Map-based cloning of *PLA1*, a heterochronic gene, in rice (*Oryza sativa* L.)

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Members of the Judging Committee :

Professor Hiroyuki Sasaki (Chair)

Professor Yasuo Nagato

ProfessorToshihiko Shiroishi

Associate Professor Tetsuji Kakutani

Associate Professor Toshiuki Takono

Supervisor :

Associate Professor Nori Kurata

Contents

General Introduction

Life cycle of higher plants can be mainly divided into three discrete phases; embryogenesis, vegetative phase and reproductive phase. In the early embryogensis, most higher plants set aside two distinctive regions, undifferentiated meristematic region called shoot apical meristem (SAM) and root apical meristem at opposite pole of the embryo. The SAM initially produces vegetative organs such as leaves or shoots. At certain point the SAM goes into the reproductive developmental phase and initiates the production of reproductive organs. It has been long known that this phase change of development is controlled by environmental and endogenous elements (Levy and Dean, 1998). The vegetative phase is further divided into two phases, the juvenile phase and adult phase.

Several characters reflecting phase change from the juvenile phase to the adult vegetative phase have been reported in maize and *Arabidopsis*. In maize, phase progression from the juvenile-to-adult phase is discerned by the change of several traits such as trichome distribution, composition of epicuticular wax and aerial roots (Poethig, 1990). It is considered in maize that the juvenile - to - adult phase transition occurs at a predictable developmental time, between phytomer 6 and 8, on the shoot. In *Arabidopsis,* the juvenile-to-adult phase transition is also recognized as a change of trichome distribution and structural characterization of leaves (Telfer *et al.*, 1997). And in *Arabidopsis*, it is known that the juvenile-to-adult phase transition occurs at the stage of 4th leaf formation where both traits of juvenile and adult vegetative phases co-express (Telfer *et al.*, 1997). In rice, the juvenile-to-adult phase change is uneasy to discern due to their gradual change, whereas transition to reproductive phase occurs in a specific stage acompanied by the change such

as the change of phyllotaxy and rapid elongation of upper four or five internodes.

Such phenomena reflecting these phase change serve as an indicator to isolate the genes associated with heterochrony. The intermediate characters reflecting co-expression of neibouring two phases suggest that these phases are specified by independently regulated and partially overlapped developmental programs (Poethig, 1988; 1990). It is known that the timing of phase transition is controlled by interaction between endogenous hormonal level, such as Gibberellin, and environmental elements such as photoperiod and temperature (Zimmerman *et al.*, 1985; Wilson *et al.*, 1992; Lawson and Poethig, 1995; Evans and Poethig, 1995). However, little is known about how phase transition is controlled by interaction between genetic and environmental factors.

Higher plants show variation in the relative timing of developmental processes, called heterochrony, during life cycle. Heterochronic mutations affecting timing of expression of developmental programs may drastically modify morphological traits. If a single mutation modifies expression of a gene that controls phase change or temporal pattern of organ development, conspicuous change of plant body organization would occur. Thus it has been long considered to be of major significance in ontogeny and evolutionary trends (Gould, 1982; Ambros and Horvitz, 1984; Lord and Hill, 1987).

Several species, such as *Arabidopsis*, maize, English ivy, *C.elegans* and rice have been reported for their distinct phase change as revealed partly by heterochronic mutations (Ambros and Horvitz, 1984; Ambros and Moss, 1994; Dudley and Poethig, 1991; Galinat, 1966; Lawson and Poethig, 1995; Evans and Poethig, 1995; 1997; Telfer *et al.*, 1997; Itoh *et al.*, 1998; Asai *et al.*, 2002). In maize, several dominant mutants such as *Teopod1* (*Tp1*),

Tp2, *Tp3* and *Corngrass1*, were characterized by the ectopic expression of juvenile traits in the adult vegetative phase and these mutants are thought to prolong the juvenile phase of development and modify plant body organization (Poethig, 1988; Bassiri *et al.*, 1992; Dudley and Poethig, 1991; Abedon and Tracy, 1996). Another recessive mutant *glossy 15* initiates or maintains the expression of juvenile epidermal traits and suppresses the expression of adult epidermal traits (Evans *et al.*, 1994; Moose and Sisco, 1994). Another semidominant mutant, *Corngrass 1* (*Cg1*), in which the juvenile vegetative phase is prolonged, is suggested as a heterochrony mutant (Abedon and Tracy, 1996). In *Arabidopsis*, it is known that distribution of abaxial trichome, which is a morphological marker to recognize the phase change from juvenile to adult vegetative phase, appears to be regulated by the age, rather than the size of plant (Telfer *et al.*, 1997). A similar morphological marker associated with phase change based on distribution of trichome is also recognized in maize. *embryonic flower* (*emf*) mutant of *Arabidopsis* associated with the switch between vegetative and reproductive phase induces reproductive phase immediately after germination with no vegetative phase (Sung *et al.*, 1992). The most important results obtained from these analyses of heterochrony is that different developmental stages are independently regulated (Conway and Poethig, 1993).

C.elegans is an excellent model to understand developmental processes because complete cell lineage has been determined and each cell can be recognized by its lineage and position. Several *C. elegans* mutants associated with heterochrony events have been isolated and profoundly studied at a molecular level. Among them, *lin-4, lin-14, lin-28, lin-29 and let-9* showed precocious or retarded cell fate determination, which resulted in

abnormal temporal transformation of the cells (Ambros and Horvitz, 1984; Ambros and Moss, 1994). Molecular mechanisms controlling the developmental timing have been profoundly studied in *lin-4, let-7* and *let-9* mutants. It has been elucidated that these genes encode small temporally expressed RNAs (stRNA) those are not translated into protein and function as antisense RNAs to suppress target genes, *let - 4* and *let - 9*, respectively (Reinhart *et al.*, 2000). The *let-7* regulatory RNA may control late temporal transitions during development across animal phylogeny (Pasquinelli *et al.*, 2000).

Recently, another heterochronic mutant, *moril*, defective in juvenile-to-adult phase transition was studied in rice (Asai *et al.*, 2002). Morphological characteristics of *moril* leaves at any position was shown to be similar to that of the 2nd leaf of wild type. The mutant maintains the 2nd leaf stage (juvenile phase) throughout development and continues to grow forever. Thus, it is suggested that *moril* is a heterochronic mutant that suppresses induction of adult vegetative phase and termination of juvenile vegetative phase (Asai *et al.*, 2002).

A recessive rice mutation, *plastochron1* (*pla1*), causing plastochron shortening and ectopic expression of the vegetative program during the reproductive phase was also reported (Itoh *et al.*, 1998). The *pla1* mutant produced many leaves compared to that of wild type in a unit time during the vegetative phase. After transition to reproductive growth phase, primordia of primary rachis branches were converted into vegetative shoots depending on the severity of the alleles (Fig. 1 A and B). However, the timing for the onset of transition to reproductive phase in the *pla1* mutant was similar to that of wild type. This indicates that termination of vegetative phase is independently regulated from the

onset of reproductive phase. *PLA1* regulates the termination of vegetative phase without affecting initiation switch to reproductive phase. Mutations resembling *pla1* have not been reported so far in any plant species. Thus, *PLA1* is the first gene that regulates a program for the duration of vegetative phase (Itoh *et al.*, 1998).

Studies about heterochrony in rice, maize, *Arabidopsis,* woody species and English ivy showed significance of temporal regulation of phase specific programs in the plant life cycle (Lawson and Poethig, 1995; Poethig, 1988 ; 1990; Telfer *et al.*, 1997). Several mutants associated with the phase change like heterochronic mutants have been reported and analyzed through genetic, anatomical and biochemical approaches. Nevertheless, it is still unknown how phase change is controlled by genetic program. Further understanding of phase change in plant development requires identification and characterization of genes having a function on heterochronic events.

Map-based cloning is a promising method to isolate a target gene without any information on a gene product, but it needs some requirements. The first requirement for this strategy is to generate a high-resolution molecular linkage map. Secondly, the availability of large insert DNA libraries such as bacterial artificial chromosome (BAC), yeast artificial chromosome (YAC) and P1-derived artificial chromosome (PAC). Rice is considered as an ideal model plant for map-based cloning and molecular approaches from some points of view. First, rice has the smallest genome size (430Mb, Martinez *et al.*, 1994) among other mono-cotyledous plants analysed so far and has synteny with other cereal plants (Ahn and Tanksley, 1993; Kurata *et al.*, 1995; Chen *at al.*, 1997; Gale and Devos, 1998). Secondly, the high-density linkage map comprising over 3,200 molecular

markers is well developed (Harushima *et al.*, 1998; http://rgp.dna.affrc.go.jp/). Third, a physical maps covering the almost entire genome with BAC, YAC and PAC clones provides efficient tools for cloning of genes, analysis of the complex genome and for genome sequencing. Finally, although the transformation efficiency of rice is low compared to other dicotyledous plants such as tobacco, *Arabidopsis,* generation of the transgenic plants by *Agrobacterium*-mediated transformation was well established (Hiei *et al.*, 1994). It can provide a sufficient number of transgenic plants for the functional analysis of isolated genes.

Genes regulating agriculturally important traits have been identified by map-based cloning strategy in several plant species, such as *Xa21* (Song *et al.*, 1995), *Xa1* (Yoshimura *et al.*, 1998), *Hd1* (Yano *et al.*, 2000) and *D1* (Ashihikari *et al.*, 1999) in rice, *Mlo* (Büschges *et al.*, 1997) in barley, *Cf-2* (Dixon *et al.*,1996) and *Pto* (Martin *et al.*, 1993) in tomato, *fad3* (Arondel *et al.*, 1992), *RPS2* (Bent *et al.*, 1994) and *GA4* (Chiang *et al.*, 1995) in *Arabidopsis*, and $Hs1^{pro\text{-}1}$ (Cai *et al.*, 1997) in sugar beet. Among them, *Hd1* that is a major photoperiod sensitivity gene on the quantitative trait loci (QTL) in rice was isolated by a map-based approach. This implies that map-based cloning could apply not only to qualitative traits but also to quantitative traits, which comprise most of the agronomically important traits. Recent accumulation of information concerning high-density genetic map, a physical map covering an entire genome, an EST map and genome sequencing released by IRGSP (International Rice Genome Sequencing Project) may enforce to identify even quantitative trait loci as well as genes related to plant development. Whole genome sequencing projects of rice have been conducted for years and very recently draft genome

sequences covering 92-93% of the entire genome were published (Yu *et al.*, 2002; Goff *et al.*, 2002).

In this thesis, I describe cloning of the *PLA1* gene through the map-based cloning strategy. In chapter 1, construction of the restriction fragment length polymorphism (RFLP) linkage map of the *PLA1* gene using mapping population is described. In chapter 2, construction of the physical map encompassing the *PLA1* gene by chromosome walking is described. In chapter 3, cloning of *PLA1* gene and complementation of *pla1* mutant revealed the *PLA1* gene was the P450. Finally, spatial and temporal expression pattern of *PLA1* is shown in chapter 4. Then the functional role of the *PLA1*gene in the vegetative growth and in phase transition from vegetative to reproductive is disscussed.

