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A genetic and physical map of the region containing *PLASTOCHRON1*, a heterochronic gene, in rice (*Oryza sativa* L.)

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Abstract The rice heterochronic gene *plastochron1*, *pla1*, shows shorter plastochron and ectopic expression of the vegetative program during the rice reproductive phase resulting in aberrant panicle formation. A genetic and physical map was constructed to isolate the causal gene for the *pla1* syndrome. Small-scale mapping was carried out to determine the approximate map position of the *pla1* locus, and then a high-resolution genetic map was made for *pla1-1*, one of the *pla1* alleles, using an F_2 population comprising 578 *pla1-1* homozygous plants. In a high-resolution genetic map, the *pla1-1* locus was found to map between RFLP markers C961 and R1738A on chromosome 10, within a 3.6-cM genetic distance. A physical map encompassing the *pla1-1* locus was constructed by overlapping Bacterial Artificial Chromosome (BAC) clones through chromosome walking. PCR-based RFLP markers from BAC-end clones were developed and mapped relative to the *pla1* locus. Physical map construction using BAC clones indicated that a BAC clone, B44A10 (167-kb), contained the *pla1* locus within 74-kb corresponding to a 0.52-cM genetic distance. Gene prediction of 74-kb region carrying the *pla1* locus suggested several candidate genes for the *pla1* gene. Identification of a candidate gene for *pla1* will be made by sequence analysis of allele variation and cDNA screening.

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Introduction

All higher plants undergo a process of several distinctly different phases comprising the life cycle: embryogenesis, a juvenile vegetative phase, an adult vegetative phase and a reproductive phase. The intermediate characters of structures produced during phase transition suggests that these phases are specified by an independently regulated and partially overlapped developmental program (Poethig 1988, 1990).

Several species, such as *Arabidopsis*, maize and English ivy have been reported for their distinct phase-change from juvenile to adult vegetative phases, as well as vegetative to reproductive phases (Poethig 1988, 1990; Lawson and Poethig 1995; Telfer et al. 1997). The transition from vegetative to reproductive development is abrupt and involves obvious changes in the character of the shoot, particularly in flowering plants. The drastic change of shoot architecture during the transition from vegetative to reproductive phases enables us to characterize more details of the mutant associated with phase transition and to identify the gene responsible for temporal regulation of the reproductive phase. However, the transition from the juvenile to adult phase of vegetative growth usually occurs gradually and is not accompanied by gross visible changes. Hence, temporal regulation in the process of the vegetative phase is still largely unknown.

Heterochronic mutations affecting the timing of developmental events may be of major significance in ontogeny and evolutionary trends (Gould 1982; Ambrose and Horvitz 1984). If a single mutation modifies the expression of a gene that controls phase change or the temporal pattern of organ development, a conspicuous change of plant body organization would occur. In several plant species, mutation-related phase transitions have been reported (Poethig 1988; Dudley and Poethig 1991; Evans and Poethig 1997). Among them, several domi-

nant mutations in maize, including *Teopod1* (*Tp1*), *Tp2*, *Tp3* and *Corngrass*, are thought to prolong the juvenile phase and change plant-body organization (Galiat et al. *1996*). Studies about heterochrony in maize, *Arabidopsis* and woody plants showed significance of the temporal regulation of the phase-specific programs in the plant life cycle (Poethig 1988, 1990; Lawson and Poethig 1995; Telfer et al. 1997).

A recessive mutation, *plastochron1* (*pla1*) of rice, causing plastochron shortening and ectopic expression of the vegetative program during the reproductive phase, was also isolated (Itoh et al. 1998). Both vegetative and reproductive phases were affected by the *pla1* mutation, the number of leaves produced in unit time during the vegetative phase of *pla1* was nearly twice that produced in the wild-type, and many primordia of primary rachis branches were converted into vegetative shoots during the reproductive growth phase. Analysis of developmental features in the *pla1* mutant indicates that *PLA1* regulates a program for the duration of the vegetative phase (Itoh et al. 1998).

Further understanding of the phase change in plant development requires identification and characterization of genes having a function on heterochrony. High-resolution RFLP linkage maps, and fine physical mapping using a BAC contig encompassing a *pla1* gene, has been developed in this study. This should accelerate positional cloning of the *pla1* gene and unravel the genetic program working in the growth phase change from vegetative to reproductive.

