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Molecular mapping of a male fertility restorer locus of *Brassica oleracea* using EST-based SNP markers and analysis of a syntenic region in *Arabidopsis thaliana* for identification of genes encoding pentatricopeptide repeat proteins

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Abstract

An F_2 population was developed from a cross between a *mur*-cytoplasmic male sterile broccoli line and a restorer Chinese kale line. Phenotypic analysis of F_2 plants indicated that the pollen fertility is controlled by two genes and segregated in a duplicate gene interaction mode with a ratio of 15:1. A total of 236 SNP markers were developed utilizing 1448 primers designed for production of EST-SNP markers of *Raphanus sativus* and analyzed by the dot-blot technique in 205 F_2 individuals. A linkage map was constructed with a total of 142 markers and these markers were assigned to nine linkage groups together with SSR markers mapped previously on the published linkage maps of *B. oleracea.* The linkage map spanned 909 cM with an average marker distance of 6.4 cM. A fertility restorer locus (*Rfm1*) was mapped on LG1, corresponding to chromosome 3, along with a flower color locus at a distance of 25 cM. SNP markers flanking the *Rfm1* locus were BoCL2642s at a distance of 2.5 cM on one side and BoCL2901s at a distance of 7.5 cM on the other side. All the SNP markers showed homology with *Arabidopsis thaliana* and *Brassica rapa* genome sequences. Three PPR genes of the P-subfamily, particularly expressed in buds of the restorer line, were identified and these genes could be potential candidate fertility restorer genes.

Keywords: Cytoplasmic male sterility, Fertility restorer locus, *Diplotaxis muralis*, Linkage map, Synteny, PPR genes

Introduction

The family Brassicaceae is an economically important source of vegetables, oilseeds and forages. *Brassica oleracea* is one of the major species in this family, which includes broccoli, cauliflower, cabbage and kale. Commercial hybrid seed production of *B. oleracea* vegetables is performed using self-incompatibility, but sometimes its instability results in contamination of selfed seeds in hybrid seeds. Cytoplasmic male sterility (CMS) together with fertility restoration is a reliable hybrid seed production tool in crop species and also an excellent model to study nuclear-cytoplasmic gene interaction. CMS is the maternally inherited inability of plants to produce functional pollen and is associated with the expression of novel chimeric open reading frames (ORFs) encoded by mitochondrial genome. The chimeric ORFs differ among the CMS systems, but often carry a recognizable segment of coding or flanking sequences of essential mitochondrial genes. The nuclear genes that suppress or compensate for mitochondrial dysfunction and restore fertility to CMS plants are designated fertility restorer (*Rf*) genes. The CMS-*Rf* system is commercially used for hybrid seed production of *Brassica napus*, *Brassica juncea*, onion, etc.

In Brassicaceae, several different cytoplasmic male sterile lines have been identified and these can be grouped under two categories, alloplasmic and autoplasmic. CMS lines have been developed in *B. napus, B. rapa* and *B. juncea,* and four sources of *Brassica* CMS, i.e., *ogu/kosena*, *pol, nap* and *tour,* have been well characterized at molecular level (Schnable and Wise, 1998). In each case, a novel open reading frame has been identified in transcripts of normal mitochondrial genes. Identification of a new CMS line and a restorer gene is always beneficial as a new source material for hybrid breeding. Each restorer gene is unique in nature and molecular study helps to elucidate the mechanism of gene action. A *Diplotaxis muralis*-based CMS line of *B. oleracea* (termed here *mur* CMS *B. oleracea*) has been developed previously and a novel open reading frame, *orf72,* associated with male sterility has been identified (Shinada et al. 2006).

For most alloplasmic CMS systems, fertility restorer genes are required to be introduced from cytoplasm donor species. Restoration is mostly governed by a single restorer gene in all the known Brassica CMS-*Rf* systems except *tour* CMS *B. napus,* in which two dominant genes, *Rft1* and *Rft2,* have been reported for fertility restoration (Janeja et al. 2003). Even a single restorer gene has been found to restore pollen fertility in two different CMS Brassica lines developed from different cytoplasms (Bhat et al. 2005). A number of restorer genes have been cloned in maize, petunia, radish, rice and sorghum, and all except *rf2* in maize (Cui et al. 1996), *Rf17* of CW-CMS rice (Fujii and Toriyama 2005) and *Rf2* in Lead rice CMS (Itabashi et al. 2011) encode pentatricopeptide repeat (PPR) proteins targeted at mitochondria (Bentolila et al. 2002; Brown et al. 2003; Desloire et al. 2003; Koizuka et al. 2003; Kazama and Toriyama 2003; Klein et al. 2005). CMS restorer genes belong to P-subfamily of PPR genes, which are categorized based on their C-terminal domain structure (Small and Peeters 2000; Lurin et al. 2004). All restorer genes cloned to date have been identified by a map-based cloning approach, in which the first step is to find DNA markers linked with a fertility restorer locus followed by fine mapping for positional cloning of the gene.

The objective of the present study was molecular mapping of

a male fertility restorer locus of *mur* CMS *B. oleracea* and analysis of an *Arabidopsis thaliana* homologous region for identification of genes encoding PPR proteins. A linkage map was constructed using EST-based SNP markers based on an F_2 population derived from a CMS line and a restorer line of *B. oleracea*. A total of 142 loci were assigned to nine linkage groups with a total coverage of 909 cM. A fertility restorer locus (*Rfm1*) was mapped on LG1, corresponding to chromosome 3 of *B. oleracea,* along with a flower color gene. Flanking EST-SNP markers for *Rfm1* were identified covering a region of 10 cM. Based on sequence homology of a region between the markers flanking the *Rfm1* locus with the genome sequences of *Arabidopsis thaliana* and *Brassica rapa*, three genes encoding PPR proteins could be identified. These genes are expressed only in buds of the fertility restorer line and are potential candidate genes for male fertility restoration.

Materials and methods

Plant materials

A CMS line of alloplasmic origin in *Brassica rapa* has been developed by substituting the nucleus of wild species *Diplotaxis muralis* with the nucleus of *B. rapa* var. *chinensis* through repeated backcrossings of an intergeneric hybrid of *D. muralis* x *B. rapa* with *B. rapa* as a pollinator for eight generations (Hinata and Konno 1979). Similarly a CMS line in broccoli (*B. oleracea* var. *italica*) harboring *D. muralis* cytoplasm has also been developed by N. Konno and K. Hinata (unpublished), named *mur* CMS *B. oleracea*. A male fertility restorer line has been developed in Chinese kale (*B. olearcea var. alboglabra*) by backcrossing with another male semi-sterile alloplasmic line of *B. oleracea* having *D. muralis* cytoplasm (Shinada et al. 2006). A segregating F_2 population generated from a cross between CMS broccoli and restorer Chinese kale was used for genotyping.

Phenotyping and genomic DNA extraction

A segregating F_2 population was grown in a greenhouse from July 2009 to March 2010 in Sendai, Japan. Pollen fertility and sterility of 205 plants were investigated at the flowering stage through visual examination of pollen grain dust and stainability with 2% acetocarmine. Seed setting ability was also examined after self-pollination in some plants. Based on pollen grain fertility, plants were categorized as male fertile (male fully fertile and male semi-fertile) or male sterile and analyzed to decipher the genetics of fertility restoration. The F_2 population also showed segregation for white and yellow flower color, and phenotypic data were obtained for molecular mapping of a flower color gene. Genomic DNA was extracted from young leaves of all the individuals using the modified CTAB protocol (Doyle and Doyle 1990). The DNA was quantified by ethidium bromide staining after electrophoresis on agarose gel and used as a PCR template in genotyping of the F_2 population with EST-SNP and SSR markers.

Development of EST-based SNP markers and detection of polymorphism

The expressed sequence tag (EST) sequences of radish published on the radish sequence database (http://radish.plantbiology.msu.edu) have been explored to design primer pairs for specific amplification of genes and to identify single nucleotide polymorphisms (SNPs) for production of EST-based SNP markers in *Raphanus sativus* (Li et al. 2011). Since *R. sativus* and *B. oleracea* belong to the same tribe Brassiceae, these radish primer sequences were utilized for production of EST-based SNP markers in *B. oleracea.* These primers were used in PCR amplification from genomic DNA of the parental lines, i.e., CMS broccoli and restorer Chinese kale. A 20 µl reaction mixture contained 10 ng of plant genomic DNA, 10 pmol of each primer, 1x *ExTaq* buffer, 2 nmol of each dNTP, and 0.5 U of *Taq* DNA polymerase (*ExTaq*, Takara Biomedicals, Japan). PCR was performed in a thermal cycler (Eppendorf) with the following cycling conditions: initial denaturation at 94°C for 5 min, 40 cycles of 94°C for 30 sec, 56°C for 30 sec and 72°C for 1 min. Five microliters of the PCR product were electrophoresed on 1.2% agarose gel, and amplified fragment sizes ranged from 300 bp to 600 bp. Single fragment PCR products were selected and sequenced by the Sanger method. High quality sequences were aligned and analyzed to identify SNPs using software SEQUENCHER version 4.7 (Gene Codes Corporation, MI, USA).

Sequences having SNPs between CMS broccoli and restorer Chinese kale were used for designing probes for dot-blot-SNP analysis, which is a cost effective and highly efficient SNP analysis method (Shiokai et al. 2010a). For each marker, a set of two oligonucleotide probes of 48 nucleotides, comprising a sequence of 17 nucleotides with an SNP in the middle, a 6-nucleotide spacer and a bridge sequence of 25 nucleotides, were designed. The hybridization conditions for the probes were predicted using DINAMelt web server (http://www.bioinfo.rpi.edu/applications/hybrid/) and slight modifications were made in hybridization temperature or salt concentration to achieve optimum dot-blot results as described by Shiokai et al. (2010b). Genomic regions having SNPs were amplified by multiplex PCR using 5 or 6 primer pairs mixed together based on annealing temperature and other parameters (Kaplinki et al. 2005). The PCR reaction was set up in a 10 µl volume consisting of 10 ng template DNA, 10 pM of each primer, 1x KAPATaq buffer (without $MgCl_2$), 1.75 mM $MgCl_2$, 0.25 mM of each dNTP and 0.25 U of KAPATaq DNA polymerase (KAPABIOSYSTEMS, Boston, MA, USA). The PCR conditions were initial denaturation at 94°C for 4 min, 35 cycles of 94°C for 30 sec, 56°C for 30 sec and 72°C for 1 min and final extension at 72°C for 5 min. PCR-amplified products were denatured by mixing an equal volume of denaturation solution containing 0.4N NaOH and 10mM EDTA before blotting onto nylon membrane by Multi-pin Blotter (ATTO, Japan). SNP detection was performed using the dot-blot-SNP analysis procedure (Shiokai et al. 2010a), except that the washing and hybridization temperature were changed as per the probe.

SSR marker analysis

SSR markers from published literature (Supplementary Table 1) were screened for polymorphism between parental genotypes to select potential anchor markers for the *B. oleracea* genetic map. PCR amplification of SSR markers was performed as per the reaction conditions given in the reference data of markers. The amplified products were resolved on 8% polyacrylamide gel in 1xTBE buffer and visualized under UV after staining with ethidium bromide. Markers polymorphic between the parents were used for genotyping of F_2 plants to assign linkage groups to those of the reference linkage map (Lowe et al. 2004; Piquemal et al. 2005;

Iniguez-Luy et al. 2008). Two SCAR markers, M3.4 and Nit-2, were also analyzed on 3% agarose gel (Supplementary Table 1).

Linkage analysis and map construction

Linkage analysis and map construction were performed using JoinMap 4.0 software (Van Ooijen, 2006) and linked loci were grouped with independent LOD parameter into nine linkage groups. The marker order was confirmed by a regression mapping algorithm on the basis of a minimum LOD score of 1.0 and a maximum recombination fraction of 0.4. The Kosambi map function was used to estimate genetic distances in cM (Kosambi 1944). The EST-based SNP markers were named <Bo> <EST name> <s> and SCAR markers were designated with <c> at the end, with names following the international nomenclature (De Vincente et al. 2004).