Chapter 1

RFLP linkage mapping of the *PLA1* locus

Introduction

Map-based cloning is a powerful approach to isolate a target gene based only on its phenotype and genomic location without any other information such as a protein product of the target gene. For map-based cloning, generation of a high-density linkage map with saturated markers on the entire genome is most essential.

The first rice RFLP linkage map was constructed in 1988 (McCouch *et al.*, 1988), following the genetic linkage map using RFLP in human (Botstein *et al.*, 1980) and in other organisms (Helentjaris, 1987; Chang *et al.*, 1988; Dietrich *et al.*, 1996). A large number of rice genetic maps locating trait markers by the crosses between cultivars and phenotypical mutants had been constructed during the three decades. Those were combined into a standard genetic map consisting of 170 phenotypical trait markers (Kinoshita, 1990). This promoted construction of higher-density molecular linkage maps (Saito *et al.*, 1991; Causse *et al.*, 1994; Kurata *et al.*, 1994; Harushima *et al.*, 1998). Recently, the high-density rice genetic map constructed with 3,267 molecular markers including EST markers was published by Rice Genome Research Program (RGP) (http://rgp.dna.affrc.go.jp/). This provides useful information to identify a gene of interest and made it possible to clone quantitative trait genes such as *Hd1* gene, which is one of the agriculturally important genes (Yano *et al.*, 2000). *Hd1* is the first gene in cloning of quantitative trait loci, which had long been considered to be difficult to access by a map-based cloning method.

In rice, the map-based cloning strategy was first applied to the agronomically important genes such as resistance genes to bacterial blight disease, *Xa21*and *Xa1*. They succeeded to isolate *Xa21* gene that encoded a receptor kinase like protein (Song *et al.*, 1995), and *Xa1* gene encoding a cytoplasmic receptor-like protein with nucleotide binding sites (NBS) and leucine-rich repeat (LRR) domains (Yoshimura *et al.*, 1998). In addition, a *Dwarf 1* gene, which regulates plant height, one of the most important agronomical traits, was shown to encode an α-subunit of a GTP-binding protein (Ashikari *et al.*, 1999).

In this chapter, construction of a high-density RFLP linkage map encompassing the *pla1* locus performed by using 578 *pla1* homozygous plants was described. An approximate map position of *pla1* was first determined by using a small-scale population mapping and then by a pooled sampling method with a large number of segregants. The exact map position of *pla1* was determined by high-resolution linkage mapping using many newly developed markers around the target gene.

Materials and Methods

Population for a small-scale genetic mapping

A mapping population was generated by crossing a Japonica variety, Fukei-71 carrying the *pla1-1* mutation (Fig. 1) and the Indica variety, Kasalath. Since homozygous *pla1-1* is sterile, heterozygous plants were used for this cross. An F_2 population of 3,256 seeds was sown and 578 *pla1-1* homozygotes, which had narrower leaves and a shorter plastochron than wild type siblings, were selected at the seedling stage and transplanted into a paddy field (Fig. 2).

Fig.1. Phenotype of pla1 mutants and wild type. A : Plastochron of *pla1* mutant was nearly half of that of the wild type. B : Panicles. Primary branches of the panicle were changed new shoot completely (*pla1-1*) or incompletely (*pla1-2*). C : Three alleles of *pla1* mutants. From left to right, WT (Nipponbare), *pla1-1*, *pla1-2* and *pla1-3*.

Fig. 2. Selected 578 *pla1* homozygous plants for genetic and physical mapping.

For a small-scale mapping, 30 plants out of 578 *pla1* homozygous plants were used for analyzing the linkage between the *PLA1* locus and 28 RFLP markers evenly distributed on all rice chromosome arms as shown in the Appendix 1. About 2g leaves from each of 30 plants were collected and genomic DNA was extracted for linkage analysis.

Population for pooled sample mapping

After the small-scale mapping, the pooled sampling method which combined leaves of five plants as one pool (Churchill *et al.*, 1993) was applied to save the labor and cost for DNA extraction. A total of 117 pools derived from 578 homozygous for *pla1-1* was generated and linkage analysis was done. At the same time, leaves of 578 *pla1* homozygous plants were collected independently and frozen in liquid nitrogen for further analysis.

A high-resolution linkage mapping of the *PLA1* locus

Individual recombinants identified in each pool used for pooled sample mapping. Genomic DNA of all identified recombinants from 117 pools was extracted indendpendently and linkage analysis was done with nine RFLP markers between R2447 and R1629.

DNA extraction

The cetylmethyl-ammonium bromide (CTAB) method (Murray and Thompson, 1980) was used with minor modifications for extracting total genomic DNA from rice leaves (Appendix 2).

RFLP markers

RFLP markers were selected in the databases and were provided by RGP (Rice Genome Research Program, Japan; http://rgp.dna.affrc.go.jp/) and Dr. S. R. McCouch (Cornell University, USA; http://genome.cornell.edu/rice).

Southern hybridization analysis

Extracted genomic DNA was digested with several restriction enzymes. Two micrograms of each digested DNA were electrophoresed on 0.8% agarose gel for 16h at 25V and then blotted onto a nylon membrane. Labeling of probes, hybridization, washing and signal detection were carried out by the ECL system (Amersham-Pharmacia) according to supplier's instruction. Chemiluminescence was detected by X-ray film after 30 minutes - 18 h exposure.

Linkage analysis

In small-scale mapping, the recombination values were calculated by the maximum likehood method (Allard, 1956) and converted into genetic distances (cM) using the Kosambi function (Kosambi, 1944). In pooled sample mapping and high resolution linkage mapping, map distance was calculated based on the formula of $r=-(1/2k)\ln[1-(yA/n)]$; r : the approximate maximum likehood estimator of genetic distance from the target locus, k : the number of individuals per pool, yA : a total number of recombinant pools, n : the number pool (Churchill *et al.*, 1993). In this experiment, the number of individuals per pool (k) is five. The number of pools (n) was 117 in pooled sample mapping and 578 in high-

resolution mapping.

Total number of recombination between *PLA1* and RFLP markers were counted by the recombinant bands of Southern hybridization shown with RFLP markers near the *PLA1* locus.

Results

A small-scale mapping of the *PLA1* locus

A small-scale mapping was performed to determine approximate map position of the *PLA1* locus on a chromosome. Genomic DNA of 30 F_2 homozygous plants of *pla1-2* derived from the cross between Fukei-71, which carries *pla1* mutation, and Kasalath carrying a wild type *PLA1* allele, was extracted. RFLP linkage analysis was carried out with 24 markers evenly distributed on all rice chromosome arms. Frame map and RFLP markers used for small-scale mapping is shown in Appendix 1. Linkage analysis indicated that the *PLA1* locus located near the marker R2174, which exhibit least recombinant between the *PLA1* and markers used on chromosome 10. Further linkage analysis was carried out with additional three markers between marker R2174 and R2447, near the centromere of chromosome 10. Genetic distances from markers to the linked *PLA1* locus were calculated 11.4cM, 1.6cM and 6.0cM for R2174, R1629 and R2447 respectively. Thus, small-scale mapping revealed that the *pla1* locus is located between the markers R1629 and R2447 extended along 7.6cM genetic distance on chromosome 10 (Fig. 3).

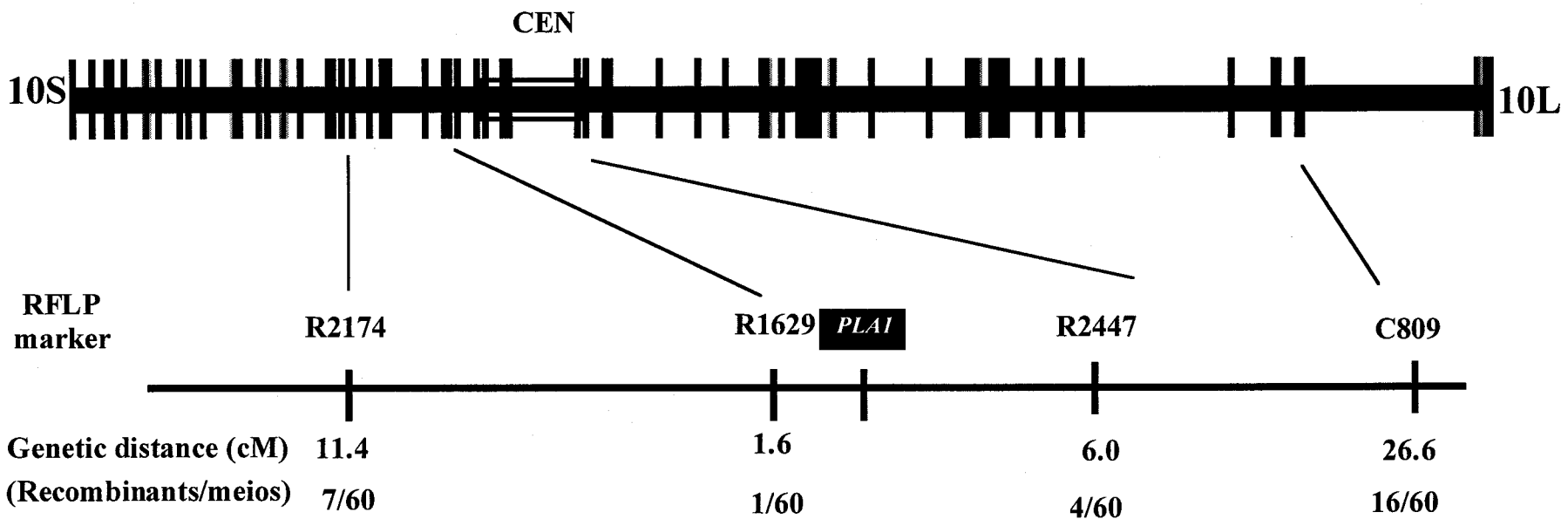

Fig. 3. Small-scale linkage map of *PLA1* on chromosome 10. The upper bar indicate the high-density linkage map of rice chromosome 10 (Harushima *et al.*, 1998). Linkage relationship of *PLA1* with RFLP marker used in small-scale mappings was shown on the lower bars.

Pooled sample mapping

All 578 *pla1-2* homozygous plants were divided into 117 pools with 5 individuals in each pool and genomic DNA was extracted. RFLP linkage analysis was carried out with additional markers between R1629 and R2447, which were the nearest markers on both sides of the *PLA1* locus in the small-scale mapping. Out of 117 pools, 9 pools and 33 pools appeared to have recombinants between the *PLA1* locus and the RFLP markers, C961 and R1738A, respectively.

In the pooled sample mapping, five plants were combined as one pool for Southern hybridization analysis but several pools were difficult to detect the presence of recombinant plants. The plants in those pools were examined individually for recombination. To determine exact map position of the *PLA1* locus, plants from the pools with ambiguous recombinants were subsequently analyzed one by one for individual recombination as shown in Fig. 4.