Materials and methods

Mapping population

A mapping population was generated by crossing a Japonica variety, Fukei-71, carrying the *pla1-1* mutation, and the Indica variety, Kasalath. Since homozygous *pla1-1* is sterile, heterozygous plants were used for this cross. A F_2 population of 3,250 seeds was sown and 578 *pla1-1* homozygotes, which had narrower and double the number of leaves and a shorter plastochron than wild-type siblings, were selected at the seedling stage and transplanted into a paddy field.

DNA extraction and Southern analysis

The cetymethyl-ammonium bromide (CTAB) method (Murray et al. 1980) was used, with minor modifications, for extracting total genomic DNA from rice leaves. Extracted DNA was digested with restriction enzymes. Two micrograms of each digested DNA were electrophoresed on a 0.8% agarose gel for 16 h at 25 V and then blotted onto a nylon membrane. Labeling of probes and signal detection were done with an ECL system (Amersham-Pharmacia) according to the supplier's instruction. Chemiluminescence was detected by an X-ray film after 1–18 h exposure. The RFLP markers used for framework genetic mapping were provided from the Rice Genome Research Program (RGP), Japan (http://rgp.dna.affrc.go.jp/), and Cornell University, USA (http://genome.cornell.edu/rice).

Genetic mapping

To determine the approximate map position of the *pla1* locus on a rice chromosome, small-scale mapping was done with 30 plants out of 578 *pla1-1* homozygous plants and 28 RFLP markers evenly distributed on all rice chromosome arms (Ahn et al. 1999). Then, the pooled sampling method (Churchill et al. 1993) was applied for fine mapping by sampling five plants as a pool to generate a total of 117 pools. To identify markers closely linked to the *pla1* locus, recombination events around four additional RFLP markers lying closer to the *PLA 1* locus were detected by pooled sample analysis. Individual recombinants were selected from each pool to construct a high-resolution RFLP linkage map. In the course of physical mapping, RFLP genetic mapping using BAC end-clones was further conducted to confirm linkage with the *pla1* gene.

Physical mapping covering the *pla1* locus

In order to identify BAC clones covering the target region, screening of the BAC library (*Hin*dIII library) made from 'Nipponbare' in Clemson University, USA, (http://www.genome.clemson.edu/) was carried out. The two RFLP markers flanking the *pla1* locus, C961 and R1738A, were used as starter probes for constructing a BAC contig by chromosome walking. High-density BAC clone filters from Clemson University were screened with RFLP marker probes using the ECL detection system (Amersham-Pharmacia). For further chromosome-walking, BAC-end DNA fragments were amplified using a slightly modified TAIL-PCR (Liu et al. 1995) and were cloned to the TA cloning vector (Invitrogen). A contig map covered with BAC clones was constructed by chromosome-walking using several end clones. Clone sequence information (CSHL, http://www.cshl.org/) of some BACs were also used to generate PCR-amplified markers for physical and genetic mapping. All selected BAC-end and PCR-amplified fragments were simultaneously used for RFLP linkage analysis to confirm recombination between those locations and the *pla1-1* gene.

Results

A high-resolution RFLP linkage map of the *pla1* region

Mapping of the *pla1* locus was performed using *pla1-1* homozygous F_2 plants (Figs. 1B, C) derived from the cross of a *pla1-1* heterozygote with Kasalath. Small-scale mapping was initially carried out to determine the approximate map position of the *pla1* locus on the chromosome. Small-scale mapping, using 30 F_2 plants with 28 RFLP markers evenly distributed on 12 chromosomes, showed that the *pla1* locus is located between RFLP markers R2447 and R1629, near the centromere of chromosome 10. The genetic distance between the two markers harboring the *pla1* locus was about 7.6-cM (Fig. 1A).

For large-scale mapping, 578 F_2 plants of *pla1* homozygotes were analyzed for their recombination between the *pla1-1* locus and additional markers by a pooled sampling method. Linkage analysis was done with nine RFLP markers between R2447 and R1629.

The RFLP markers C961 and R1738A appeared to have the least number of recombinants, nine with the former and 33 with the latter, out of a total of 117 pools (Fig. 1A). Genomic DNA of 210 individual recombinants identified from the 117 pools were extracted and linkage analyses were done with nine markers used for large-scale mapping to construct a high-resolution *pla1* linkage map. The genetic distance from the *pla1* locus to RFLP marker C961 was 0.78 cM, and to R1738A was