Sequence comparison with *Arabidopsis thaliana* and *Brassica rapa* genomes

The sequences of EST-based SNP loci on a linkage map were aligned with the genome sequences of *A. thaliana* (TAIR; http://www.arabidopsis.org) and *B. rapa* (*Brassica rapa* Genome Sequencing Project Consortium) using the BLAST tool of the Brassica database BRAD (http://brassicadb.org/brad) (Feng et al. 2011) and homologous regions were searched. The E-value, a statistical significance threshold for reporting matches against database sequences, was set at 0.01 for sequence alignment. The sequences of marker loci were regarded as homologous to the genomes with a threshold value of $E<10^{-10}$. The regions having at least three loci with conserved collinearity with *A. thaliana* and *B. rapa* were considered to be homologous syntenic regions. A single non-collinear homologue in the syntenic region was ignored.

Identification of PPR genes and expression analysis

EST-SNP markers flanking *Rfm1* showed sequence collinearity with the *B. rapa* and *A. thaliana* genome sequences. A selected syntenic region of the *A. thaliana* sequence was searched for PPR genes in the browser (http://www.plantenergy.uwa.edu.au/gb2/gbrowse/atbrowser/) and primers were designed based on PPR-encoding genes of *A. thaliana* (O'Toole et al. 2008). A homologous *B. rapa* sequence region was also analyzed by GENSCAN (http://genes.mit.edu/GENSCAN.html/) to predict genes, and deduced amino acid sequences were aligned with known sequences of *A. thaliana* proteins using the BLASTP program of NCBI. Predicted peptides having high similarity to *A. thaliana* PPR proteins were identified. Genes encoding PPR proteins were selected to design specific primers for PCR (Supplementary Table 5). Genomic DNAs of CMS broccoli and restorer Chinese kale were used as templates in PCR amplification. The 20 µl reaction mixture contained 10 ng of plant genomic DNA, 10 pmol of each primer, 1x *ExTaq* buffer, 2.5 nmol of each dNTP and 0.5 U of *Taq* DNA polymerase (*ExTaq*, Takara Biomedicals, Japan). PCR was performed in a thermal cycler with the following cycling conditions: initial denaturation at 94°C for 4 min, 40 cycles of 94°C for 30 sec, annealing temperature (as given in Supplementary Table 5) for 30 sec and 72°C for 45 sec. PCR products were resolved on 1.5% agarose gel and visualized under UV after staining with ethidium bromide.

RNA was extracted from young leaves and buds of CMS broccoli and restorer Chinese kale using SV Total RNA Isolation System (Promega Corp.) as per the manufacture's instructions. First strand cDNA was synthesized from 1 µg total RNA using Pd(N)6 primer and reverse transcriptase of the first strand cDNA synthesis kit (GE Healthcare, UK). RT-PCR was performed using PPR gene-specific primers with the same PCR conditions as used in amplification of genomic DNA. The actin gene primer set was used as a positive control of RT-PCR. The amplified products were separated on 1.5% agarose gel and visualized under UV after staining with ethidium bromide.

Results

Phenotypic analysis of pollen fertility

An F_2 population segregating for pollen fertility was used for linkage map construction and gene mapping. Phenotypic differences were clearly distinguishable between fully fertile and sterile flowers, as the sterile flowers had comparatively short filaments with stunted anthers having a small quantity of pollen, whereas fully fertile flowers had long filaments positioned above a stigma and a high quantity of pollen (Fig. 1). The semi-fertile flowers had medium size filaments and a comparatively less quantity of pollen than fertile ones. Pollen grain stainability showed a clear difference between semi-fertile and fertile, which was reconfirmed by visual examination. The pollen grain fertility

Fig. 1. Flowers of *Diplotaxis muralis* male sterile (A), semi-fertile (B) and fertility-restored *B. oleracea.*

Table 1. Segregation of male sterility and flower color in F_2 population of the CMS broccoli line and the Chinese kale restorer line

Traits	Chinese kale type	CMS broccoli type	$\chi 2$	p
Pollen fertility	Fertile (semi-fertile and fully fertile)	Sterile	$\chi 2$ (15:1)	
	191	14	0.117	0.73
Flower color	White flower	Yellow flower	$\chi 2$ (3:1)	
	153	52	0.014	0.90

Table 2. Frequency of SNPs identified between the CMS broccoli line and the Chinese kale restorer line

Description	Number	Frequency
Primer pairs used	1448	
Amplified as single fragment	729	50.3%
Sequenced	720	
Aligned sequence fragments	633	
Total length of aligned sequence	298,732 bp	
Polymorphic alignments	236	37.2%
Variant bases	1113	1/268 bp
SNP bases	930	1/325 bp
Indel	183	1/1632 bp
SNP sites	752	
Indel sites	85	

was analyzed in 205 individuals, and 191 plants were classified as fertile (170 full fertile and 21 semi-fertile) and 14 as sterile. The phenotypic segregation fitted well with the 15:1 ratio (χ^2 value 0.117), revealing that fertility restoration was controlled by two genes under the duplicate gene interaction mode (Table 1). The genetic mode of fertility restoration was sporophytic and all the plants in F_1 progeny were fully fertile. In the F_2 population, there were 153 white flower plants and 52 yellow flower plants, suggesting that white petal color (from Chinese kale) was dominant over yellow petal color (broccoli) and is under monogenic control (segregation ratio 3:1). In this segregation analysis, we were unable to detect linkage between the genes for fertility restoration and a flower color gene. Reciprocal crosses made between *B. oleracea* cultivars of yellow petal color and *B. oleracea var alboglabra* of white petal color yielded the identical results, hence confirming that the inheritance of flower color is not influenced by the cytoplasm.

SNP analysis and molecular mapping of a restorer gene

A total of 1,448 primer pairs derived from radish EST-sequences were used in PCR amplification of DNAs from parental lines and 729 (~50%) of these primer pairs yielded single band amplification. Out of these, 720 amplified products were sequenced and data showing a sequence quality score >90% were analyzed for SNP identification. In 633 DNA fragments, sequence data were obtained from both CMS broccoli and restorer Chinese kale and aligned properly, while 87 sequences were obtained either from one of the parental lines or were poorly aligned. The aligned sequence data of 633 fragments covering ~300 kb showed 1,113 nucleotide variations between the parental lines. A total of 236 fragments having SNPs were identified by comparing parental sequences. The frequency of variable bases, i.e., SNPs and indels, was 1/268 bp and the frequency of SNP was 1/325 bp (Table 2). Sequences having SNPs between the parental lines were used for designing probes of SNP markers. SNPs of 204 markers, which have been developed for QTL analysis using an F_2 population obtained from a cross between cabbage and broccoli (Kifuji et al. unpublished), were also screened and 48 SNP markers were found to be polymorphic between CMS broccoli and restorer Chinese kale. Among 122 SSR markers surveyed for polymorphism, 15 single-locus SSR markers covering all the linkage groups showed segregation in the F_2 population.

In total, 163 markers segregated in the 205 F_2 plants genotyped. By linkage analysis with JoinMap 4.0 software, 142 markers (125 EST-SNPs, 15 SSR, 1 SCAR and one flower color) could be assigned to nine linkage groups, designated LG1-LG9, and the others remained ungrouped. Primer sequences, probe sequences and hybridization conditions of the mapped SNP markers are given in Supplementary Table 2. One to three SSR markers were assigned to each linkage group and on this basis the linkage groups were designated C1-C9 as per the reference linkage map of *B. oleracea* (Fig. 2). The linkage map spanned 909 cM with an average distance between markers of 6.4 cM, a minimum distance of 0.2 cM and a maximum distance of 23 cM. The largest linkage group (LG1) comprised 30 markers and had length of 182.4 cM and the smallest group (LG9) had 8 loci with 44.6 cM. Based on the estimated physical length of 596 Mb in *B. olercaea* (Johnston et al. 2005), the average physical distance between markers for this map is estimated to be 4.9 Mb (estimated 1 cM=766 kb).

Linkage analysis of phenotypic data by JoinMap 4.0 assigned the *Rfm1* locus to LG1 (chromosome 3) of *B. oleracea*. We were unable to locate the exact position of another restorer locus (*Rfm2*) on the linkage map, but comparing segregation distortion for markers in sterile and semi-fertile plants, it seemed to be positioned on LG3 between markers BoCL6818s and BoCL7968s. SNPs flanking the *Rfm1* locus were BoCL2642s on one side and BoCL2901s on other side at a distance of 2.54 cM and 7.48 cM, respectively. The flower color locus was also assigned to LG1 with a distance of 25 cM from *Rfm1* locus. The closest marker to the flower color locus was BoCL3107s, which was identified at a distance of 5.88 cM.

Syntenic relation with *A. thaliana* and *B. rapa*

All the EST-SNP markers used in the present study showed homology with *A. thaliana* and *B. rapa* genome sequences in BLAST analysis with the BRAD software. Loci homologous to *A. thaliana* at a significance threshold $E < 10^{-10}$ were 115 and were distributed over all the nine linkage groups. Collinearity was interrupted by the presence of markers showing homology to other regions of the *B. oleracea* map at a significant E value. Matching nucleotide lengths, E values and chromosome names are given in Supplementary Table 3. Based on criteria of three or more (continuous) collinear markers, we identified 11 regions syntenic

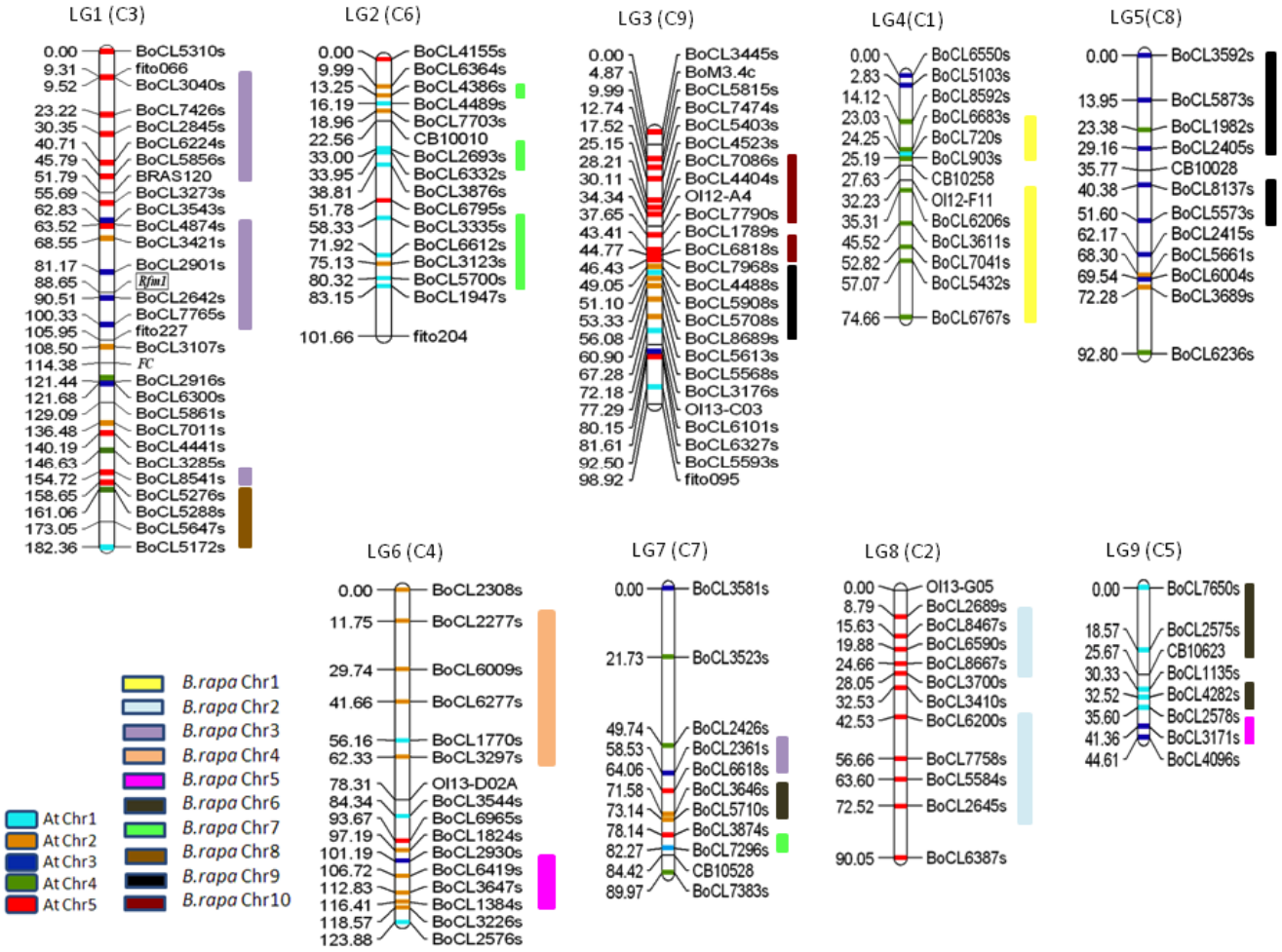

Fig. 2. Genetic linkage map of *B. oleracea* based on EST-SNP markers with comparative maps of *A. thaliana* and *B. rapa*. The linkage groups are labeled as LG1–LG9 in the order of length and their correspondence to the C genome chromosome (C1-C9). Marker positions (in cM) are shown on the left side with the corresponding marker names on the right side of each LG. Each locus was tested for homology with *A. thaliana* and *B. rapa* and is represented in the horizontal bar within LGs (colored according to Parkin et al. 2005) and verticals bars shown to the right of LGs (colored as per given at the bottom of the figure) for *A. thaliana* and *B. rapa* chromosomes respectively. FC on LG1 represents flower color.