Consequently, pooled sample mapping could drove the *PLA1* gene into the region between C961 and R1738A (Fig. 5). Five markers, C1166, G1082, S11069, R2447 and R1738A that were mapped at the same position in the previous high-density linkage map were shown to have different numbers of recombinant in this mapping population. The order of these markers was determined to be R1738A (33 recombinant pools), S11069 and C1166 (36 recombinant pools), G1082 (37 recombinant pools) and R2447 (41 recombinant pools).

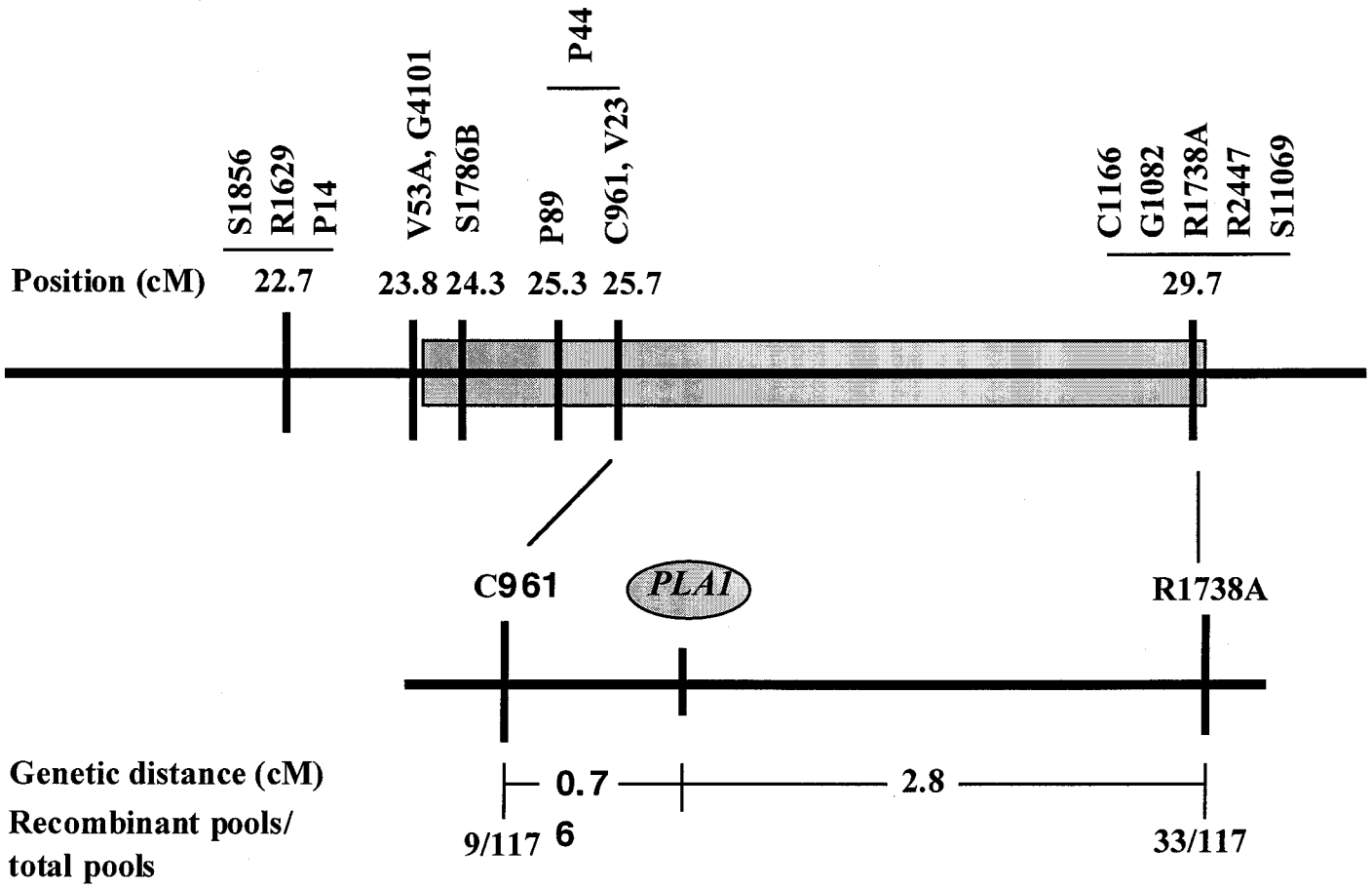

Fig. 4. RFLP linkage map of the *PLA1* on chromosome 10 constructed by pooled sampling method. The high-density linkage map of centromere region on chromosome 10 was shown in the upper bar (Harushima *et al.*, 1998). Previously presumed centromere region was indicated in shaded box. The lower bar indicates the linkage relationship between the *PLA1* and RFLp markers constructed by pooled sampling method.

High-resolution RFLP linkage mapping of the *PLA1* gene

Using the pooled sample mapping, 42 pools containing recombinants with the nearest markers were identified. To determine the exact map position of the *pla1* allele, genomic DNA of 249 individual plants (49 pools×5 plants and 1 pool ×4 plants) from 50 pools including 8 pools which showed ambiguity in discerning recombinants, out of a total of 117 pools was extracted. The linkage analysis through Southern hybridization was further performed with all markers between markers R1629 and R2447. A high-resolution linkage map showed that the *PLA1* gene is located between markers C961 and R1738A with a genetic distance of 3.6cM, near the centromere on chromosome 10 (Fig. 6). The five markers located at the same position on chromosome 10 in the reported map showed different genetic distance from the *PLA1* locus in the present map. The order of the five markers are R1738A with 2.87cM distance from the *PLA1*, S11069 and C1166 with 3.1cM, G1082 with 3.2cM and R2447 with 3.6cM.

Consequently, the high-resolution linkage mapping indicated that the marker C961, which was tightly linked to the *PLA1* gene with a genetic distance of 0.76cM, was appropriate as a starting marker for chromosome walking. A large F_2 population of 578 homozygous *pla1-2* plants could resolve larger number of loci and determine fine order of markers. Forty-two recombinant plants detected by the markers of C961 and R1738A were used for construction of a physical map using BAC contigs encompassing the *PLA1* gene.

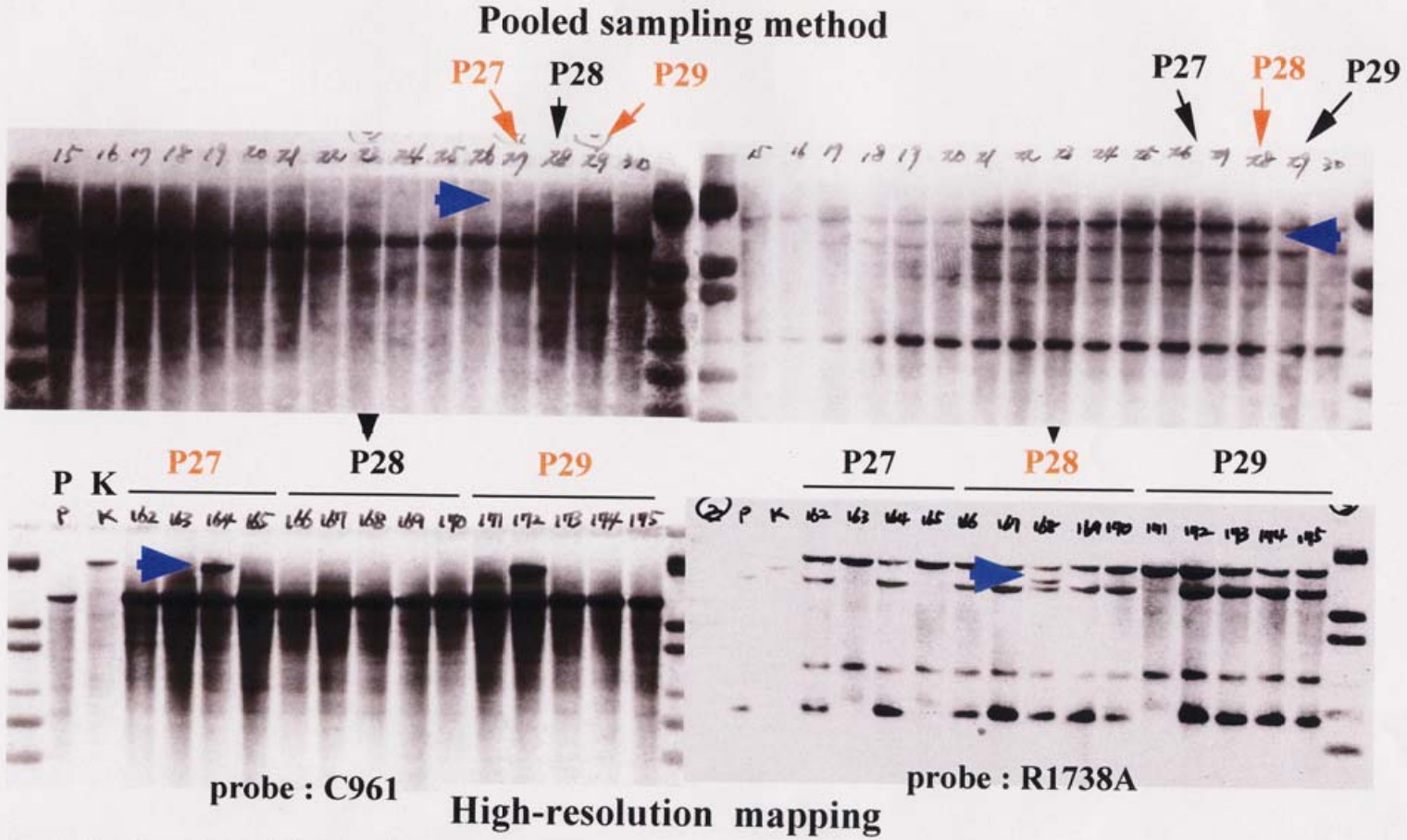

Fig. 5. Southern hybridization in pooled sampling mapping (upper panels) and high resolution mapping (lower panels) with markers linked C961 and R1738A. Five plants in each recombinant pools were used for Southern hybridization and for determination of individual recombination as shown in the lower panels. Recombinant pools were indicated in the red letter. P : *pla1*, K : Kasalath.