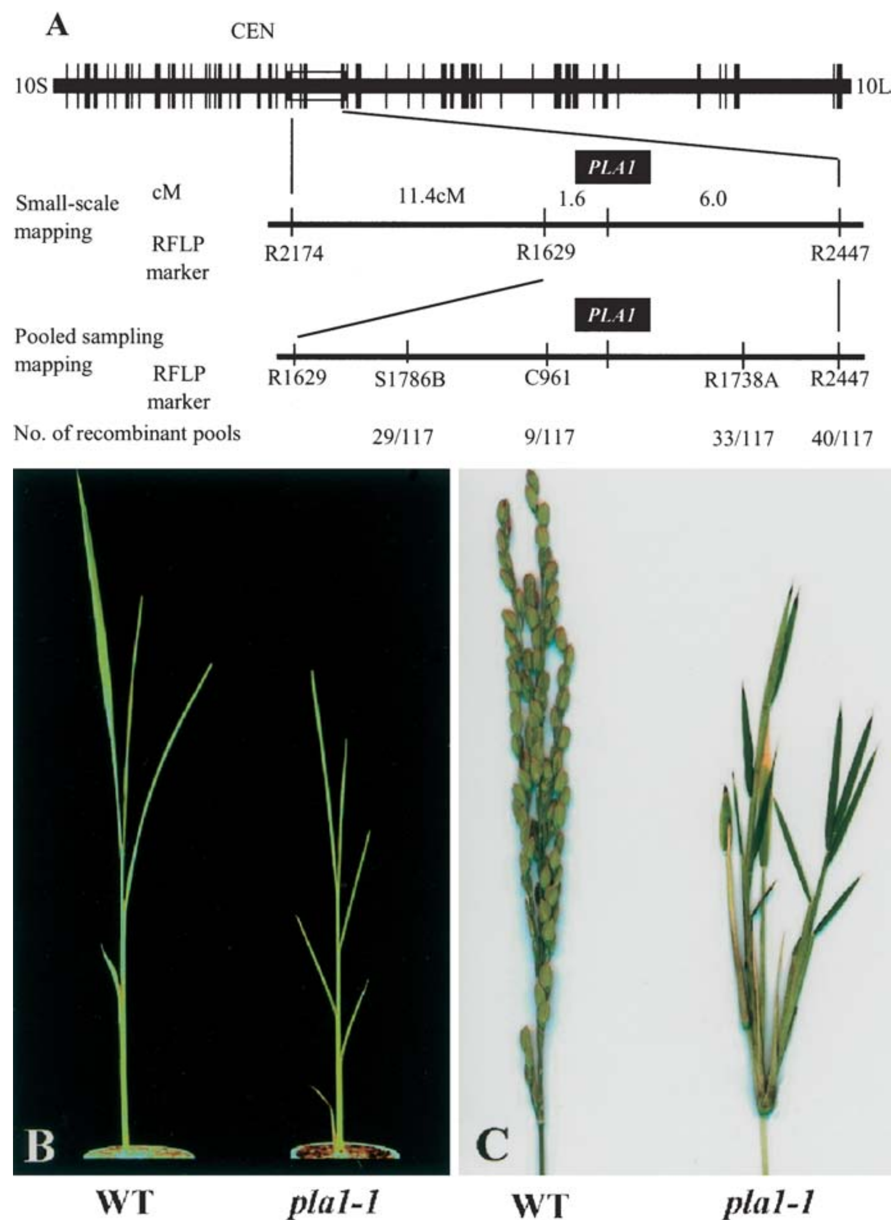

Fig. 1A–C **A** Small-scale mapping of the *pla1* locus located between the RFLP markers R1629 and R2447. The *upper bar* shows the high-density RFLP linkage map (Harushima et al. 1998). *Lower bars* show the small-scale mapping and the pooled sampling mapping encompassing the *pla1* locus. **B** and **C** Phenotypes of the *pla1-1* mutant. **B** Phenotypes of the seedling stage. The *pla1-1* mutant shows dwarfism and has double the number of leaves and a shorter plastochron compared to the wild-type (WT: cultivar Nipponbare) 2 weeks after germination. **C** Phenotypes of panicles (reproductive phase). The *pla1-1* mutant has multiple shoots at positions where primary rachis branches emerge in wild-type plants

2.86 cM (Fig. 2A). Five RFLP markers, R1738A, C1166, G1082, R2447 and S11069, which were very near the *pla1* locus, were previously mapped at the same locus on chromosome 10 (Harushima et al. 1998). Our mapping results using 578 *pla1* homozygous plants showed that those markers could be assigned to the order of R1738A, C1166 or S11069, G1082 and R2447 (Fig. 2A) in the range of 0.7-cM genetic distance.