with the genome of *A. thaliana*. LG8 was completely collinear with chromosome 5 of *A. thaliana,* whereas LG1 and LG3 were homologous to a 55 cM region and a 40 cM region of *A. thaliana* chromosome 5, respectively. LG7 had segmental homology to all five chromosomes of *A. thaliana.* LG9 was homologous to chromosome 1 of *A. thaliana*. Homology of each locus to the *B. rapa* genome sequence was searched for by the BLAST tool of BRAD (Brassica database) for the recently published *B. rapa* genome sequence (*Brassica rapa* Genome Sequencing Project Consortium) and a total of 24 syntenic segments were identified by comparison with our linkage map of *B. oleracea* based on criteria of three or more (continuous) collinear markers. LG1 had four collinear regions, three of which were collinear with chromosome 3 of *B. rapa*. LG2 (C6), LG4 (C1), LG5 (C8) and LG8 (C2) showed synteny with *B. rapa* chromosome 7, 1, 9 and 2, respectively (Fig. 2).

Identification of PPR genes in the syntenic region and expression analysis

The EST-SNP markers BoCL2901s and BoCL2642s flanking *Rfm1* showed sequence homology with regions of 608,752 - 609,089 bp and 2,439,089 - 2,439,370 bp, respectively, on chromosome 3 of *A. thaliana* and homology with regions of 14,577,244 - 14,577,510 bp and 15,565,034 - 15,565,334 bp, respectively, on chromosome 3 of *B. rapa*. The homologous segment of *A. thaliana* (~3 Mb of chromosome 3) contained 18 genes encoding pentatricopeptide repeat (PPR) proteins. Nine of them were P-subfamily PPR genes, which encode PPR proteins targeted to mitochondria or plastid (Supplementary Table 4). The region of *B. rapa* ca. 1 Mb in size was analyzed by GENSCAN and a total of 216 genes were predicted. These were searched to detect identity with *A. thaliana* PPR proteins by the BLASTP program and deduced amino acid sequences showed identities with 16 PPR proteins. The highest identity (87%, e value 0) was between the *B. rapa* predicted peptide and AtPPR_3g06920. Interestingly, AtPPR_3g06920 was one of the 18 identified PPR genes in the region of ~3 Mb on *A. thaliana* chromosome 3. In most cases, the CMS restorer genes encode PPR proteins of the P-subfamily targeted to mitochondria and these *Rf* PPR genes are present in clusters together with other non-restorer PPR genes (Brown et al. 2003; Koizuka et al. 2003; Barr and Fishman 2010). We utilized the *A. thaliana* sequence information for designing eight specific primer pairs for

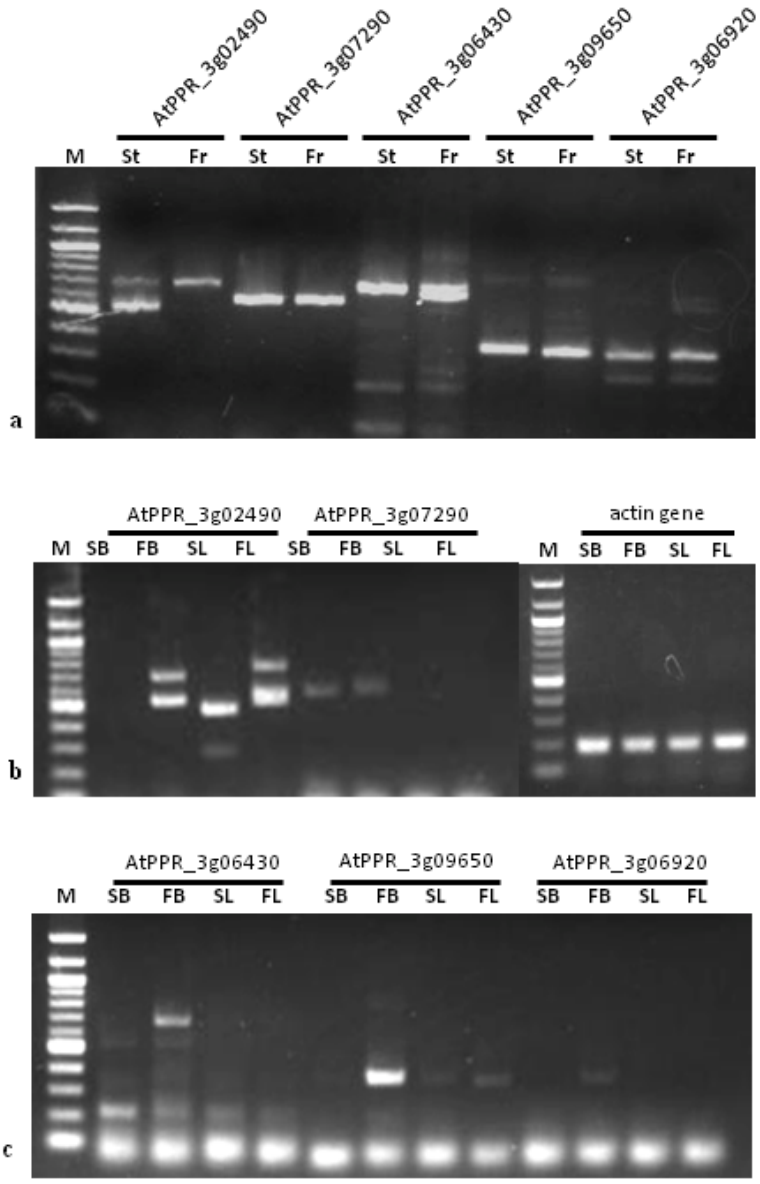

Fig. 3. Analysis of PPR genes. (a) PCR amplification of PPR genes in genomic DNA of CMS broccoli (St) and restorer Chinese kale (Fr). (b and c) Expression analysis of PPR genes using RT-PCR in buds and leaves of CMS and restorer plants. SB, CMS bud; FB, restorer bud; SL, CMS leaf; FL, restorer leaf; M, 100 DNA ladder. The actin gene was used as a control.

P-subfamily PPR genes. In PCR using a primer pair designed from the sequence of AtPPR_3g02490, two faint bands were amplified in parental genomes with slight variation in size. Amplified products having expected sizes were obtained from genomic DNAs of both CMS broccoli and restorer Chinese kale by the primer pairs of AtPPR_3g07290, AtPPR_3g06430, AtPPR_3g049650 and AtPPR_3g06920, whereas there was no amplification by the primer pairs of AtPPR_3g02650, AtPPR_3g04130 and AtPPR_3g09060 (Supplementary Table 5). PCR products of parental genotypes had no size polymorphism and showed monomorphic bands (Fig. 3).

RT-PCR was performed using total RNA from leaves and buds of CMS broccoli and restorer Chinese kale. By the primer pair of AtPPR_3g02490, two bands (approximately 500 bp and 700 bp) were obtained in the buds and leaves of the restorer line by RT-PCR and a single band was detected in the leaves of CMS lines, whereas there was no amplification in the CMS buds. The primer pair of AtPPR_3g07290 generated faint amplification of 500 bp in the buds of both the CMS and restorer lines. By the primer pair of AtPPR_3g06430 and AtPPR_3g06920, products having sizes of 700 bp and 350 bp, respectively, were amplified exclusively in the restorer buds with no amplification in the CMS buds nor in the leaves of the CMS and restorer lines. The primer pair of AtPPR_3g09650 generated a product of 350 bp in the restorer buds and faint amplification in the leaves of the CMS and restorer lines. RT-PCR was replicated and consistency of results was confirmed (Fig. 3).

Discussion

In most *Brassica* CMS systems, a single gene restores pollen fertility, e.g., *pol* CMS of *B. napus* (Feng and McVetty, 1989) and *mori* CMS of *B. juncea* (Prakash et al. 1998), whereas fertility

restoration in *mur* CMS *B. oleracea* was found to be under the control of two loci functioning in a duplicated mode of gene interaction. Janeja et al. (2003) have also reported two dominant genes, i.e., *Rft1* and *Rft2,* required for fertility restoration in *tour* CMS *B. napus*, in which *Rft1* alone can restore pollen fertility completely.

Developing a linkage map of DNA markers is a prerequisite for molecular mapping of important agronomic traits and facilitating marker assisted breeding. Most of the restorer genes cloned so far have been identified by a map-based cloning approach, in which the first step is to find DNA markers linked with an *Rf* locus followed by fine mapping and synteny analysis with known genomes. Although the *Rf2* gene of CMS T-maize encoding a mitochondrial aldehyde dehydogenase has been identified by a transposon tagging strategy (Cui et al. 1996), molecular tagging and fine mapping of restorer loci in other species, e.g., petunia, radish and rice, have been performed for the cloning of genes. We constructed a linkage map of *B. oleracea* using EST-based SNP markers with a segregating F_2 population of CMS and restorer parents. In the primers designed from *R. staivus* EST sequences, 50% primers yielded single band amplification in the parental lines, and 236 ESTs having SNPs (37%) were identified. This result proved the transferability of EST information to other Brassicaceae species and the utility of resources in marker development. *Rfm1* of *mur* CMS *B. oleracea* was mapped on LG1 (*B. oleracea* chromosome 3) with flanking markers at a distance 2.54 cM on one side and 7.48 cM on the other side. For the *tour* Brassica restorer gene, Trendelkamp et al. (1999) have identified 11 AFLP markers linked with the restorer gene, whereas Janeja et al. (2003) have found two AFLP markers by using NILs. Two AFLP markers and one close (0.6 cM) SCAR marker linked to the fertility restorer gene have been developed in *mori* CMS *B. juncea* (Ashutosh et al. 2007). Since AFLP markers could not be used directly for map-based cloning of genes, they must be converted to sequence-tagged markers, such as SCAR, CAPS or SNP markers. Furthermore, AFLP markers frequently reside in intergenic regions, which is not suitable for synteny analysis. The present EST-SNP linkage map would be informative for identification and positional cloning of agronomically important genes in *B. olercaea*.

Chromosome 5 of *A. thaliana* showed synteny to the complete LG8 (*B. oleracea* chromosome 2), a 55 cM LG1 segment (*B. oleracea* chromosome 3) and a 40 cM LG3 segment (*B. oleracea* chromosome 9). Repeated long syntenic regions have been reported for *B. oleracea* corresponding to chromosome 2, 3 and 5 of *A. thaliana* genome by comparing RFLP and EST sequences (Lan et al. 2000; Babula et al. 2003; Parkin et al. 2005) but not for whole linkage group. We also found repeated long regions in *B. oleracea* chromosomes syntenic to *A. thaliana* chromosome 2, 3 and 5, whereas a single syntenic region for chromosome 1 and 4 of *A. thaliana*. *Brassica* species has 87% sequence identity in coding regions with *A. thaliana* (Cavell et al. 1998) and some genes have counterparts. Other genes have no apparent counterparts, but synteny is preserved between Brassica and *A. thaliana*. Transcriptome mapping followed by homology analysis between *B. oleracea* and *A. thaliana* have revealed extensive collinearity of the genomes and duplication mostly of chromosome 1 and 5 of *A. thaliana* (Li et al. 2003). Furthermore, in the present study, sequence homology analysis identified 24 syntenic segments in the recently published *B. rapa* genome. LG1 had four collinear regions, three of which showed synteny with chromosome 3 of *B. rapa.* These results indicate the suitability of sequence-based markers for comparative genomic studies. Radish EST sequences have been utilized extensively to generate an SNP linkage map of *R. sativus* and comparative studies have revealed high homeology among *Brassica* species (Li et al. 2011).