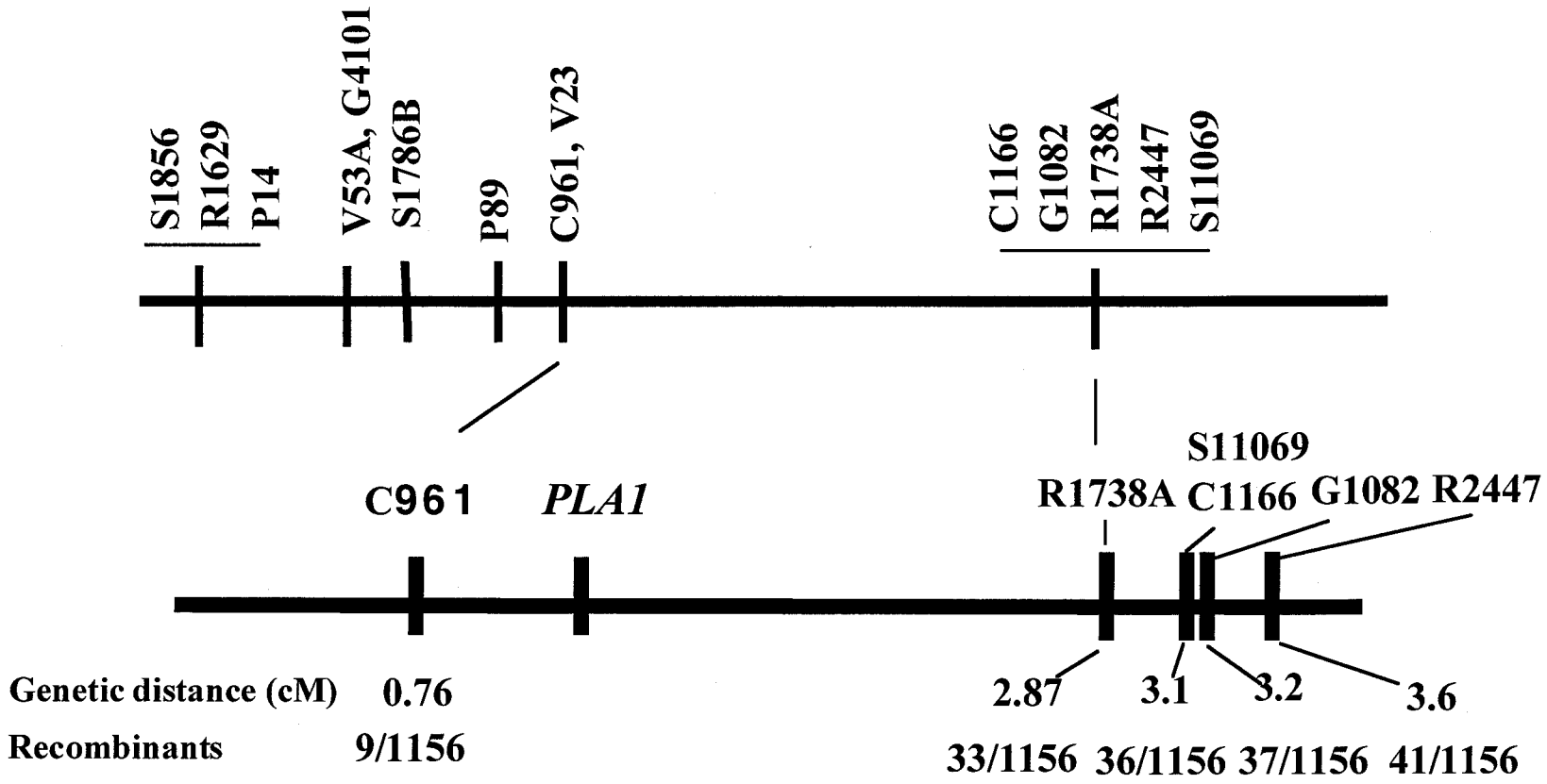

Fig. 6. High resolution linkage map of the *pla1* gene. The high density linkage map around *PLA1* locus on chromosome 10 was shown. The linkage among five markers in the previously map was desected into four separate marker loci in the present study.

Discussion

Approximate map position of the *PLA1* locus on a chromosome was determined using 30 *pla1* homozygous plants. The small-scale mapping indicated that the *PLA1* locus is located between markers R1629 and R2447 on chromosome 10 with 7.6cM genetic distance. In rice, a population size of 30 plants appeared to be sufficient to map most genes approximately. The genome size of rice is estimated 430Mb and the total genetic map was about 1530 cM (Kurata *et al.*, 1994). This indicates that 1cM corresponds to 281Kb in average. For fine mapping of a gene within a few dozens kilobases, which corresponds to 0.1cM and seems adequate to designate one or two candidate genes, it is needed to use above 500 plants of F_2 population. In case of *PLA1* gene mapping, I selected 578 homozygous *pla1-2* plants from 3,265 F_2 progenies to skip heterozygous genotyping.

After small-scale mapping, 578 *pla1-2* homozygotes were used for further fine mapping of *PLA1* gene. The high-resolution mapping followed by the pooled sample mapping indicated that the *PLA1* locus was located between the markers C961 and R1738A at a genetic distance of 3.6 cM (Fig. 6). Though there is no additional available marker linked to the *PLA1* gene between the markers C961 and R1738A, these two markers seemed good starting points for chromosome walking toward the *PLA1*.

Five RFLP markers, R1738A, C1166, G1082, R2447, and S11069, which were located very near to the *PLA1* locus, were previously mapped at the same locus on chromosome 10 (Harushima *et al.*, 1998). In this study, mapping results using 578 *pla1* homozygous plants showed that these five markers could be assigned to the order of R1738A, C1166 or S11069, G1082 and R2447 in a range of 0.7 cM genetic distance. In

addition, 3.63 cM distance between markers C961 and R1738A could be divided by 42 recombination events in this population (0.086 cM/recombination). This indicates that the mapping resolution of this population should be high enough for further fine mapping.

Chapter 2

Physical mapping of the *PLA1* gene

Introduction

An essential step of the map-based cloning next to fine mapping is the construction of a physical map encompassing a target gene. Libraries with large insert fragments of genome DNA, such as a bacterial artificial chromosome (BAC) library, a yeast artificial chromosome (YAC) library and a P1-derived artificial chromosome (PAC) library serve as excellent resources to construct a physical map harbouring a gene of interest.

The first large insert library of rice DNA was constructed by an YAC. The YAC library of rice composed of about 7,000 clones with an average insert size of 350Kb, was estimated to cover six times of a haploid rice genome (Umehara *et al.*, 1995). The YAC library further led to the construction of physical maps to cover entire genome by landing of YAC clones on all chromosomes. (Kurata *et al.*, 1997; Wang *et al.*, 1996; Antonio *et al.*, 1996; Saji *et al.*, 1996; Shimokawa *et al.*, 1996; Umehara *et al.*, 1996; Tanoue *et al.*, 1997; Koike *et al.*, 1997; Saji *et al.*, 2001). Recently, the advanced YAC-based expression sequence tag (EST) map of rice, which comprises 364 YAC contigs with 6,591 EST sites was developed by Wu *et al* (2002).

Another vector capable to clone large genome DNA fragments is a BAC vector (Shizuya *et al.*, 1992). The BAC vector has several advantages over the YAC vector, such as easy manipulation, high cloning efficiency and stable maintenance of inserted DNAs (Woo *et al.*, 1994; Song *et al.*, 2000; Lijavetzky *et al.*, 1999). BAC libraries of several plant

species including rice have been constructed (Wang *et al.*, 1995; Zhang *et al.*, 1996; Nakamura *et al.*, 1997; Zhang and Wing, 1997). In addition, a P1-derived artificial chromosome (PAC) system that could introduce large DNA fragments of 80 - 350Kb was developed (Ioannou *et al.*, 1994) and used for the construction of the library of a Japonica variety, Nipponbare (http://rgp.dna.affrc.go.jp/).

These large insert DNA libraries have been used for map-based gene cloning not only in rice but also in other cereals through comparative genetic mapping between rice and other cereals (Kilian *et al.*, 1995; Dunford *et al.*, 1995). The large insert clones can also facilitate structural genome analysis and genome sequencing. During my gene cloning approach of this study, a part of genome sequences became available from International Rice Genome Sequencing Project (IRGSP) and Monsanto company, USA (http://www.rice-research.org/). Very recently, two projects of whole rice genome sequencing have published draft sequences covering 92-93% of the entire genome (Yu *et al.*, 2002; Goff *et al.*, 2002).

This chapter describes the construction of the physical map of the *PLA1* locus using a BAC library and identification of a candidate BAC clone encompassing the *PLA1* locus. Molecular markers generated by the use of BAC sequences published in the course of this study could narrowed the *PLA1* locus into 74Kb region in the candidate BAC clone.

Materials and Methods

BAC library and DNA markers

A BAC library constructed with genomic DNA of a Japonica cultivar, Nipponbare, (Zhang *et al.*, 1996) was obtained from Clemson University Genome Institute. The BAC

library has 36864 clones of 130Kb average size and so covers about 10 times of the genome. DNA markers including available RFLP markers and end clones of BAC inserts that are located around the *PLA1* gene on chromosome 10 were used for screening of BAC clones.

TAIL-PCR and Cassette PCR for BAC end cloning

To generate a probe for chromosome walking, BAC end DNA fragments were amplified using slightly modified TAIL-PCR (Liu *et al.*, 1995) or Cassette-PCR, and used for the screening of BAC clones. The procedures were illustrated in Appendix 4-1 and 4-2.

Generation of SSR (simple sequence repeat) markers

Four SSR markers closest to the RFLP marker C961 were generated using a rice genome sequence published by Monsanto Company, USA, and used as probes for chromosome walking. Primers were designed to amplify the SSR sequences and detected polymorphisms. PCR products were electrophoresed on a 10% polyacrylamide gel electrophoresis at 150V for 1 hour.

BAC clone Screening and contig formation

High-density BAC clone filters obtained from Clemson University (URL: http://www.genome.clemson.edu/) were hybridized with labeled probes at a concentration of 10ng/ml according to the ECL detection system (Amersham-Pharmacia). BAC clones hybridized to these probes were selected and cultured for overnight in a TB medium (12 g tryptone, 24 g yeast extract, 8 ml 50% glycerol/L) containing 12.5μg/ml Chloramphenicol.

The DNA from clones was extracted using QIAGEN plasmid extract kit according to supplier's instruction (QIAGEN, USA). BAC contig information published by Clemson University and BAC clone sequence around the *PLA1* locus on chromosome 10 developed by Cold Spring Harbor Laboratory (CSHL), USA, were also used for construction of the physical map around the *PLA1* gene (http://www.cshl.org/). Confirmation of BAC clones to have the same sequence as the RFLP marker was carried out by Southern hybridization or PCR experimennt.

Pulse field gel electrophoresis

PFGE (Pulsed field gel electrophoresis) was carried out to know the insert DNA size of BAC clones mapped around the *PLA1* locus. One μg of a BAC DNA was digested with *NotI* and electrophoresed on a 1% agarose gel in a 0.5×TBE buffer for 14 hours at 14℃ in a PFGE apparatus according to supplier's instruction (BIO-RAD).

Results

Construction of a BAC contig using the closest RFLP markers to the *PLA1* locus on both sides

A rice BAC library was used to construct a physical map encompassing the *PLA1* gene. The first colony hybridization was carried out with the RFLP marker C961 tightly linked to the *PLA1* locus with genetic distance of 0.76 cM as a probe. Eleven BAC clones (B30O24, B33C23, B36C20, B44J21, B45H24, B45P01, B50L02, B50N08, B91A22, B91B23, B95M16 and B95N15) were identified with C961 (Fig. 7).