Construction of BAC contigs

For further construction of a detailed physical map, we also utilized a BAC library constructed from a Japonica variety, Nipponbare (CUGI, http://www.genome.clemson.edu/). The BAC library on high-density filters was screened with the two closest RFLP markers, C961 and R1738A, to the *pla1* locus, to construct BAC contigs covering the *pla1* locus. Ten BAC clones including B50N08 were identified with C961, and 20 BAC clones including B22C14 were selected with R1738A by colony hybridization and then confirmed by Southern hybridization analysis (data not shown). End sequences of those BAC clones were cloned by a slightly modified TAIL-PCR method (Liu et al. 1995). Because all BAC-ends of ten BAC clones selected with C961 contained repeated DNA sequences, it was impossible to walk along to the *pla1* locus from the C961 locus. Out of 20 BAC clones selected with another closest marker, R1738A, both of the closer ends of the two BAC clones, B17I12R and B22C14R, appeared to locate in the region within a 2.5 cM genetic distance to *pla1* (Figs. 2A, B).

We continued to walk with the probe B22C14R and identified two positive clones, B8F14 and B18H18.

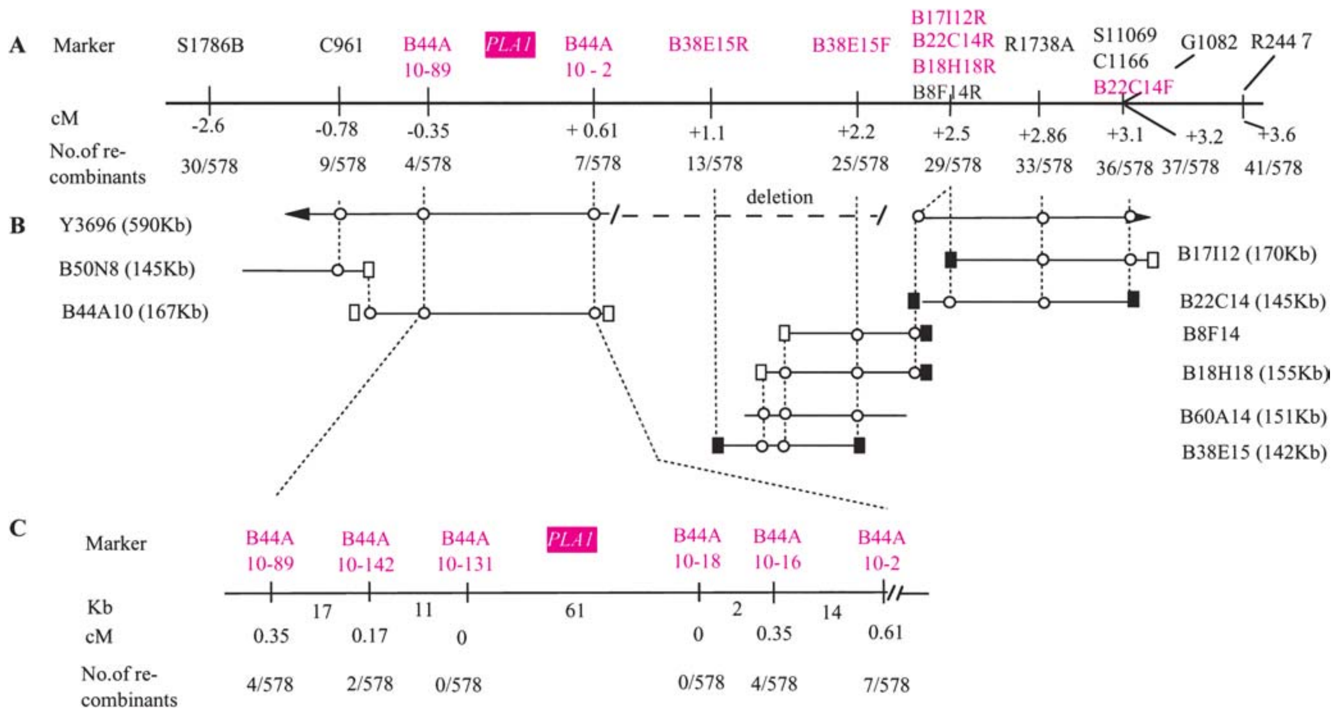

Fig. 2A–C Genetic and physical maps around the *pla1* locus on chromosome 10. **A** A high-resolution RFLP linkage map of the *pla1* locus. **B** BAC contigs around the *pla1* locus. **C** A fine map around the *pla1* locus on the B44A10 BAC clone. *Filled rectangles* indicate single-copies and *empty rectangles* indicate multi-copies of end clones. *Empty circles* show the presence of marker sequences on indicated BAC and YAC clones. Clone designations with Y and B at the top represent YAC and BAC clones, respectively. Marker with R and F letters designate right (SP 6 promoter) and left (T7 promoter) ends of BAC clones. Other markers are derived from the high-density genetic map (Harushima et al. 1998). Markers developed on B44A10 are indicated by names with B44A