A. thaliana-derived markers have been utilized in high density mapping of the *Rfp* restorer locus of *B. napus* and have supported the extended collinearity between the *B. napus Rfp* region and an orthologous segment of *A. thaliana* genome with a single exception (Formanova et al. 2010). Cloning of the restorer genes of the radish CMS systems 'Ogura' and 'Kosena' has been performed utilizing the close synteny between radish and *A. thaliana* genomes following a map-based approach, and *Rf*-encoded PPR genes have been revealed to be present in clusters together with other non-restorer PPR genes (Brown et al. 2003; Desloire et al. 2003; Koizuka et al. 2003). The flanked markers of the *Rfm1* gene showed homology with the *A. thaliana* region containing 18 genes encoding PPR proteins and nine of these belong to the P-subfamily of the PPR genes. One of the PPR genes, AtPPR3g06920, was also found in a syntenic *B. rapa* region around the *Rfm1* locus. Analyzing syntenic genomic regions from *A. thaliana* and *B. rapa,* Geddy and Brown (2007) have shown that the location and direction of PPR genes are less conserved in collinear regions and often appear in different chromosomal contexts. PPR regions were aligned for a small region, but in the present studies we considered a large region of ca. 3 Mb for analysis and found conserved PPR genes. Kato et al. (2007) have reported that the *Rf-1* locus of rice contains several duplicated copies of the restorer gene. Furthermore, the gene order between clusters from different species is conserved, suggesting that the *Rf1* locus may have been generated by homologous recombination. The PPR protein-encoding genes in *B. oleracea,* similar to AtPPR_3g06430, AtPPR_3g09650 and AtPPR3g06920, which were deduced from the syntenic region of *A. thaliana,* showed gene expression exclusively in fertile buds. All three PPR genes belong to the P-subfamily category in *A. thaliana.* A target organelle of protein encoded by AtPPR_3g06920 is unknown, whereas AtPPR_3g06430 and AtPPR_3g09650 encode proteins targeted to plastids as per Predotar prediction (Lurin et al. 2004). Based on findings of the present study, we consider these genes to be potential candidate restorer genes, but additional molecular analysis is required to confirm their relevance and functional role in fertility restoration.

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Supplementary Data

Supplementary Table 1. Primer pair sequences of polymorphic SSR and SCAR markers and their mapped reference chromosomes of *B. oleracea*

Supplementary Table 2. Sequences of primer pairs and probes of SNP markers and hybridization and washing conditions

Supplementary Table 3. Analysis of homology with *Arabidopsis thalaina* and *Brassica rapa* genome using blast tool of BRAD

Supplementary Table 4. List of PPR genes in the homologous region of *Arabidopsi*s and subcellular localization of PPR proteins

Supplementary Table 5. Primer sequences of PPR genes derived from *Arabidopsis thaliana* sequences for amplification of genomic

DNA in *B. oleracea*

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Supplementary Table 1. Primer pair sequences of polymorphic SSR and SCAR markers and their mapped reference chromosomes of *B. oleracea*

Name of markers	Primer sequences		Reference chromosomes of *B. olercaea*	References
	Forward	Reverse		
SSR markers				
FITO66	AGCCCATTTACCTGCTGA	GAAAGACGATGCTTAGGGT	C3	Iniguez-Luy et al. (2008)
BRAS120	AAAAATAAATACAGCGAACC	ACCTTTAGCAGCTAATCATC	C3	Piquemal et al. (2005)
FITO227	GTAACAGCAGAAGCAGAAGCA	CAGGTTCACGATACACAAGA	C3	Iniguez-Luy et al. (2008)
CB10010	TTATCTTTGAATGAGCATCT	ACCCTGTTCCTTCTACTAT	C6	Piquemal et al. (2005)
FITO204	TCTGATGGAGAAGAAGAAGAC	ATTGAAGAGGAAGAAGGAGAA	C6	Iniguez-Luy et al. (2008)
Ol12-A04	TGGGTAAGTAACTGTGGTGGC	AGAGTTCGCATACTCTGGAGC	C9	Lowe et al. (2004)
Ol13-C03	GATCGGAGATGCGATGAGAG	GCATGCACCAGTGAAAAACTC	C9	Lowe et al. (2004)
FITO95	AGATTTCATCCACAGCCTC	TTTGATTCTTGCGTTCTCTC	C9	Iniguez-Luy et al. (2008)
CB10258	ATGATGCCTAGCATGTCC	AAGCTAAAGCGAAAGAAGC	C1	Piquemal et al. (2005)
Ol12-F11	AAGGACTCATCGTGCAATCC	GTGTCAGTGGCTACAGAGAC	C1	Lowe et al. (2004)
CB10208	ACTACTGTTGCGGTTGGA	GGCATTCATTACGTCTGC	C8	Piquemal et al. (2005)
Ol13-D02A	TTCTCCACACCAAGCAACAC	TACAGGCTTGGTCGTTTTCC	C4	Lowe et al. (2004)
CB10528	ATGCTTTCTTTGCACGAG	ACCAGACTGATGGTGTGC	C7	Piquemal et al. (2005)
Ol13-G05	GTGTGCAGGAAACGATGTTC	GGGAGTTTGAAGAGAAAGCG	C2	Lowe et al. (2004)
CB10623	GAGATCGAAGGTCTCGGT	GAGTCGAAACAGTGGTGG	C5	Piquemal et al. (2005)
SCAR markers				Assession No.
M3.4	CGCTAACTCAAGGCTTCGAA	CTAAATCACTGAGCCGGTGA		AF136223
Nit2	TCAAAGCCAGTACTCCTGGT	TGTCCAGCCGGGTCTTTTAA		AF1380304

Supplementary Table 2. Sequences of primer pairs and probes of SNP markers and hybridization and washing conditions

Marker name *	*B. oleracea*		Primer sequence (5'-3')		Probe sequence**		Hybridization and washing conditions	
	Linkage group	Position (cM)					Temperature	SSC
BoCL5310s	1	0	Forward primer	CAACGAGAATCCAGATGCTGAG	Chinese kale	GTCGCTGACGCTCTCTT	55	0.5
			Reverse primer	TTCAAGACCAGTCCCATAAGCA	Broccoli	GTCGCTGATGCTCTCTT	50	0.5
fito066	1	9.309						
BoCL3040s	1	9.523	Forward primer	AGCGTTTGCAGGAATCATAGGT	Chinese kale	GTTCAGATACCAACAGA	45	0.5
			Reverse primer	AGCTCGGTCAAGAAACTCTGCT	Broccoli	GTTCAGATTCCAACAGA	45	0.5
BoCL7426s	1	23.222	Forward primer	TAGTCCCTTCTCCGATAGCACA	Chinese kale	CTCATGGGGTCTCTCAC	45	0.1
			Reverse primer	GATTCAAGGAGGTGGGATCAT	Broccoli	CTCATGGGATCTCTCAC	45	0.5
BoCL2845s	1	30.346	Forward primer	AACCAAGCAGCTTAATCGGTTC	Chinese kale	CTTGATGCTGAAGAAGA	45	0.5
			Reverse primer	TTGCCTGAAACAGTTCTCCAAG	Broccoli	CTTGATGCGGAAGAAGA	45	0.5
BoCL6224s	1	40.713	Forward primer	ACGACAGAGCTCGTTTAACCAC	Chinese kale	TCAACCAAAAACTATAG	40	0.5
			Reverse primer	TTGACCAAGAACGAAGATGGTG	Broccoli	TCAACCAAGAACTATAG	40	0.5
BoCL5856s	1	45.788	Forward primer	GAAAGGAGCATTGGAAGGACAC	Chinese kale	AAAGTTCCAACTGCGAA	50	0.5
			Reverse primer	GTAATGGTGTGGCGGCATATAA	Broccoli	AAAGTTCCGACTGCGAA	50	0.2
BRAS120	1	51.787						
BoCL3273s	1	55.691	Forward primer	CAAGCGAGATTGTCGTGAAAGA	Chinese kale	AAGTACTTAAGGTGTCT	40	0.5
			Reverse primer	CCAAAGATTTTGCACTGTCAGC	Broccoli	AAGTACTTTAGGTGTCT	40	0.5
BoCL3543s	1	62.828	Forward primer	TTAAGGGTCTTCCTTCCATGCT	Chinese kale	CAGCCTTGACATCACTG	55	0.5
			Reverse primer	GAGGTCAAGATGGAGCAAGGTT	Broccoli	CAGCCTTGGCATCACTG	50	0.5
BoCL4874s	1	63.523	Forward primer	ACTGAAGAGGAGCACGCTTTTT	Chinese kale	GGTAAAAACGGATGGAA	45	0.5
			Reverse primer	AAGCAAAACCGAACCTCTCAAG	Broccoli	GGTAAAAATGGATGGAA	40	0.5
BoCL3421s	1	68.55	Forward primer	ATTTGCAGCGCCTGTTGTAG	Chinese kale	AAGTTGCTAGGTTGGCC	50	0.5
			Reverse primer	AGTGTGCAAACAGCAAGCAG	Broccoli	AAGTTGCTCGGTTGGCC	50	0.2
BoCL2901s	1	81.172	Forward primer	AGTATGGTGCAAAGAAGCTCCA	Chinese kale	TCCTTAGGAAAATCATC	40	0.5
			Reverse primer	GACCAAGAATGTCAACCACCAA	Broccoli	TCCTTAGGCAAATCATC	40	0.2
Rfm1	1	88.645						

BoCL2642s	1	90.513	Forward primer	CCAGGTCTCTGTTTTTCTGCTG	Chinese kale	CAAGGGAATCATCGGCT	50	0.5
			Reverse primer	AGACGTAGGCAAGCATTTGACA	Broccoli	CAAGGGAAGCTTCGACT	50	0.5
BoCL7765s	1	100.328	Forward primer	ACAAGGATGAAAGTTGCAGCAG	Chinese kale	GCTCTCACAGGACAGCT	50	0.5
			Reverse primer	TTCTGTTCTCGAGTTGGTTTCG	Broccoli	GCTCTCACGGGACAGCT	50	0.2
fito227	1	105.951						
BoCL3107s	1	108.496	Forward primer	TGGACGGATTGACTATGGAGAA	Cabbage	TACATATCAGTCTTTGG	45	0.2
			Reverse primer	AAACCCAAAAGAGGGTCAAAGC	Broccoli	TACATATCTGTCTTTGG	45	0.5
FC	1	114.382						
BoCL2916s	1	121.435	Forward primer	ACGAGTTTCAAAAAGGGAGCAG	Chinese kale	ACTGTAGCGGCAGTGGC	50	0.5
			Reverse primer	GTAGAAGATGCCTTTGGCGTTT	Broccoli	ACTGTAGCTGCAGTGGC	50	0.5
BoCL6300s	1	121.68	Forward primer	CATGCAAAGACCCATCACAAGT	Chinese kale	GGACTCAATCCGCCGCA	55	0.5
			Reverse primer	AAGCAAGTCATCGGGAATAGGA	Broccoli	GGACTCAACCCGCCACA	55	0.5
BoCL5861s	1	129.091	Forward primer	ACGCTCCGACTCAATAGCATCT	Chinese kale	ATCGTCCCGAGGTTCAA	50	0.2
			Reverse primer	TTCGGACAACAAGATGAGGAGA	Broccoli	ATCGTCCCTAGGTTCAA	50	0.5
BoCL7011s	1	136.484	Forward primer	AAGCGTAAAGATGCGGTCACTA	Chinese kale	TCACACCATACACGGTG	50	0.5
			Reverse primer	GTTTCGCCTTGTCGACTTCATT	Broccoli	TCACACCAAACACGGTG	50	0.5
BoCL4441s	1	140.187	Forward primer	GGAAAGGACACGACTTTGAGGT	Chinese kale	AGGTGAAGTAATGGAGA	45	0.5
			Reverse primer	AGACTCCGCTTCTCATCTTTCC	Broccoli	AGGTGAAGCGATGGAGA	50	0.5
BoCL3285s	1	146.629	Forward primer	ATCTGAAGGTTGGTCACTGCAA	Chinese kale	GCATGTCTATCATGGTG	45	0.5
			Reverse primer	CAAAACCACTGTCCAAAACTCG	Broccoli	GCATGTCTTTCATGGTG	45	0.5
BoCL8541s	1	154.717	Forward primer	GAGAAGAGCTTCCAAAGGCAAC	Chinese kale	GTCCGTTGATTCCTGCG	50	0.5
			Reverse primer	TCCTTGGTTTCACCCATCTCTT	Broccoli	GTCCGTTGGTTCCTGCG	50	0.5
BoCL5276s	1	158.646	Forward primer	TGAGAACGTAGGAGGCAAAATG	Chinese kale	GTGATTCCTTGCATCAA	45	0.5
			Reverse primer	ACTCGTGAGCAACCACAACAAC	Broccoli	GTGATTCCGTGCACCAA	50	0.1
BoCL5288s	1	161.063	Forward primer	GTTCAACTCCAGGGGAGATGTT	Chinese kale	CTTGACTTTGCTACAGA	45	0.5
			Reverse primer	CTCCATAATCCCACCATCAAAG	Broccoli	CTTGACTTCGCTACAGA	45	0.5
BoCL5647s	1	173.046	Forward primer	GCAAGCATGGATGATGATGAAC	Chinese kale	TCGACCGTAGTAGTGAA	50	0.5
			Reverse primer	AAGACGCTGTCGAAAACGTAGA	Broccoli	TCGACCGTGGTAGTGAA	50	0.5

