar beet sterile lines and maintainer lines.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- TableS1.xlsx