However, two RFLP markers derived from the newly identified BAC-ends, B8F14R and B18H18R, also mapped at 2.5 cM from *pla1*, and the other two markers, B8F14F and B18H18F, could not be mapped because of their repetitiveness.

The end-sequencing project of BAC clones of the ‘Nipponbare’ library was almost completed, and BAC contigs based on fingerprints of the cloned genomic DNA have been published together by CUGI, USA (http://www.genome.clemson.edu/). Because a BAC contig based on fingerprint analysis agreed well with results of our chromosome walking, we next selected clones B38E15, B33C3, B60A14 and B76L8 as candidate clones connected to the *pla1* locus. Among them, one clone, B38E15, was mapped nearest to the *pla1* locus. The right-end clone B38E15R was mapped at 1.1 cM, while the left end clone B38E15F was mapped at 2.2 cM, from the *pla1* locus. However, we again failed to walk further toward the *pla1* locus because of the repetitive nature of the BAC clones selected with the B38E15R end-clone probe (Fig. 2B).

A candidate YAC clone covering the *pla1* region, Y3696 (590 kb), was previously mapped (Saji et al. 2001) on the same region as the BAC contig. Southern hybridization analysis using C961 and R1738A as probes showed that a YAC clone, Y3696, apparently covered the *pla1* region of chromosome 10 (Fig. 2B). However, an internal deletion of Y3696, the YAC clone between markers B44A10-2 and B18H18R, was detected by Southern hybridization (data not shown).

PLA1 locus restricted to 74-kb region in a BAC clone

Meanwhile, sequencing of BAC clones around the centromere region of rice chromosome 10 has been advanced by Cold Spring Harbor Laboratory, USA, in cooperation with IRGSP (International Rice Genome Sequencing Project). Among these clones, two BAC clones of B50N08 (annotation finished) and B44A10 were almost sequenced. A homology search between B50N8 and B44A10 showed that these clones overlapped each other. Southern hybridization using C961 as a probe yielded evidence that B50N08 contained the C961 sequence, but B44A10 did not hybridize with C961. We designed several primers to generate RFLP probes using the published sequence of the B44A10 clone. Linkage analysis with amplified probes helped to map two new highly linked, flanking RFLP markers, B44A10-89 by 0.35 cM and B44A10-2 by 0.61 cM, on either side of the *pla1* locus (Fig. 2B).

Therefore, we concluded that the B44A10 clone contained the *pla1* locus. To further restrict the *pla1* locus, we used several other PCR-amplified RFLP markers designed within sequences of B44A10. Linkage analysis with those markers revealed that the RFLP markers B44A10-142 and B44A10-16 could be mapped very near to the *pla1* locus with a genetic distance of 0.17 cM and

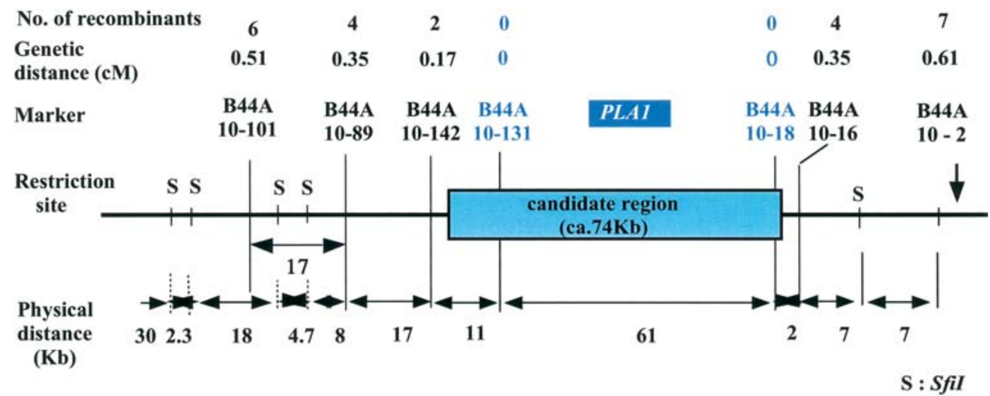

Fig. 3 Physical map encompassing the *pla1* locus. Physical lengths were calculated from the sequence of B44A10 published by Cold Spring Harbor Laboratory, USA. *Vertical arrows* indicate gap positions (based on sequence data published by Cold Spring Harbor Laboratory) in B44A10. *S*: *SfiI*