BoCL5172s	1	182.362	Forward primer	TCCCATATCGAAGGCAAGCTAT	Chinese kale	CGAAATGTCATTTACAT	45	0.5
			Reverse primer	AACGCATACACTTGTCCCCAGT	Broccoli	CAAAATGTAATTTACAT	45	0.5
BoCL4155s	2	0	Forward primer	GCTAAATCGAGCAAAGCTGGTT	Cabbage	AGGAAGACATAAAGGAC	50	0.5
			Reverse primer	GCATTTCTTCCCAGTTTCTTGG	Broccoli	AGGAAGACGTAAAGGAC	50	0.5
BoCL6364s	2	9.987	Forward primer	AAATACACTCAAGGGTGCAAGG	Chinese kale	TTTGATGACCTCGATGA	45	0.5
			Reverse primer	AGAGCTGCTCACTGTGGCTAAA	Broccoli	TTTGATGATCTCGATGA	45	0.5
BoCL4386s	2	13.246	Forward primer	ACAACTTGTGTGATTGGGAACG	Chinese kale	CAAGAAGTCGATGGGCT	50	0.5
			Reverse primer	TCCAACATTGTAGCCTGACCAC	Broccoli	CAAGAAGTTGACGGGCT	50	0.5
BoCL4489s	2	16.194	Forward primer	GCTATGCCCTTCCTCATGCTAT	Chinese kale	CGGTGCCCGGAAGTCCT	55	0.5
			Reverse primer	CGGTGATCTCTTTGCTCATACG	Broccoli	CGGTGTCCAGAAGTCCT	50	0.5
BoCL7703s	2	18.96	Forward primer	GGGAAGTGAGAGGAAAGCAATC	Chinese kale	AACAGAAATTCGTGAGT	45	0.5
			Reverse primer	TAGCATTCTGCCTCTTGCCTTT	Broccoli	AACAGAAACTCGTGAGT	45	0.5
CB10010	2	22.555						
BoCL2693s	2	32.996	Forward primer	CCAAGCTAGGTAAAAGCGAGGA	Chinese kale	ACGCTGGCCAGGGTTTC	55	0.5
			Reverse primer	AATACGCAGTTACGGGTCGAGT	Broccoli	ACGCTGGCTAGGGTTTC	55	0.5
BoCL6332s	2	33.949	Forward primer	TCATCTCTCCTTGCGTCTTCTG	Chinese kale	GCAGCGGCGGTGTTTGT	60	0.5
			Reverse primer	GTAGCGGCAGACAAGAACTTCA	Broccoli	GCAGCGGCCGTGTTTGT	60	0.5
BoCL3876s	2	38.81	Forward primer	CGCACAAGGAGGGAGATACTTT	Chinese kale	TGAAGCCGGCATCACTT	55	0.5
			Reverse primer	CGGCTTTCCAATGTAACCTCTT	Broccoli	TGAAGCCGCCATCACTT	55	0.5
BoCL6795s	2	51.779	Forward primer	GCAAATGCAAGATCTGAACAGG	Chinese kale	TTCTTTTGCCTGTTGTA	45	0.5
			Reverse primer	CATCCAACCATCAAGGACCAA	Broccoli	TTCTTTTGGCTGTTGTA	45	0.5
BoCL3335s	2	58.327	Forward primer	ACACAGACAAAGCAAAGGCAAG	Cabbage	ACCAGACCAAAGAGATA	45	0.5
			Reverse primer	CATTAGAGGCAACGGGAAGAAC	Broccoli	ACCAGACCGAAGAGATA	45	0.5
BoCL6612s	2	71.916	Forward primer	GATTAGCAGCGCAGTTTCACAG	Chinese kale	ATCTCCTAGTCTCCTTG	45	0.5
			Reverse primer	TGAGCTTTCTCCAACTCTGCTT	Broccoli	ATCTCCTACTCTCCTTG	45	0.5
BoCL3123s	2	75.13	Forward primer	TCGCTCCTACAAACCTAAACGA	Chinese kale	TGATGGAAACCGTAAGA	45	0.5
			Reverse primer	TTCATCCGACCTCTCCTTTTTC	Broccoli	TGATGGAAGCCGTAAGA	45	0.2
BoCL5700s	2	80.321	Forward primer	TGCGCATTAGCCGTATTCTGTA	Chinese kale	AGTCGACTACTCAGGGG	50	0.5
			Reverse primer	AGTCTCACCGAGCTCAACATCT	Broccoli	AGTCGACTCCTCAGGGG	50	0.5
BoCL1947s	2	83.148	Forward primer	GATTGACGAGAACCGTACTGGA	Cabbage	AGATTCTCCGGTACTCA	45	0.5

			Reverse primer	CTCGATCGGATGGTACAAACAA	Broccoli	AGATTCTCAGGTACTCA	40	0.2
fito204	2	101.657						
BoCL3445s	3	0	Forward primer	GCATGCAACTGCTAGTCTCGTT	Chinese kale	AAAGGTCACATGAAAAG	40	0.2
			Reverse primer	TTGTGGGAACAGCTTCCCTTAG	Broccoli	AAAGGTCAAATGAAAAG	40	0.2
BoM3.4c	3	4.869						
BoCL5815s	3	9.988	Forward primer	AGCGACGCTAATGGCTACAACT	Chinese kale	ATGGTTCCCGTTCTTTA	50	0.5
			Reverse primer	TGGCAATCCACTTTACCCTTCT	Broccoli	ATGGTCCCGGTTCTTTA	50	0.5
BoCL7474s	3	12.742	Forward primer	GGGGATCTCTCGATAGCTGAAT	Chinese kale	CTAAGCTACACGATAAT	40	0.2
			Reverse primer	CAGAGCGTGTTCAACCGTACTT	Broccoli	CTAAGCTAGACGATAAT	40	0.5
BoCL5403s	3	17.523	Forward primer	ATTGGAGCATTTCAAGCTACCG	Chinese kale	TTCGGAGACAAAAGCTC	50	0.5
			Reverse primer	TCCAGCTACGTCTCTGCAAGTA	Broccoli	TTCGGAGAAAAAGGCTC	50	0.5
BoCL4523s	3	25.145	Forward primer	AATGAATTCTTCTGGGGTGCTC	Chinese kale	AGGTTCATGTAAAGTCA	40	0.5
			Reverse primer	CTTGCTCTTGTCACCAAGCTCT	Broccoli	AGGTTCATCTGAAGTCA	40	0.1
BoCL7086s	3	28.207	Forward primer	AGTGTTTGGGGAAGAGAAGGTG	Chinese kale	GTGGATGTACCGTTTCC	50	0.1
			Reverse primer	TCTGCTCGACGTTCTTTCTCAG	Broccoli	GTGGATGTGCCGTTTCC	50	0.5
BoCL4404s	3	30.106	Forward primer	AGAGAAGACCAACTGGGCAAAG	Cabbage	GCTAAGTTCTGCGAGAC	45	0.2
			Reverse primer	CATCACATGCACGGTTCTAACA	Broccoli	GCTAAGTTTTGCGAGAC	45	0.2
Ol12-A4	3	34.336						
BoCL7790s	3	37.646	Forward primer	GTGTGGATCGATTCCATAACCA	Cabbage	AAGTTTTTGTATTCATG	35	0.5
			Reverse primer	GGAGCTGTGCTTGGATCTTCTT	Broccoli	AAGTTTTTTGTATTCAT	35	0.5
BoCL1789s	3	43.407	Forward primer	GTTTCCGAGACCTTCCGGTTAT	Chinese kale	AAAGTCAAGGGTGAGGA	45	0.5
			Reverse primer	ATCATCAAAGGGTGAACGGAGT	Broccoli	AAAGTCAACGGTGAGGA	45	0.5
BoCL6818s	3	44.767	Forward primer	GAGGTTGCGGTACTCTGCATAA	Cabbage	TTTGGATATTTTTGTTT	35	0.5
			Reverse primer	GGCCAACCCTTGTGTAATCATA	Broccoli	TTTGGATTTGTTTGTTT	35	0.5
BoCL7968s	3	46.425	Forward primer	ACAAGACGCATCAATGTCACCT	Cabbage	GAGCTATCATGGAGGTG	45	0.2
			Reverse primer	GAAACCCCCTTAGCCTCTTTTG	Broccoli	GAGCTATCGTGGAGGTG	45	0.2
BoCL4488s	3	49.046	Forward primer	CGGTCATGTCTCCCTCTTTTTC	Chinese kale	ACTTATCCACAACAAGT	45	0.5
			Reverse primer	TAAGGTGTACGGCGAGTTCGTA	Broccoli	ACTTATCCGCAACAAGT	45	0.5