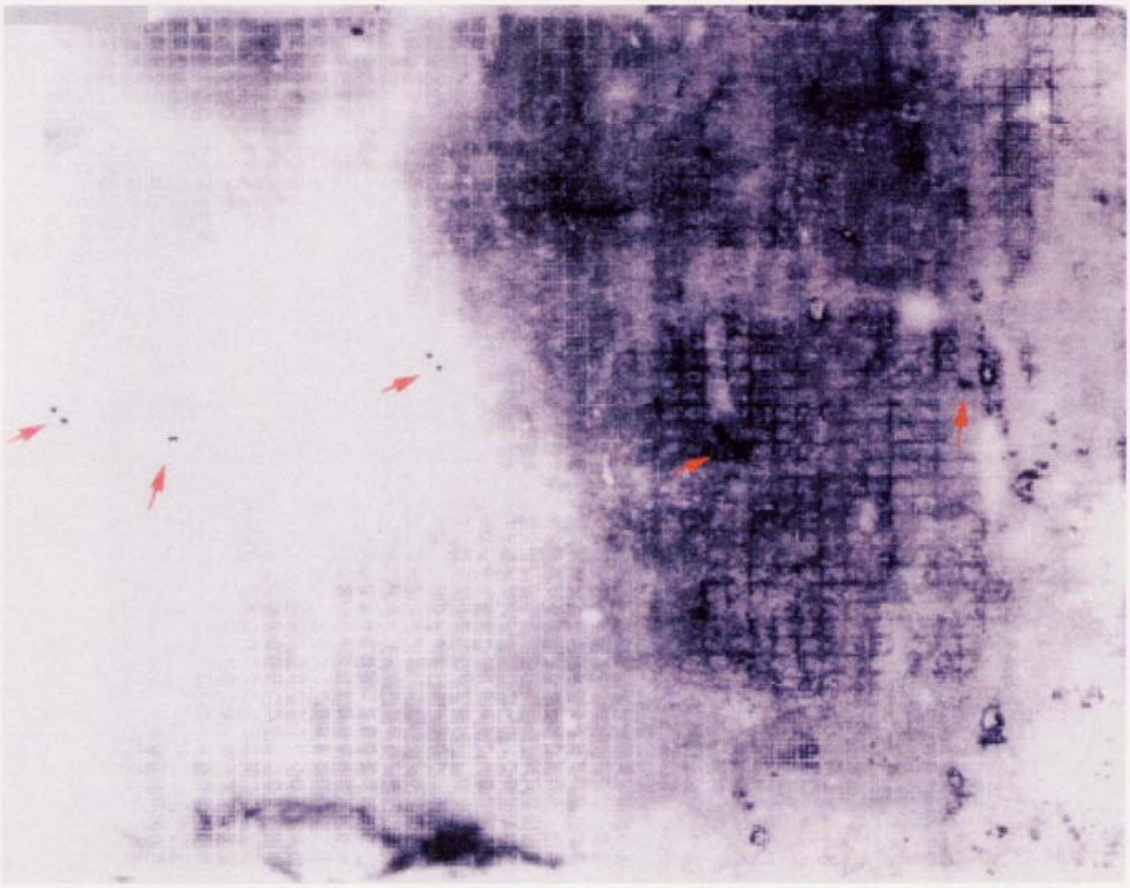

Fig.7 Screening of positive clones by colony hybridization with marker C961 as a probe.

These BAC clones were confirmed to contain the same sequence as that of the marker C961 by Southern hybridization except B91A22 (data not shown). All probes for chromosome walking were directly generated from identified BAC clones using TAIL-PCR or Cassette PCR. PCR-based amplified BAC end sequences were further used for detection of recombination between the *PLA1* and those end sequences through Southern hybridization of all 42 recombinant plants. In this experiment, both end clones, B30O24F and B45P01R, were mapped at the same position as the marker C961, whereas opposite end clone could not be mapped because of repetitive nature of the sequence (Fig. 8B). All other PCR-based end-clones (F and R); B33C23, B36C20, B91B23, B95M16, B50N08, B45P01F, B50L02R and B45H24F, amplified by TAIL-PCR or Cassette PCR were shown to contain repetitive sequences by Southern hybridization, and they could not be used for the mapping (part of those clones were shown in Fig. 8).

The BAC library was screened again with B45P01R and B30O24F sequences and several clones (B17C18, B10E03, B36O13, B43C04 and B44A10) were identified. PCR-based end-clones of identified BAC clones were generated in the same manner. Among them, four end-clones, B17C18R, B10E03R, B36O13R and B43C04R, were again mapped at the same position as C961. Four end-clones of the opposite ends, B17C18F, B10E03F, B30O13R and B43C04R, could not be used as probes for chromosome walking due to the repetitive nature. Both end-sequences of B44A10 were not amplified by PCR (those clones were not shown in Fig. 8). Because of the repetitive nature of these end-sequences, it was difficult to walk toward the *PLA1* locus from the side of C961. These results suggested that

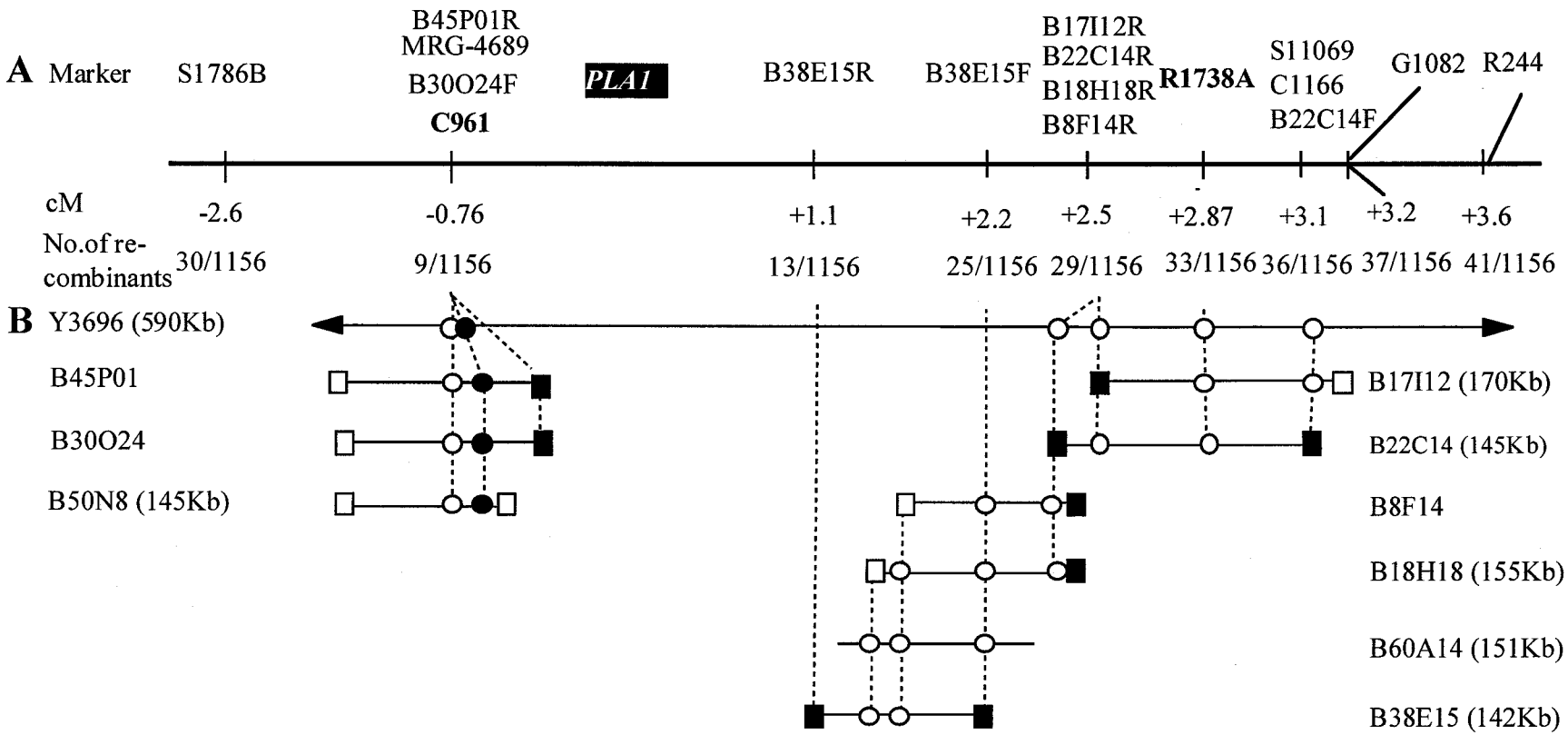

Fig. 8. High resolution linkage map and BAC contig around the *PLA1* locus. Open rectangules indicate end clones with repetitive sequences. Closed rectangules indicate end clone or developed markers used for chromosome walking. Open circles indicate markers or sequences contained in BACs or YAC clone. Closed circles indicate SSR marker.

the genome region around the *PLA1* locus is rich in repetitive sequences and recombination might be suppressed around the C961 locus.

Starting from another RFLP marker R1738A, which was located at the opposite side beyond the *PLA1* locus, chromosome walking toward the *PLA1* was also carried out. The marker R1738A, which was the second nearest marker 2.87 cM apart to the *PLA1* locus, could select and confirm about twenty clones including B8F14, B17I12, B18H18 and B22C14. Both end-fragments from these BAC clones were amplified to generate probes and used to detect polymorphism between the mapping parents. Results showed that four end-clones, B8F14R, B17I12R, B18H18R and B22C14R, were mapped at the same position with 2.5 cM genetic distance from the *PLA1* locus (Fig. 8B). B22C14F, which was an opposite end clone of B22C14R, was also mapped at the same genetic distance of 3.1cM where both RFLP markers S11069 and C1166 located. Three opposite end clones, B8F14F, B17I12F and B18H18F, could not be mapped due to their repetitive sequences. However, these results at least showed that the end clone B22C14R located nearer to the *PLA1* locus than the opposite end clone B22C14F.

As a next step to walk to the *pla1* locus, the B22C14R seemed most useful for selecting neighboring BACs. Two positive clones, B8F14 and B18H18, were screened and two end-clones, B8F14R and B18H18R, were mapped at 2.5cM genetic distance (Fig. 8B). Though the clones B8F14F and B18H18F were not mapped, F ends were thought to be closer to the *PLA1*.