0.35 cM and with two and four recombinants, respectively. Two markers, B44A10-131 and B44A10-18, cosegregated with the *pla1* locus with no recombinants (Fig. 2C). The physical distance between the RFLP marker B44A10-142, which detected two recombinants, and B44A10-131 which cosegregated with the *pla1* locus, was 11 kb. The physical distance between two markers of B44A10-16, with four recombinants, and B44A10-18, with no recombinant, was only 2 kb (Fig. 2C). On the basis of the B44A10 physical map and the Pulsed Field Gel Electrophoresis (PFGE) analysis (data not shown), the region between B44A10-142 and B44A10-18 was shown to carry the *pla1* locus and spanned about 74 kb in length (Fig. 3).

Discussion

A positional cloning strategy was applied to identify the location of the *pla1* gene, which is considered to prolong (adult) the vegetative program during the reproductive growth phase. A high-resolution genetic map showed that the *pla1* locus was located between markers C961 and R1738A on chromosome 10, and that the genetic distance between the *pla1* locus and each marker was 0.78 cM and 2.86 cM, respectively (Fig. 2A).

The repetitive sequences of the end-clones of almost all BAC clones screened led to many difficulties in construction of a BAC contig harboring the *pla1* gene. However, we were able to construct the BAC contig, including clone B44A10 that carried the *pla1* locus, with the help of the genome sequencing effort (Fig. 2B). The region mapped with the *pla1* locus on chromosome 10 was almost sequenced, and published by the Cold Spring Harbor Laboratory, USA. Published information was very useful for the construction of BAC contigs and could be very helpful for cloning a candidate gene by the map-based method.

The chromosomal region between C961 (25.7 cM) and R1738A (29.8 cM) mapped with the *pla1* locus appeared to be previously assigned to the centromere region of chromosome 10 (Harushima et al. 1998). Recently, it was demonstrated that the centromere position on chromosome 10 is located between markers G1125 (15.4 cM) and C489 (15.9 cM) using the centromere-specific probe pRCS2 and by fluorescence *in situ* hybridization (FISH) mapping with BAC clones anchored by these two RFLP markers (Cheng et al. 2001).

As shown in Fig. 2, the genetic distance from C961 to R2447 was 4.4 cM, whereas the physical distance between them was deduced to cover about 660–790 kb, based on the length of the BAC clones of B44A10 (167 kb), B38E15 (142 kb) and B22C14 (145 kb). The average recombination-event frequency up-calculated from these data was one recombination at a 12-24 kb interval for B44A10 (167 kb/1.39 cM), B38E15 (142 kb/1.1 cM) and B22C14 (145 kb/0.6 cM) (Fig. 2A, B).

As for the *pla1* region on the B44A10 clone, however, the physical map indicated that the ratio of genetic to physical distance varied along the clone (Fig. 3). For example, the number of recombinants between markers B44A10-89 and B44A10-131 were four, and the physical distance of the region was 28 kb, suggesting one recombination per 7-kb interval. Recombinants between markers B44A10-18 and B44A10-16 were four and the physical distance was only 2 kb (Fig. 3), showing one recombination per 0.5 kb interval. These facts imply that there were hotspot regions for recombination on both sides of the *pla1* locus. In contrast, the *pla1* locus spanned about 74-kb distance with no recombination. The mechanism, or a reason, for suppression of recombination for a 74-kb length around the *pla1* locus and the hotspot occurrence on both sides is not known. However, this region seems to provide a good example for analysis.

This region was almost sequenced and was searched for genes using several gene-annotation programs (http://genes.mit.edu/GENSCAN and http://rgp.dna.affrc.go.jp/ Analysis). Gene prediction of the 74-kb region containing the *pla1* locus by the gene-annotation program predicted several candidate genes such as Cytochrome P450, the putative GTPase regulator protein and genes of unknown function. Identification of a candidate gene for *pla1* by sequence analysis of three alleles and cDNA screening is now in progress. Once the *pla1* gene is isolated, it will reveal important information about how the *PLA1* gene experiences a heterochrony mutation in the phase transition of plant developmental processes at the molecular level.

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