BoCL5908s	3	51.104	Forward primer	TCTACGATCAACCAACCCTCCT	Chinese kale	CTCCTGCGCCCATCAAC	50	0.1
			Reverse primer	GAGGTTCTCGATGATGACGTTG	Broccoli	CTCCTGCGACCATCAAC	50	0.2
BoCL5708s	3	53.325	Forward primer	GCGCTTCGAATGAATCTCTCTT	Chinese kale	GCTGTTTCTTGGAAAAC	45	0.5
			Reverse primer	AGCTGAATCACTTGCAGCTCCT	Broccoli	GCTGTTTCGTGGAAAAC	45	0.2
BoCL8689s	3	56.08	Forward primer	CCTTCCTTCATTTGACGCTTG	Chinese kale	GGTTGAAATGGATGTTC	45	0.5
			Reverse primer	GGAGTTTTCTTCTCTGCCTCCA	Broccoli	GGTTGAAAAGGATGTTC	45	0.5
BoCL5613s	3	60.896	Forward primer	GAAGGGTGGAAAACACTCCACT	Chinese kale	GCTTCCAGTTCCTCCGG	50	0.5
			Reverse primer	CATTTGTTAACGGCTGGGTCTC	Broccoli	GCTTCCAGGTCCTCCGG	50	0.5
BoCL5568s	3	67.283	Forward primer	AATAAACCTCTGGCGGTTATCG	Chinese kale	GATGAGAACGGGAAAGG	45	0.5
			Reverse primer	AGCGAACACACCAACAACACTC	Broccoli	GATGAGAATGGGAAAGG	45	0.5
BoCL3176s	3	72.184	Forward primer	TCAGATCTCCTGAGGTTGTTCG	Chinese kale	GAGTGTGCTTCTATAAA	45	0.5
			Reverse primer	GTCATTCTCTCCCCACCCTTTA	Broccoli	GAGTGTGCCTCTATAAA	45	0.5
Ol13-C03	3	77.286						
BoCL6101s	3	80.149	Forward primer	CACTTCAAGAATCCAGCCAAGA	Cabbage	TTTAATTAGTCGTTCTA	35	0.1
			Reverse primer	GAGCAACGCAAAAGTCAATCAC	Broccoli	TTTAATTATTCGTTCTA	35	0.1
BoCL6327s	3	81.615	Forward primer	AACAGCCAACTTCATCTTCGTG	Chinese kale	CAGCTTATGCGTTTGCT	45	0.2
			Reverse primer	TCGAGCATATGACGAGCTTCTT	Broccoli	CAGCTTATACGTTTGCT	45	0.5
BoCL5593s	3	92.504	Forward primer	TCCATCTCTTCACCCACTTCTG	Chinese kale	TTCCTCGTCGTCCCACG	50	0.2
			Reverse primer	TGTCGATTCCGACATGGTTATC	Broccoli	TTCCTCGTTGTCCCACG	50	0.5
fito095	3	98.919						
BoCL6550s	4	0	Forward primer	GTCTTCTCCGATCCGATTCTTC	Chinese kale	GGAGACGGCGGCGCGAG	60	0.5
			Reverse primer	GAGTAAGAGCCAACGCCATAGA	Broccoli	GGAGACGGTGGCGCGAG	60	0.5
BoCL5103s	4	2.825	Forward primer	AAAGCATTCAAGGGCTACATCG	Chinese kale	CAGATTTGCTCCCTTTA	45	0.5
			Reverse primer	GATGCCATCCCATGATGAAAC	Broccoli	CAGATTTGTTCCCTTTA	45	0.5
BoCL8592s	4	14.122	Forward primer	CTGCAAACATATCCTCGGTTTC	Chinese kale	TTAATTACAATATTTAA	30	0.5
			Reverse primer	ATGGATTGGTGTGGATAAGAGG	Broccoli	TTAATTACTTTATTTAA	30	0.5
BoCL6683s	4	23.025	Forward primer	GAAGAAAGTCGAAATGCGTGTG	Cabbage	TTGCCAAAGCTAAACAG	40	0.5
			Reverse primer	GATTCCACGCAAACTCTCAATG	Broccoli	TTGCCAAACCTAAACAG	40	0.5
BoCL720s	4	24.253	Forward primer	CAAAAAGGAAGATCTGGTGCAG	Cabbage	CTGAAGCACTTTTGTTA	40	0.5

			Reverse primer	GGAACATGCCCATTATCAGACA	Broccoli	CTGAAGCATTTTTGTTA	40	0.5
BoCL903s	4	25.192	Forward primer	ACGGCTCTTCGGGAACATATAC	Cabbage	CTCGCATGTAACGGTTT	45	0.5
			Reverse primer	CTCTCTCTCACTGTCGGCAAAA	Broccoli	CTCGCATGCAACGGTTT	50	0.5
CB10258	4	27.625						
Ol12-F11	4	32.229						
BoCL6206s	4	35.309	Forward primer	TGCCACTCGTAAAGGTATGTGG	Chinese kale	AGACGACATGGCATCGG	50	0.5
			Reverse primer	AGTCAACCACCTTTGCACATGA	Broccoli	AGACGACACGGCATCGG	50	0.2
BoCL3611s	4	45.517	Forward primer	AATATCGAAAGCACCAGGCTTC	Chinese kale	AGGGAGATTCCTGCGTA	50	0.5
			Reverse primer	CTTCTTAACCGGAGCAATGACC	Broccoli	AGGGAGATCCCTGCGTA	50	0.2
BoCL7041s	4	52.822	Forward primer	AAGCAGGTAAAGACTCCGTGCT	Chinese kale	GGCGCTGCAAAGCTCAC	55	0.5
			Reverse primer	CAAGAACAAGGCCCAGAACACT	Broccoli	GGCGCTGCTAAGCTCAC	55	0.5
BoCL5432s	4	57.068	Forward primer	TCAGGCTTCGTCGAGTACATTC	Chinese kale	ATGCAGAGATTGTGATA	45	0.5
			Reverse primer	AGCTCTCATAGCAACACCATCG	Broccoli	ATGCAGAGGTTGTGATA	45	0.2
BoCL6767s	4	74.664	Forward primer	CATCCAAGAAGCATGCAACACT	Chinese kale	ATCTCAGGGGAAGACTT	45	0.2
			Reverse primer	GGAGGCATTAAGCATCTCTTCC	Broccoli	ATCTCAGGAGAAGACTT	45	0.2
BoCL3592s	5	0	Forward primer	GCTGATCAACTTCCATCTCCAA	Chinese kale	AACAAGATTGTCCTATT	45	0.5
			Reverse primer	GATATGGGTGATGGTTGGGTTT	Broccoli	AACAAGATGGTCCTATT	45	0.5
BoCL5873s	5	13.946	Forward primer	GCTTCCCTTTCTCGTTTTCTCA	Chinese kale	CTCCGTTAACGTGGGGA	55	1
			Reverse primer	CAATGTTCTTCAATCCCAGCAC	Broccoli	CTCCGTTACCGTGGGGA	55	0.5
BoCL1982s	5	23.375	Forward primer	CTTTTTCCCAGTGAAAGCTTGG	Cabbage	AGAGCTACTACTTGCTA	40	0.5
			Reverse primer	AAGTTGTGCCTGAACCTGAACC	Broccoli	AGAGCTACCACTTGCTA	45	0.5
BoCL2405s	5	29.158	Forward primer	TCACTGCCACTACTTGCAAAGC	Chinese kale	TTATGCTCAGCTACGCT	50	0.5
			Reverse primer	AGCGAGTGCATCAGAACGTTTA	Broccoli	TTATGCTCGGCTACGCT	50	0.2
CB10028	5	35.769						
BoCL8137s	5	40.383	Forward primer	AACCTCCCTGTGACTTCCTTCA	Chinese kale	ATGTGCGTGTCTATGAG	45	0.2
			Reverse primer	ATATTGCCTTGACCACATGCAC	Broccoli	ATGTGCGTATCTATGAG	45	0.5
BoCL5573s	5	51.595	Forward primer	ATGTGCATGGACAATCGCTTAG	Chinese kale	CTGGATGGGTTGTGTGA	45	0.2
			Reverse primer	CATTCCTTTGAGAGGGAGGCTA	Broccoli	CTGGATGGATTGTGTGA	45	0.5

BoCL2415s	5	62.172	Forward primer	CTGCATTACCTTCACGTCTTCC	Chinese kale	AAGAGTTCGCTGCTTGC	50	0.5
			Reverse primer	CGCTCTCATGAACCGATAATCT	Broccoli	AAGAGTTCACTGCTTGC	50	1
BoCL5661s	5	68.303	Forward primer	ACGAACGTTGTACCCAATGTGA	Chinese kale	CATTACGCGTGTCTGAT	45	0.2
			Reverse primer	CTGCTTTCTTTCCCAATTCCAC	Broccoli	CATTACGCTTGTCTGAT	45	0.5
BoCL6004s	5	69.542	Forward primer	AAGAGACAAGCCCACGAATCAT	Chinese kale	AAGTAAGGGAAGAGGAG	40	0.5
			Reverse primer	GTCCTAAAGACCCATCGCAATC	Broccoli	AAGTAAGGAAAGAGGAG	40	0.5
BoCL3689s	5	72.275	Forward primer	AACCTCCACAAAAACCTCATCC	Chinese kale	TGGAACAGGGACTTCAT	45	0.2
			Reverse primer	AGGAGCATCATCAGGGGAAT	Broccoli	TGGAACAGAGACTTCAT	45	0.5
BoCL6236s	5	92.8	Forward primer	CAAATGCTCCAAGAACTGAACC	Chinese kale	GACAACGGGGTAGCTCT	55	0.5
			Reverse primer	ATCTTCAATCCTGGGCAAACTC	Broccoli	GACAACGGCGTAGCTCT	55	0.5
BoCL2308s	6	0	Forward primer	GAGACTGCATCTGGATTTGGTG	Chinese kale	TGTAAACAGAGTGCAAC	45	0.5
			Reverse primer	TTCACAGGAAGAAACCATGACC	Broccoli	TGTAAACAAAGTGCAAC	45	0.5
BoCL2277s	6	11.754	Forward primer	AGAAGCCGAGCATTGTGTTG	Chinese kale	CCTGGAGGTCTCTTGGG	50	0.5
			Reverse primer	AGTCCCCTGGATTCCTTGAA	Broccoli	CCTGGAGGACTCTTGGG	50	0.5
BoCL6009s	6	29.743	Forward primer	TGTGAGCAAGGTTACCGTCTTG	Chinese kale	ACCTGGTTGCTAGATAA	40	0.1
			Reverse primer	TTACCATGGCTTCCTCATCTTG	Broccoli	AACTGGTTACTAGATAA	40	0.5
BoCL6277s	6	41.657	Forward primer	CCGATATGGTGGAGATGGTACT	Chinese kale	ATACTGCTCTTTGTCTT	45	0.5
			Reverse primer	CAACGTCCAAAACACACTATGC	Broccoli	ATACTGCTGTTTGTCTT	45	0.5
BoCL1770s	6	56.159	Forward primer	GCTTCCTTTCACATGCTCCTCT	Cabbage	ATGACGATATGCATGAT	40	0.1
			Reverse primer	CCTGGAATCGTGCTTGATGTT	Broccoli	ATGACGATCTGCATGAT	40	0.1
BoCL3297s	6	62.334	Forward primer	ATAGCGAGAGCGCAAGAGAGAT	Chinese kale	GTTCCTGTCTCTCTGTT	45	0.5
			Reverse primer	ATCAGCTGCATTTCTGCAAGAC	Broccoli	GTTCCTGTATCTCTGTT	45	1
Ol13-D02A	6	78.313						
BoCL3544s	6	84.338	Forward primer	GGATCCACGAAAACCCACTAAA	Chinese kale	CTCGTTCTTGTTTCGGC	45	0.5
			Reverse primer	TAACTCCGGGGACAACGTTAAT	Broccoli	CTCGTTCTAGTTTCGGC	45	0.5
BoCL6965s	6	93.674	Forward primer	CCGCTTCTAAAATTCCTCTCCA	Chinese kale	CATCGAGGTCATCTCTC	50	0.5
			Reverse primer	CGGAATCAATCTTGTTGCTACG	Broccoli	CATCGAGGCCATCTCTC	50	0.5
BoCL1824s	6	97.187	Forward primer	GGAACTTCCCTCGAGAGTCAAA	Cabbage	AAGATTGTTAAGCTCGA	40	0.5
			Reverse primer	AAACTTCAGTTCAGGGCATGG	Broccoli	AAGATTGTGAAGCTCGA	45	0.5
BoCL2930s	6	101.19	Forward primer	TTCGGTTTCGATTCTCCTCTTC	Chinese kale	TCTCTGATCCATCTCAC	40	0.2