In addition, B18H18R was also used to screen the BAC library. Four positive clones, B33C3, B38E15, B60A14 and B76L08, were identified and their end-clones were

amplified by TAIL-PCR or Cassette PCR. Amplified end-clones were investigated for their polymorphism between two mapping parents. Among them, both end fragments of B38E15F and B38E15R were mapped closest to the *PLA1* locus. B38E15R was mapped at genetic distance 1.1cM and B38E15F was at genetic distance of 2.2cM from the *PLA1* locus. Three other clones, B33C3, B60A14 and B76L08, could not be mapped due to the repetitive sequences. The marker B38E15R was used to walk further. Several clones identified were end-amplified and sequenced in the same way. However, further walking toward the *PLA1* locus from the marker B38E15R was not successful again due to the repetitive nature of the end-sequences.

SSR (Simple sequence repeat) mapping

Another strategy was considered to construct BAC contigs to overcome the difficulties of chromosome walking from the markers C961 and B38E15R. About 7000 simple sequence repeats (SSR) markers were published by Monsanto Company, USA. Four SSR markers (No. MRG 0859 (AT×17), MRG 1000 (AT×19), MRG 4689 (AAT×17) and MRG 5124(CGC×8)) which are linked to C961, and two, which are linked to R1738A (No. MRG 2096 and MRG2495), were selected through the database of Monsanto Company, USA (http://www.rice-research.org/). Four SSR markers around C961 were investigated for their possibilities to walk further to the *PLA1* locus. The primers flanking simple sequence repeats were designed, and used for PCR to detect polymorphisms between parent DNAs. One SSR marker 〔No. MRG 4689 (AAT×17)〕 detected a polymorphism and was investigated for recombinant. However, the MRG 4689 (AAT×17)

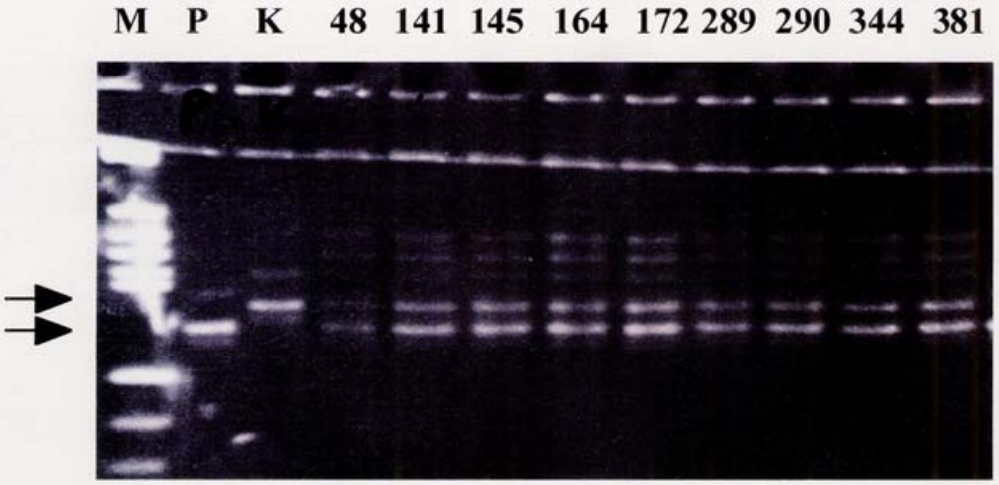

Fig. 9. SSR(Simple sequence repeat) mapping. SSR marker MRG4689 detected polymorphism between crossed parents of pla1-2 (P) and Kasalath (K). Numbers indicate recombinants detected by the RFLP marker C961. M : marker.

was also mapped at the same genetic position as that of the marker C961 (Fig. 9). Newly released sequence data for B50N08 (145Kb, DDBJ Accession No. AC021891) showed that the clone has both MRG 4689 (AAT×17) and C961 sequences and that MRG 4689(AAT×17) is only 5Kb closer to the *PLA1* locus than C961 (Fig. 8B). It is considered that no recombination took place in this 5Kb region in the mapping population used.

Construction of a BAC contig encompassing the *PLA1* locus

During the experiments of BAC contig construction, sequencing of BAC clones around both markers C961 and R1738A on rice chromosome 10 had been progressed and published by Cold Spring Harbor Laboratory, USA, in cooperation with the International Rice Genome Sequencing Project (IRGSP). Among those clones, two BAC clones, B50N08 (annotated) and B44A10 (annotation is in progress by CSHL), were almost completely sequenced. Comparison of these two sequences showed that these clones partially overlapped each other. Southern hybridization using C961 showed that B50N08 contained the C961 sequence, but B44A10 did not hybridize with C961. Though B44A10 was not yet annotated, several primers were designed using the published sequence to generate RFLP or PCR-based polymorphic markers. Linkage analysis revealed that the PCR-amplified marker, B44A10-89 or B44A10-2, were located on the different sides of the *PLA1* locus with 0.35cM and 0.61cM genetic distance, respectively (Fig. 10). Therefore, it was concluded that the B44A10 clone contained the *PLA1* locus. To refine the *PLA1* locus, several other primers were designed within the sequence of B44A10. Linkage analysis between these markers and the *PLA1* revealed that the RFLP markers B44A10-142 and B44A10-16 were

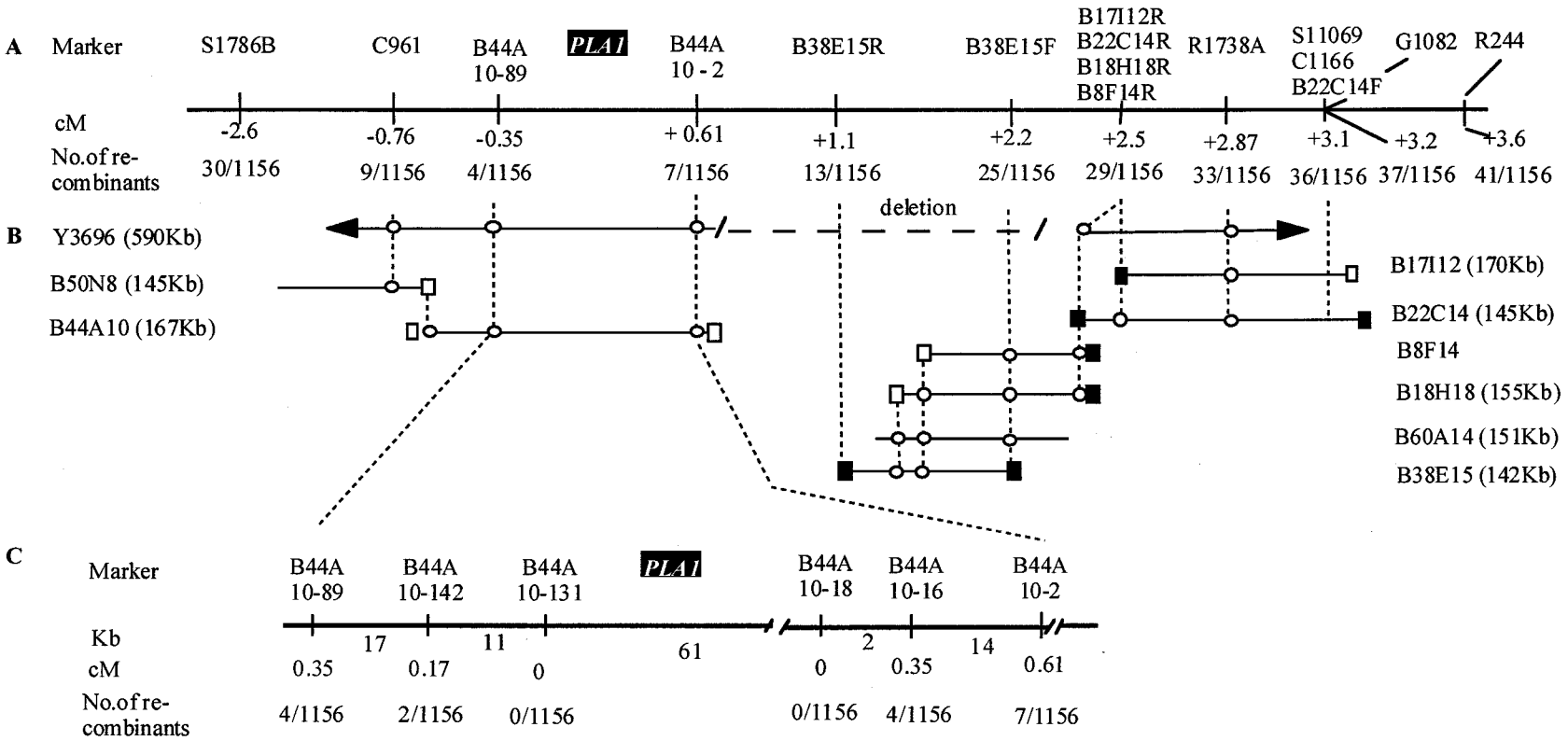

Fig.10. Genetic and physical maps around the *pla1* locus on chromosome 10. (A) A high-resolution RFLP linkage map of the *PLA1* locus. (B) BAC contigs around the *PLA1* locus. (C) A fine map around the *PLA1* locus on the B44A10 BAC clone. Filled rectangles indicate single copy and empty rectangles indicate multicopy 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. Markers with R and F letters designate right (SP 6 promoter) and left (T7 promoter) end of BAC clones. Other markers are derived from the high-density genetic map (Harushima *et al.*, 1998). Double oblique line indicate the gap position in B44A10.

mapped very near to the *PLA1* locus with genetic distances of 0.17cM and 0.35cM, representing two and four recombinants, respectively (Fig. 11 A, B and Fig. 12). Two markers, B44A10-131 and B44A10-18, co-segregated with the *PLA1* locus with no recombinant (Fig. 10). Physical distance between the RFLP markers B44A10-142, which detected two recombinants, and B44A10-131, which co-segregated with the *PLA1* locus, was 11Kb. The distance between two markers, B44A10-16 with four recombinants, and B44A10-18 with no recombinant, was only 2Kb (Fig. 11).

As the published sequence of B44A10 had several gaps, a pulsed field gel electrophoresis (PFGE) analysis was carried out to examine the accuracy of the physical length of B44A10. DNA of B44A10 was extracted and digested with a *SfiI* restriction enzyme. Comparison between a PFGE pattern and the physical map around *PLA1* locus showed complete agreement of the two results (Fig. 11B), confirming the accuracy of the physical map of B44A10 clone.

The exact physical distance between the *PLA1* locus and markers were calculated based on the sequence of the B44A10. The physical distance between B44A10-142 and B44A10-131 was 11Kb, between B44A10-18 and B44A10-16 was 2Kb. This exact physical distance between the markers B44A10-142 and B44A10-16 could drive the *PLA1* gene into 74Kb in the B44A10 (Fig. 11A).