			Reverse primer	TGATTAACCGGAAGGCTCCTAA	Broccoli	TCTCTGATTCATCTCAC	40	0.2
BoCL6419s	6	106.718	Forward primer	CGCAAATCCTACCAAGACCTTC	Chinese kale	GCTGCGTCCGCTGTTTC	55	0.5
			Reverse primer	ATCGAGGAGGTCTGGATTCTCA	Broccoli	GCTGCGTCTGCTGTTTC	55	0.5
BoCL3647s	6	112.827	Forward primer	TGTCACTTGGAGGTACACACGA	Chinese kale	GACCAGCGGGTGGTGAA	55	0.2
			Reverse primer	TGCATCGGCCATATAAGAGTTG	Broccoli	GACCAGCGAGTGGTGAA	55	0.2
BoCL1384s	6	116.413	Forward primer	GAAGAACAAAGTGGCGGCTATT	Cabbage	CAGCGTCGTATTGTTAG	40	0.5
			Reverse primer	CATGGTTGATGGCTTCATACG	Broccoli	CAGCGTCGGATTGTTAG	45	0.2
BoCL3226s	6	118.569	Forward primer	CATCCGACGATAGAATGGTGAG	Chinese kale	TGGGATCTTGGAGATGG	50	0.5
			Reverse primer	CTACATCCCATGCCGGTTAAGT	Broccoli	TGGGATCTCGGAGATGG	50	0.5
BoCL2576s	6	123.876	Forward primer	AGATTGCGTTGAAGTGTTAGGC	Chinese kale	GCTCTTGCTTTGGTTAA	45	0.5
			Reverse primer	GGGAAACTTCACACTTCGTTTC	Broccoli	GCTCTTGCGTTGGTTAA	45	0.2
BoCL3581s	7	0	Forward primer	TCCTCTGATGTTGTTCCTGTGC	Chinese kale	ACTTTTGTATCATTAAT	35	0.5
			Reverse primer	TTACACATTTCCCCACCTTGTG	Broccoli	ACTTTTGTCTCATTAAT	35	0.2
BoCL3523s	7	21.726	Forward primer	GAAGTCATGACCAGATCGATGG	Chinese kale	AGACGGGAGGGGCGAAC	55	0.2
			Reverse primer	GACCTTGACAAAAACGCTACCA	Broccoli	AGACGGGACAGGCGAAC	55	0.2
BoCL2426s	7	49.737	Forward primer	TTGTCCAGAGCATCTTTTGCAG	Cabbage	ACTATGTCAGCTAGAGA	40	0.5
			Reverse primer	TATCCATTACATTCGCGTGGTC	Broccoli	ACTATGTCTGCTAGAGA	40	0.5
BoCL2361s	7	58.532	Forward primer	TCTAGATAACCACGTGGCGTTG	Chinese kale	CGGTACATAGCTGGGCT	50	0.5
			Reverse primer	CACGGATAGCAAGCTTCACAGT	Broccoli	CGGTACATTGCTGGGCT	50	0.5
BoCL6618s	7	64.064	Forward primer	GTCGATGCCTAGCGTTGTGATA	Chinese kale	ACGTGGGTCGGGACTAG	55	0.5
			Reverse primer	CCGGTTCACAAACTCCAAAATC	Broccoli	ACGTGGGTGGGGACTAG	55	0.5
BoCL3646s	7	71.583	Forward primer	TATGCTCAAGATGGTTCAGTGG	Chinese kale	CTCATTGGTGCTGTCTT	50	0.5
			Reverse primer	AGTAAATGCCGGAGAAACAAGC	Broccoli	CTCATTGGAGCTGTCTT	50	0.5
BoCL5710s	7	73.135	Forward primer	CAAGGCATGTCCGTAACGTAAG	Cabbage	TTAGTTGAATTTAACGT	35	0.2
			Reverse primer	GGGTCTCGCATTTACATACACG	Broccoli	TTAGTTGAGTTTAACGT	35	0.2
BoCL3874s	7	78.137	Forward primer	ACGGGAAGCCAGTTTCAAGA	Cabbage	AGAAAAAAGATTGTTCT	35	0.5
			Reverse primer	TAACGAAAACCAGAGGATCAGC	Broccoli	AGAAAAAAAATTGTTCT	35	0.5
BoCL7296s	7	82.274	Forward primer	TCCGGCAAGAAGTGTCTGTT	Chinese kale	AGTGCATGCCCGAAACT	50	0.5
			Reverse primer	TGGTCATGTTCATGCCTACGA	Broccoli	AGTGCATGTCCGAAACT	50	0.5
CB10528	7	84.423						

BoCL7383s	7	89.971	Forward primer	AGTTCTTGACGAATTGCCTTCC	Chinese kale	TGACAAGTAGGAGGCGA	50	0.5
			Reverse primer	ACTCAAGCCCATCGACTACGTT	Broccoli	TGATAATTGGAAGGCGA	45	0.5
Ol13-G05	8	0						
BoCL2689s	8	8.792	Forward primer	TCGCAGGCATCTTAGAATACGA	Chinese kale	TGCCTCAGAAGGTTGAT	50	0.5
			Reverse primer	GTAACGCAGCACCATCAAGTTC	Broccoli	TGCCTCAGGAGGTTGAT	50	0.5
BoCL8467s	8	15.628	Forward primer	CAAACCGGTTCTGTGAAATCTG	Chinese kale	GCAGAGACCGTCTTCCA	50	0.5
			Reverse primer	CACGCCTCAGAATAGCAATCAA	Broccoli	GCAGAGACTGTCTTCCA	50	0.5
BoCL6590s	8	19.88	Forward primer	GTCTTCATTGGAGCCTCTGGAT	Chinese kale	AGTAAAGCCTACATTTT	40	0.5
			Reverse primer	ACCGAGGCTCTTTCTTCTATCG	Broccoli	AGTAAAGCATACATTTT	40	0.5
BoCL8667s	8	24.663	Forward primer	CCCGGAAAATTCTCAGCTTCTA	Chinese kale	TGTTTCTTTCTGACACC	45	1
			Reverse primer	CATAACGTTGACGGTCTCTTCC	Broccoli	TGTTTCTTGCTGACACC	45	0.5
BoCL3700s	8	28.05	Forward primer	AGTGAGGATGACCACAATCCAA	Chinese kale	AGGCTCGCCTCAAGGTT	50	0.2
			Reverse primer	TTGTCTGTCTCTCCCTTCATCG	Broccoli	AGGCTCGCATCAAGGTT	50	0.5
BoCL3410s	8	32.529	Forward primer	CGATCAACAACGTCTCCTTGTC	Chinese kale	GGGATCGTGGTGGACGT	50	0.5
			Reverse primer	CATATCTTAACGCCGTCCATCC	Broccoli	GGGATCGTTGTGGACGT	50	0.5
BoCL6200s	8	42.533	Forward primer	GGTTGGAAAGCAATTGGTGAAC	Chinese kale	AGAAGGAATGAGAAGTC	45	0.5
			Reverse primer	GGTTCGACACACAAAGAAACCA	Broccoli	AGAAGGAACGAGAAGTC	45	0.5
BoCL7758s	8	56.663	Forward primer	TTCCTAAGACGGTGTCTCAAGC	Chinese kale	AACCGTGCTTACTTGCG	50	0.5
			Reverse primer	CTTCTTGATTCAGCTGCGTTTG	Broccoli	AACCGTGCCTACTTGCG	50	0.2
BoCL5584s	8	63.599	Forward primer	CAAGAGCACAATCTCGGTCCTA	Cabbage	GGTACCACTCAGGAGAA	45	0.5
			Reverse primer	ATGACACGCGTTTACACTCTGC	Broccoli	GGTACCACACAGGAGAA	45	0.5
BoCL2645s	8	72.522	Forward primer	TACGGATCGGGTCAAATAAACC	Chinese kale	TACGGACCGTCGACCTA	55	0.5
			Reverse primer	CAAGATGGGACTCCTCACAAGA	Broccoli	TACGGACCTTCGACCTA	55	1
BoCL6387s	8	90.05	Forward primer	TTGATGCGCTTAAAGGTGGTC	Chinese kale	TGGCAGGCAGCTACAAG	55	0.5
			Reverse primer	CCCTGATCTCTTCTGTTGCTTC	Broccoli	TGGCAGGCGGCTACAAG	55	0.5
BoCL7650s	9	0	Forward primer	AAGTTCCTGGCTGCAGCTCTAT	Cabbage	AAGAAGAACGGAAAGAA	40	0.5
			Reverse primer	AATGGTGGAACCGAGTTCTGTC	Broccoli	AAGAAGAATGGAAAGAA	40	0.5
BoCL2575s	9	18.574	Forward primer	TCGTTCAACATGGTTCATAGCC	Chinese kale	AGATAGATGTCATTCAA	40	0.5
			Reverse primer	CCCCTGTCTCCAAATGCAATA	Broccoli	AGATAGATCTCATTCAA	40	0.5
CB10623	9	25.668						

BoCL1135s	9	30.335	Forward primer	TACAAGTACCGGCCATAGGTGA	Cabbage	TTCATATTGGAACGGCT	40	0.5
			Reverse primer	GCATGCTGAAAGATTCTCTGTG	Broccoli	TTCATATTTGAACGGCT	40	0.5
BoCL4282s	9	32.523	Forward primer	ACTAAACCCTGGTGGTGTTTCC	Chinese kale	GTTGCACCAGTTTCTGT	50	0.5
			Reverse primer	CATCAGATCAGCCATCATGACA	Broccoli	GTTGCACCTGTTTCTGT	50	0.5
BoCL2578s	9	35.597	Forward primer	CAACACATCTCTTCCCAAAACC	Chinese kale	CCAACACCTGCCATAGC	50	0.5
			Reverse primer	TGTGGAAGTGGGTAAAGGGTTA	Broccoli	CCAACACCGGCCATAGC	50	0.2
BoCL3171s	9	41.361	Forward primer	TAGCCCTAATCTCATGGGTGGT	Chinese kale	AGAACCGCGATAAGCCA	50	0.5
			Reverse primer	GATCGCCGAAACCCAATAGTAA	Broccoli	AGAACCGCAATAAGCCA	50	0.5
BoCL4096s	9	44.609	Forward primer	CAGATGACCTCTTCTCCGGAAT	Chinese kale	AAAATCCTCTTGGAGGC	50	0.5
			Reverse primer	CATCATCCCAGGAGGGAAATTA	Broccoli	AAAATCCTGTTGGAGGC	50	0.5

* SCAR and SSR markers primer sequences are given in Supplementary Table 1

** The oligonucleotide probes were designed as bridge probes (Shiokai et al. 2010b). Sequences excluding bridge sequence are shown. A sequence, TATATTTACATTCGCAATTAAGAGGCTTCGT designated as SCR-27, and a sequence, TATATTCCCTCCGTCAGCGGATC designated as ds-52, were added to allele-specific sequences of Chinease kale and broccoli, respectively.

Supplementary Table 3 . Analysis of homology with *Arabidopsis thalaina* and *Brassica rapa* genome using blast tool of BRAD*

S/n	LG	Locus	Significant alignment with *Arabidopsis thaliana* (Chr no., identity, e value)	Significant alignment with *Brassica rapa* (Chr no., identity, E-value)
1	1	BoCL5310s	Chr5 385 e-106	A03 650 0.0
2	1	fito066		
3	1	BoCL3040s	Chr5 92 2e-17	A03 509 e-143
4	1	BoCL7426s	Chr5 226 5e-58	A03 452 e-125
5	1	BoCL2845s	Chr5 281 8e-75	A03 650 0.0
6	1	BoCL6224s	Chr5 135 9e-31	A03 272 1e-71
7	1	BoCL5856s	Chr5 88 2e-16	A03 533 e-150
8	1	BRAS120		
9	1	BoCL3273s	Chr5	A03 482 e-135
10	1	BoCL3543s	Chr3 147 3e-34	A05 739 0.0
11	1	BoCL4874s	Chr5 523 e-147	A03 775 0.0
12	1	BoCL3421s	Chr2 60 4e-08	A03 281 1e-74
13	1	BoCL2901s	Chr3 149 9e-35	A03 505 e-142
14	1	*Rfm1*		
15	1	BoCL2642s	Chr3 250 2e-65	A03 484 e-135
16	1	BoCL7765s	Chr3 402 e-111	A03 551 e-155
17	1	fito227		
18	1	BoCL3107s	Chr2 113 4e-24	A06 521 e-146
19	1	FC		
20	1	BoCL2916s	Chr4 270 3e-71	A08 670 0.0
21	1	BoCL6300s	Chr3 345 5e-94	A03 678 0.0
22	1	BoCL5861s	Chr4 301 8e-81	A08 825 0.0
23	1	BoCL7011s	Chr2 78 2e-13	A07 406 e-112
24	1	BoCL4441s	Chr5 456 e-127	A06 642 0.0
25	1	BoCL3285s	Chr4 442 e-123	A08 630 e-179
26	1	BoCL8541s	Chr5 478 e-134	A03 581 e-164
27	1	BoCL5276s	Chr5 309 2e-83	A03 498 e-139
28	1	BoCL5288s	Chr4 287 1e-76	A08 418 e-115
29	1	BoCL5647s	Chr1 167 2e-40	A08 234 3e-60
30	1	BoCL5172s	Chr1 305 3e-82	A08 505 e-142
31	2	BoCL4155s	Chr5 58 3e-07	A06 577 e-163
32	2	BoCL6364s	Chr3 103 5e-21	A07 650 0.0
33	2	BoCL4386s	Chr3 402 e-111	A07 712 0.0
34	2	BoCL4489s	Chr1 198 7e-50	A03 125 2e-27
35	2	BoCL7703s	Chr3 62 2e-08	A07 396 e-109
36	2	CB10010		
37	2	BoCL2693s	Chr1 521 e-147	A07 702 0.0
38	2	BoCL6332s	Chr1 331 9e-90	A07 638 0.0
39	2	BoCL3876s	Chr1 311 6e-84	A07 581 e-164
40	2	BoCL6795s	Chr5 141 2e-32	A06 607 e-172
41	2	BoCL3335s	Chr1 264 2e-69	A07 755 0.0
42	2	BoCL6612s	Chr1 80 5e-14	A07 236 8e-61
43	2	BoCL3123s	Chr2 202 6e-51	A07 599 e-170