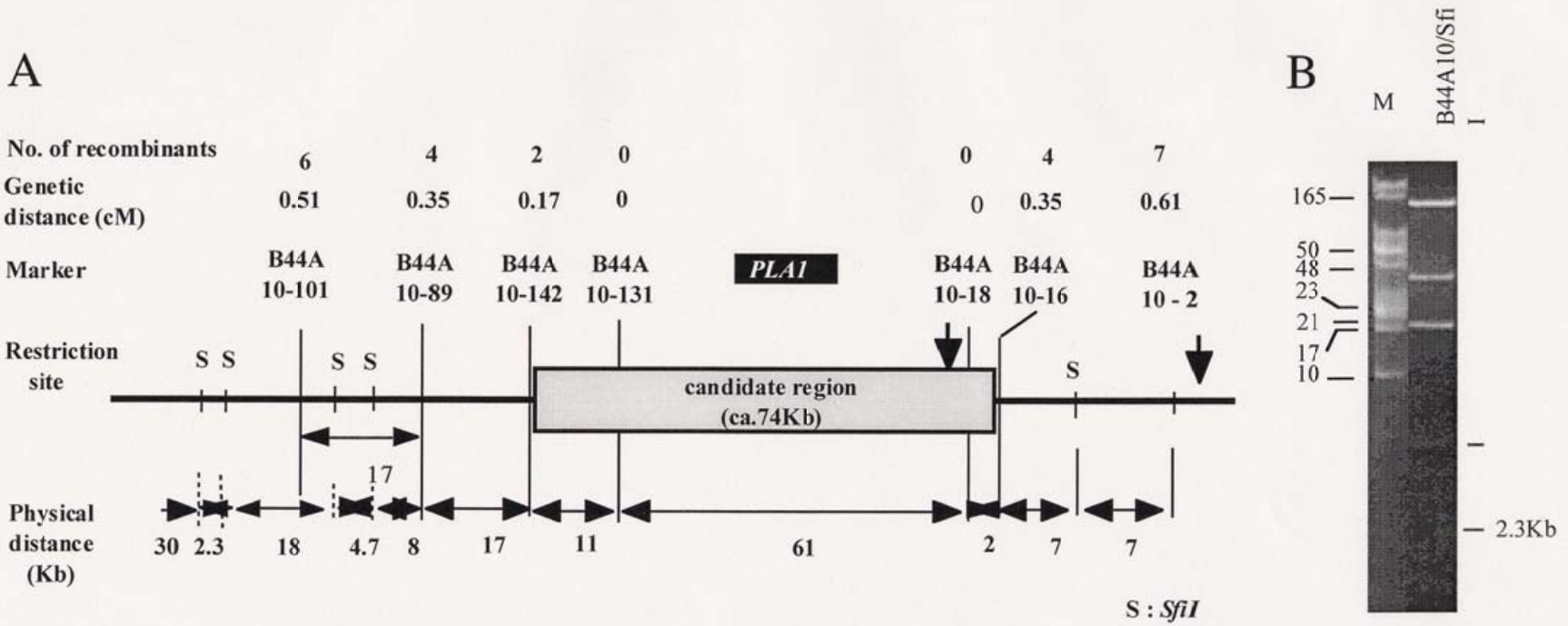

Fig. 11. A physical map of the B44A10 clone and it's PFGE analysis. A : Physical length were calculated from the sequence of B44A10 (published by CSHL, USA). Vertical arrows indicate sequence gaps in the B44A10. B : Pulse field gel elcctrophoresis of B44A10. S : *SfiI.* , M : marker.

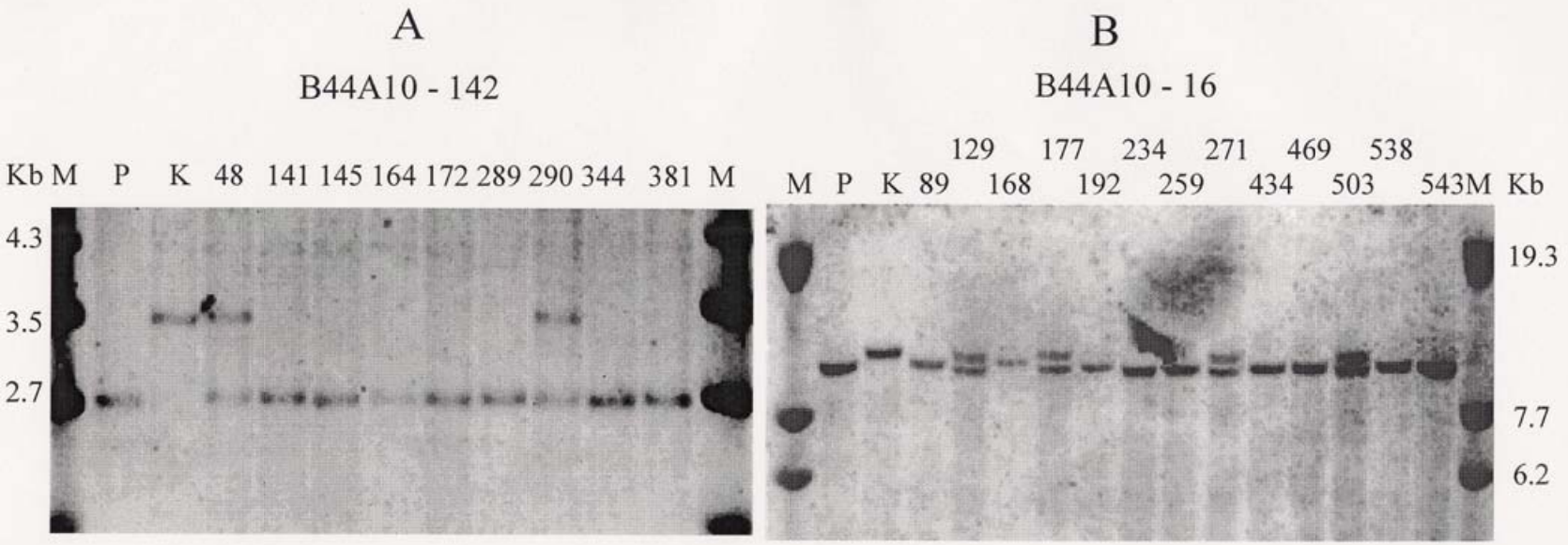

Fig. 12. Southern hybridization using two markers nearest linked to *pla1*. A : The marker B44A10-142 detected two recombinants with genetic distance 0.17cM from the *pla1*. B : The marker B44A10-16 detected four recombinants with genetic distance 0.34cM.

Discussion

Repetitive sequences of end-clones of almost all BAC clones led to difficulties in construction of a BAC contig harboring the *PLA1* gene. The region between marker C961 at 25.7cM and R1738A at around 29cM encompassing the *PLA1* locus was previously considered to be very near to the centromere on chromosome 10 (Harushima *et al.*, 1998). Recently, it was elucidated that centromere region is located between the markers G1125 (15.4cM) and C489 (15.9cM) by using the centromere specific probe pRCS2 and fluorescence *in situ* hybridization (FISH) mapping with BAC clones anchored by RFLP markers on chromosome 10 (Cheng *et al.*, 2001). Therefore, it is considered that genomic regions near the *PLA1* locus appeared to carry a fair amount of repetitive sequences but not centromeric repetitive sequences.

A candidate clone, B44A10 (167Kb), carrying *PLA1* gene was identified and the *PLA1* locus was finely mapped between markers B44A10-142 and B44A10-16 with a genetic distance of 0.52 cM (Fig. 11). Physical distance between the nearest two makers was estimated about 74Kb based on the sequence information.

BAC contigs based on the fingerprints of insert DNA was published by Clemson University Genomic Institute (CUGI), USA, helped me to construct BAC contigs. A series of BAC contigs developed in this study by chromosome walking with newly generated markers well coincided with the BAC contig based on the fingerprint pattern published by the CUGI.

The yeast artificial chromosome (YAC) library is known to have some limitations such as chimaeric clones, occasional instability of insert DNA and difficulties in DNA

manipulation and low transformation efficiency (Sizuya *et al.*, 1992; Ioannou *et al.*, 1994). Southern hybridization analysis using C961 and R1738A markers showed that a YAC clone, Y3696 (590Kb) apparently covered the *PLA1* region of chromosome 10. However, after construction of a physical map, the Y3696 was found to have an internal deletion in a region between markers, B44A10-2 and B18H18R, as indicated in Fig. 10 (though the experiment has not been shown here). The repetitive sequences in the YAC clones are known to be easily deleted. The deletion in Y3696 might correspond to the repetitive sequences, which also gave difficulties in chromosome walking.

In general, it is considered that 1 cM of genetic distance correspond to 250 – 350Kb of physical distance in rice (Kurata *et al.*, 1994; Causse *et al.*, 1994). Genetic distance from C961 to R2447 was 4.4cM, whereas physical distance between them was deduced more than 450Kb, by calculation of BAC clone length of B44A10 (167Kb), B38E15 (142Kb) and B22C14 (145Kb) through PFGE analysis (data not shown). Average recombination frequency calculated from these data was one recombination at every 12-24Kb interval for B44A10 (167Kb/1.39cM), B38E15 (142Kb/1.1cM) and B22C14 (145Kb/0.6cM) (Fig. 10). As for the *PLA1* region on the B44A10 clone, however, the physical map indicated that the ratio of genetic to physical distance (167Kb) varied along the clone (Fig.11). For example, recombinants between markers B44A10-89 and B44A10-131 were four, and physical distance of the region was 28Kb, suggesting one recombination per 7Kb interval. Recombinants between markers B44A10-18 and B44A10-16 were four and physical distance was only 2Kb (Fig. 11), showing one recombination in 0.5Kb interval. These imply that there are hotspots for recombination just outside the *PLA1* locus. The

mechanism for recombination suppression for 74Kb length flanking the *PLA1* and presence of hotspot next to it awaits for clarification.

Chapter 3

Cloning and identification of *PLA1* gene

Introduction

Physical mapping revealed that the *PLA1* locus was restricted in a 74Kb region of the B44A10 (167Kb) clone. Most part of the B44A10 have been sequenced by CSHL, USA, but with one gap flanking to the marker B44A10-18 (Fig. 11A). To select candidate cDNAs corresponding to *PLA1*, colony hybridization with the B44A10 clone (100Kb) probe was conducted against cDNA libraries derived from young shoot and young panicle stages. Only one cDNA was identified and it was located not in the 74Kb region but outside of the covered by region of B44A10 (not shown data).

Rice genome draft sequences published by two research groups (Yu *et al.*, 2002; Goff *et al.*, 2002) predicted that the number of rice gene was between 32,000 and 55, 000. It is considered that rice gene would be distributed at every 8-13Kb interval on the genome in average. This indicates six to nine genes in 74Kb.

To elucidate candidate gene corresponding *pla1* syndrome, gene prediction programs GENESCAN (http://genes.mit.edu/GENESCAN) and HMM (http://www.rgp.-dna.affrc.go.jp/Analysis) were used to predict genes existing in the 74Kb region of B44A10. Seven genes were predicted as candidate genes and those genes were examined to identify expression in the transcription level by RT-PCR.

To clarify mutations in all four alleles of *pla1* mutants, sequencing analysis and nucleotide comparison between four mutant alleles and their wild type strains were conducted for three candidate genes. A gene complementation experiment to prove the

PLA1 gene was also carried out.