44	2	BoCL5700s	Chr1	276	3e-73	A07	450	e-125
45	2	BoCL1947s	Chr1	301	7e-81	A07	611	e-173
46	2	fito204						
47	3	BoCL3445s	Chr5	276	6e-73	A10	440	e-122
48	3	BoM3.4c						
49	3	BoCL5815s	Chr5	484	e-135	A10	722	0.0
50	3	BoCL7474s	Chr5	198	8e-50	A10	565	e-160
51	3	BoCL5403s	Chr5	351	8e-96	A10	654	0.0
52	3	BoCL4523s	Chr5	113	4e-24	A10	287	3e-76
53	3	BoCL7086s	Chr5	377	e-103	A10	555	e-157
54	3	BoCL4404s	Chr5	250	4e-65	A10	373	e-102
55	3	Ol12-A4						
56	3	BoCL7790s	Chr5	54	4e-06	A10	345	2e-93
57	3	BoCL1789s	Chr5	363	2e-99	A10	728	0.0
58	3	BoCL6818s	Chr5	285	6e-76	A10	488	e-136
59	3	BoCL7968s	Chr5	373	e-102	A10	549	e-155
60	3	BoCL4488s	Chr2	188	8e-47	A09	696	0.0
61	3	BoCL5908s	Chr1	190	2e-47	A09	601	e-170
62	3	BoCL5708s	Chr2	123	5e-27	A09	434	e-120
63	3	BoCL8689s	Chr2	92	2e-17	A09	618	e-176
64	3	BoCL5613s	Chr2	135	1e-30	A09	523	e-147
65	3	BoCL5568s	Chr2	351	7e-96	A09	490	e-137
66	3	BoCL3176s	Chr1	472	e-132	A09	710	0.0
67	3	Ol13-C03						
68	3	BoCL6101s	Chr3	168	1e-40	A07	771	0.0
69	3	BoCL6327s	Chr5	367	e-100	A09	1070	0.0
70	3	BoCL5593s	Chr1	240	3e-62	A06	779	0.0
71	3	fito095						
72	4	BoCL6550s	Chr3	90	4e-17	A01	287	2e-76
73	4	BoCL5103s	Chr3	56	9e-07	A03	337	4e-91
74	4	BoCL8592s	Chr4	174	1e-42	A01	327	3e-88
75	4	BoCL6683s	Chr4	184	1e-45	A01	809	0.0
76	4	BoCL720s	Chr1	68	2e-10	A01	371	e-101
77	4	BoCL903s	Chr4	147	3e-34	A01	617	e-175
78	4	CB10258						
79	4	Ol12-F11						
80	4	BoCL6206s	Chr4	238	9e-62	A01	517	e-145
81	4	BoCL3611s	Chr4	212	5e-54	A01	299	7e-80
82	4	BoCL7041s	Chr4	163	4e-39	A01	432	e-120
83	4	BoCL5432s	Chr4	402	e-111	A01	533	e-150
84	4	BoCL6767s	Chr4	323	1e-87	A01	480	e-134
85	5	BoCL3592s	Chr3	115	9e-25	A09	704	0.0
86	5	BoCL5873s	Chr3	90	3e-17	A09	333	3e-90
87	5	BoCL1982s	Chr2	125	8e-28	A09	486	e-136
88	5	BoCL2405s	Chr3	391	e-107	A09	559	e-158
89	5	CB10028						
90	5	BoCL8137s	Chr3	357	2e-97	A09	644	0.0
91	5	BoCL5573s	Chr3	278	1e-73	A09	504	e-141
92	5	BoCL2415s	Chr3	196	3e-49	A06	567	e-160

93	5	BoCL5661s	Chr2	361	1e-98	A04	678	0.0
94	5	BoCL6004s	Chr3	161	3e-38	A09	440	e-122
95	5	BoCL3689s	Chr2	232	5e-60	A04	416	e-115
96	5	BoCL6236s	Chr4	291	7e-78	A01	636	0.0
97	6	BoCL2308s	Chr2	167	3e-40	A01	369	e-100
98	6	BoCL2277s	Chr2	264	2e-69	A04	492	e-138
99	6	BoCL6009s	Chr2	90	8e-17	A04	599	e-170
100	6	BoCL6277s	Chr2	159	6e-38	A04	410	e-113
101	6	BoCL1770s	Chr1	182	5e-45	A04	454	e-126
102	6	BoCL3297s	Chr2	186	3e-46	A04	289	6e-77
103	6	Ol13-D02A						
104	6	BoCL3544s	Chr1	72	2e-11	A08	56	2e-06
105	6	BoCL6965s	Chr5	280	3e-74	A03	722	0.0
106	6	BoCL1824s	Chr2	232	7e-60	A07	420	e-116
107	6	BoCL2930s	Chr3	72	2e-11	A04	264	5e-69
108	6	BoCL6419s	Chr2	133	4e-30	A05	737	0.0
109	6	BoCL3647s	Chr2	117	3e-25	A05	327	4e-88
110	6	BoCL1384s	Chr2	157	4e-37	A05	301	2e-80
111	6	BoCL3226s	Chr2	365	e-100	A07	587	e-166
112	6	BoCL2576s	Chr1	250	3e-65	A09	462	e-128
113	7	BoCL3581s	Chr3	222	4e-57	A09	517	e-145
114	7	BoCL3523s	Chr4	163	4e-39	A06	103	7e-21
115	7	BoCL2426s	Chr4	100	5e-20	A03	722	0.0
116	7	BoCL2361s	Chr3	420	e-116	A03	571	e-161
117	7	BoCL6618s	Chr5	141	2e-32	A06	515	e-145
118	7	BoCL3646s	Chr2	258	8e-68	A06	375	e-102
119	7	BoCL5710s	Chr2	46	7e-04	A06	232	1e-59
120	7	BoCL3874s	Chr5	153	5e-36	A07	238	3e-61
121	7	BoCL7296s	Chr1	157	3e-37	A07	636	0.0
122	7	CB10528						
123	7	BoCL7383s	Chr4	56	1e-06	A10	494	e-138
124	8	Ol13-G05						
125	8	BoCL2689s	Chr5	333	2e-90	A02	605	e-171
126	8	BoCL8467s	Chr5	301	7e-81	A02	658	0.0
127	8	BoCL6590s	Chr5	103	2e-21	A02	287	2e-76
128	8	BoCL8667s	Chr5			A02	280	7e-74
129	8	BoCL3700s	Chr5	230	3e-59	A02	561	e-158
130	8	BoCL3410s	Chr5	196	3e-49	A10	222	1e-56
131	8	BoCL6200s	Chr5	226	4e-58	A02	418	e-115
132	8	BoCL7758s	Chr5	432	e-120	A02	763	0.0
133	8	BoCL5584s	Chr5	133	4e-30	A02	402	e-111
134	8	BoCL2645s	Chr5	222	8e-57	A02	833	0.0
135	8	BoCL6387s	Chr5	375	e-103	A03	761	0.0
136	9	BoCL7650s	Chr1	357	2e-97	A06	513	e-144
137	9	BoCL2575s	Chr1	176	3e-43	A06	617	e-175
138	9	CB10623						
139	9	BoCL1135s	Chr1	254	1e-66	A06	511	e-144
140	9	BoCL4282s	Chr1	270	4e-71	A06	710	0.0
141	9	BoCL2578s	Chr1	198	5e-50	A06	432	e-120

142	9	BoCL3171s	Chr3	486	e-136		A05	771	0.0
143	9	BoCL4096s	Chr3	339	2e-92		A05	537	e-151

* Reference: Feng C, Shengyi L, Jian W, Lu F, Silong S, Bo L, Pingxia L, Wei H, Xiaowu Wa (2011) BRAD, the genetics and genomics database for Brassica plants. BMC Plant Biology 11:136. Doi:10.1186/1471-2229-11-136.

Supplementary Table 4. List of PPR genes in the homologous region of *Arabidopsis* and subcellular localization of PPR proteins¶

SNo	Gene Name	PPR subclass	Target P prediction of localization	Predotar prediction of localization
1	AtPPR_3g01580	E+	M (rc: 4) (Length of targeting peptide: 14)	-
2	AtPPR_3g02010	DYW	M (rc: 2) (Length of targeting peptide: 15)	-
3	AtPPR_3g02330	E+	M (rc: 2) (Length of targeting peptide: 106)	possibly plastid
4	**AtPPR_3g02490**	P	M (rc: 5) (Length of targeting peptide: 37)	Mitochondrial
5	**AtPPR_3g02650**	P	C (rc: 5) (Length of targeting peptide: 76)	Mitochondrial
6	AtPPR_3g03580	DYW	C (rc: 4) (Length of targeting peptide: 79)	possibly plastid
7	**AtPPR_3g04130**	P	M (rc: 4) (Length of targeting peptide: 74)	Mitochondrial
8	AtPPR_3g04750	E+	M (rc: 2) (Length of targeting peptide: 18)	Mitochondrial
9	AtPPR_3g04760	P	C (rc: 2) (Length of targeting peptide: 78)	possibly plastid
10	AtPPR_3g05240	E	none (rc: 4) (Length of targeting peptide:)	Possibly Mitochondrial
11	AtPPR_3g05340	E+	M (rc: 5) (Length of targeting peptide: 120)	Possibly Mitochondrial
12	**AtPPR_3g06430**	P	C (rc: 1) (Length of targeting peptide: 36)	Plastid
13	**AtPPR_3g06920***	P	none (rc: 2) (Length of targeting peptide:)	None
14	**AtPPR_3g07290**	P	M (rc: 3) (Length of targeting peptide: 89)	Mitochondrial
15	AtPPR_3g08820	DYW	none (rc: 3) (Length of targeting peptide:)	Plastid
16	AtPPR_3g09040	E+	M (rc: 3) (Length of targeting peptide: 30)	Possibly Mitochondrial
17	**AtPPR_3g09060**	P	none (rc: 5) (Length of targeting peptide:)	Mitochondrial
18	**AtPPR_3g09650**	P	C (rc: 2) (Length of targeting peptide: 65)	Plastid

M- Mitochondria, C- Chloroplast

PPR genes indicated in bold letter were analyzed in PCR amplification and used for identification of candidate genes

*The PPR gene showing 87% identity with a predicted peptide derived from the *B. rapa* sequence.

¶The table derived from the references Small and Peeters 2000; Lurin et al. 2004; O'Toole et al. 2008.

Supplementary Table 5. Primer sequences of PPR genes derived from *Arabidopsis thaliana* sequences for amplification of genomic DNA in *B. oleracea*

Gene Name	Forward primer (5' – 3')	Reverse primer (5' – 3')	Annealing temperature (°C)	PCR amplification of genomic DNA	
				CMS broccoli	Chinese kale Restorer line
AtPPR_3g02490	ATGAGGTATCAATGGCGATCGC	ATTAGCTGAAACACCATGCCCT	56	+	+
AtPPR_3g02650	TGTAGATCGCAAAATGCGTCTC	CTACGTGTATCACTAGCAATTG	52	-	-
AtPPR_3g04130	TCGCATTGGAAACACCTTGTTA	AAGCGTATCGAGCAGTAAGTTC	52	-	-
AtPPR_3g07290	ATGCTGCTGATACATATCAGAA	CATTGACAATGGTTCTGTAATC	52	+	+
AtPPR_3g09060	ATGGTCGTGTTCCCAAAGTC	GAAGTAGGCGAACAAGGACT	52	-	-
AtPPR_3g06430	ATGGCGTCAATGTCTCTCTCCT	TGTAAGCAGCGAGAAGAGCAGT	56	+	+
AtPPR_3g09650	ATGAACATTCTCCGACCTCCGA	GCCTAGATTGACCAACCAAAGA	53	+	+
AtPPR_3g06920	ATCCGCTCATTAGTTTGATCCT	GGAACTTGAACTTCCTCATCAT	53	+	+