Material and Methods

Plant material

Four alleles of *pla1* mutants and their wild type strains were used (Table1). *pla1-2*, *pla1-3* and *pla1-4* mutants were crossed to *pla1-1* mutant in heterozygous condition and were examined for mutant segregation in their progenies for allelism test. The results showed that these four *pla1* mutation were allelic (data not shown).

Similarity search and gene prediction

Database searches for exact matching sequences were carried out against the public database of DDBJ using the BLAST algorithm. Gene prediction programs, GENESCAN and HMM, were used to predict genes existing in the 74Kb region of B44A10.

DNA extraction and Southern hybridization

DNA extraction and Southern hybridization protocols were same as those described in Chapter 1.

Direct DNA sequencing

Three candidate genes including a member of P450 gene family and two genes for GTPase regulator protein were amplified by PCR with several combinations of primers from four alleles of *pla1* mutants and their wild type background strains. PCR products

amplified from each genomic DNA were subjected to agarose gel electrophoresis, followed by elution and purification using Geneclean kit. The PCR products were directly amplified using 10-30 ng template DNA and FS Ready Reaction kit (Perkin-Elmer Applied Biosystems, CT, USA) by Big dye terminator cycle sequencing system. The reaction products were sequenced by ABI 3100 automatic DNA sequencer according to the manufacture's instruction (ABI 3100 automatic sequencer, USA).

Construction of the binary vector plasmid

To clone a genomic DNA fragment corresponding to the P450 gene from the B44A10 clone, restriction enzyme sites were searched against B44A10 sequence using GENE-MAX program. DNA of B44A10 clone was digested with *SpeI* and a 6Kb fragment containing *PLA1* was isolated. The 6Kb fragment was cloned into a *XbaI* site of a binary vector pBGH1 and the resultant plasmid pBGH1-*PLA*1 was introduced into *Agrobacterium tumefaciems* EHA101. Transformed colonies were cultured in 2×YT medium containing kanamycin (50 μg/ml) and hygromycin (50 μg/ml) at 28℃ overnight and the plasmids were extracted. PCR and restriction enzyme digestion confirmed that pBGH1-*PLA1* maintained the correct structure without any structural change.

Complementation test

Transgenic plants were generated through *Agrobacterium*-mediated transformation following the procedure with a slight modification (Hiei *et al.*, 1994). A flowchart of transformation experiment is shown in Appendix 5. *pla1-2* seeds, which could set seeds

on a homozygous plant, were dehulled, sterilized in 70% EtOH for 1 minute and then in 2% NaOCl for 30 minutes followed by rinsing with sterilized water for several times. Calli from *pla1-2* homozygous seeds were generated in N6 medium supplemented with 10mg/L 2,4-D for 4 weeks. *Agrobacterium* transformed with the binary vector pBGH1-*PLA1*(6Kb) or the pBGH1 was cultured at 28℃ for 3days in solid LB medium containing kanamycin (50μg/ml) and hygromycin (50μg/ml), and a small piece of a colony was suspended in AA medium containing 10μg/ml acetosyringone. The calli were treated in the *Agrobacterium* suspension medium, then transferred onto N6 medium containing 10μg/ml acetosyringone and incubated at 28℃ in the dark for 3 days. After co-cultivation for 3 days, the calli were rinsed three times to remove the surplus *Agrobacterium* in sterilized water containing claforan (500μg/ml). The calli were cultivated on N6SE (2,4-D 2mg/l) medium that contained claforan (500μg/ml) and hygromycin (50μg/ml) for 3 weeks. The calli were transferred to regeneration medium (NAA 1mg/l and BAP 2mg/l) that contained claforan (250μg/ml) and hygromycin (50μg/ml) and incubated at 28℃. The calli were transformed to fresh medium every 3 weeks until shoots were regenerated. The regenerated shoots were transplanted to rooting medium (MSHF) supplimented with claforan (200μg/ml) and hygromycin (50μg/ml). After rooting on the MSHF medium, transgenic plants were transplanted to pots and grown in greenhouse.

As the number of *pla1-2* homozygous seeds for the genetic complementation experiment was not sufficient, heterozygous seeds were also applied. Calli were produced from seeds of *pla1-2* heterozygous plants and subjected to CAPS (Cleaved amplified polymorphic sequence) analysis (Appendix 6). The mutant *pla1-2* allele has an identical

HhaI site in the most promising candidate gene of P450, but wild type allele did not have. Calli were induced from 800 *pla1-2* heterozygous seeds for 3 weeks and DNAs were isolated from each callus, amplified by PCR and were digested with *NheI*. The homozygous calli of *pla1-2* were identified with specific CAPS and were used for the complementation test as described above. Transgenic plants were confirmed by PCR with primers specific to the binary vector and by Southern hybridization with the *hpt* (hygromycin phospho-transferase) gene as a probe.

Result

Identification of the *PLA1* gene

Fine mapping revealed that the *PLA1* gene was located between markers B44A10-142 and B44A10-16 in a 74Kb region as shown in Chapter 2 (Fig. 11A). Gene annotation programs were used to predict a causal gene corresponding to *pla1* based on the published sequence of B44A10. Seven candidate genes were predicted by using two gene annotation programs, GENESCAN and HMM. The candidate genes detected in the 74Kb region were a cytochrome P450 gene, two putative GTPase regulator protein genes and five transposon-like sequences (Fig. 13).

RT-PCR was carried out to investigate the expression of the seven candidate genes. Based on the sequence predicted by the gene annotation program, the primers encompassing an intron were designed for all seven candidate genes. RT-PCR using RNAs from young seedlings which was the most probable tissue for *PLA1* expression revealed

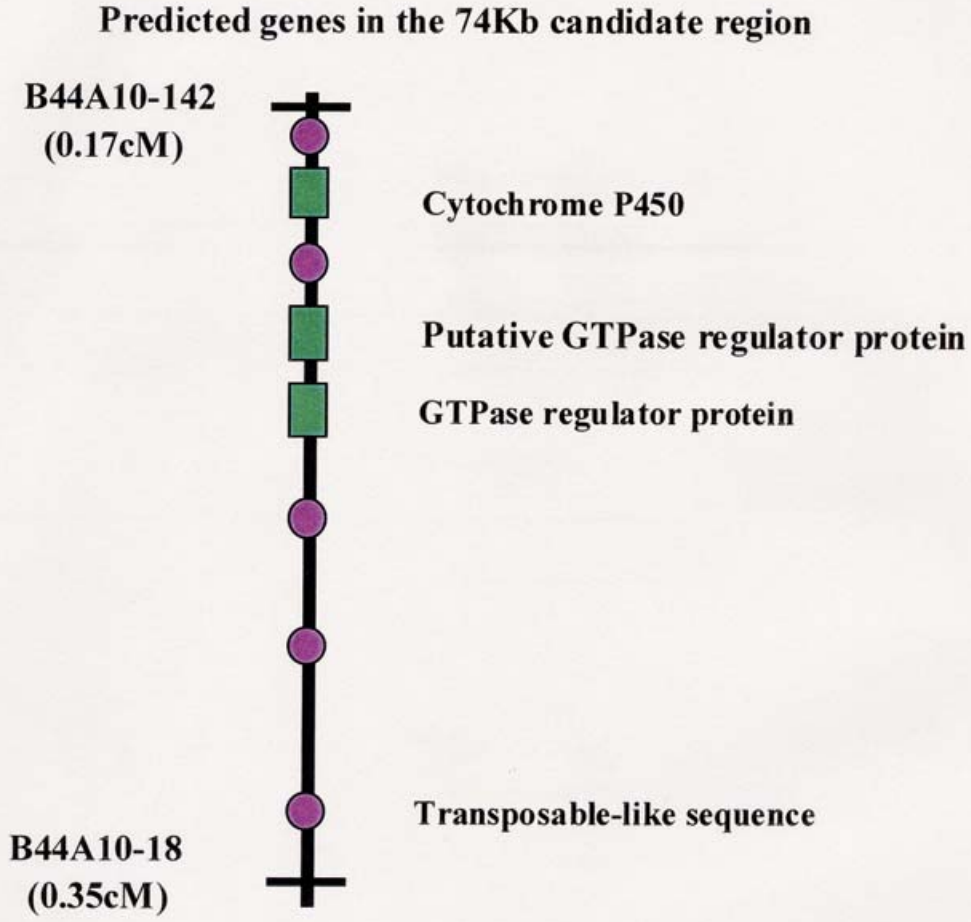

Fig.13. Prediction of a candidate gene corresponded to the *PLA1* gene in 74Kb region of BAC 44A10 clone Genescan (MIT,USA) and HMM (RGP, Japan) were used for Prediction gene.

Table 1. Plant materials used in this study

Alleles	Background Strain	Mutatgen	Severity of phenotype
pla1-1	Fukei-71	γ -ray	Very severe
pla1-2	Kinmaze	MNU	Severe
pla1-3	T65	MNU	weak
pla1-4	T65	MNU	very severe

that three candidate genes of P450 and two GTPase regulator proteins were expressed (data not shown, but protocols are given in the Materials and Methods of Chapter 4).

To detect mutation in the three candidate genes, the coding regions of the three genes were amplified from four alleles of the *pla1* mutants and from wild type allele by PCR with gene specific primers, and all PCR products were directly sequenced. Nucleotide sequence analysis indicated that all four alleles of the *pla1* mutants carried mutations in the P450 gene (Fig. 14) but other two genes had no mutation in all four alleles. The *pla1-1* is the most severe allele and had a one bp deletion at 1137bp position in the first exon, resulting in frameshift mutation. The *pla1-2* carried a substitution of alanine to valine due to the nucleotide change at 1462 bp of the second exon near the steroid binding domain. The *pla1-3* carried a substitution of proline to serine due to the nucleotide change at 1687bp of the second exon in the midpoint of conserved heme binding domain. The *pla1-4*, which was isolated recently, carried a substitution mutation of guanine to adenine at the splicing donor site (Fig. 14 and Fig. 15). Among the four alleles of *pla1*, *pla1-1* and *pla1-4* showed severe phenotype and had the mutation that resulted in a frameshift, while *pla1-2* and *pla1-3* with weaker phenotypes had single amino acid substitutions. These sequencing results strongly suggest that the *PLA1* gene encodes a cytochrome P450 protein.

Fig. 14. Nucleotide and amino acid sequences confirmed in the P450 gene of *PLA1*. All alleles of *pla1* mutants were sequenced and compared with its background cultivars; *pla1-1* vs Fukei 71, *pla1-2* vs Kinmaze, *pla1-3* vs T65 and *pla1-4* vs T65. Intron is indicated in small letters. Each domain conserved in other P450 family is shown in blue letters. *pla1-1* had a mutation of frameshift in the first exon. *pla1-2* and *pla 1-3* brought amino acid transitions in the second exon. *pla1-4* had a mutation in splicing donor site.

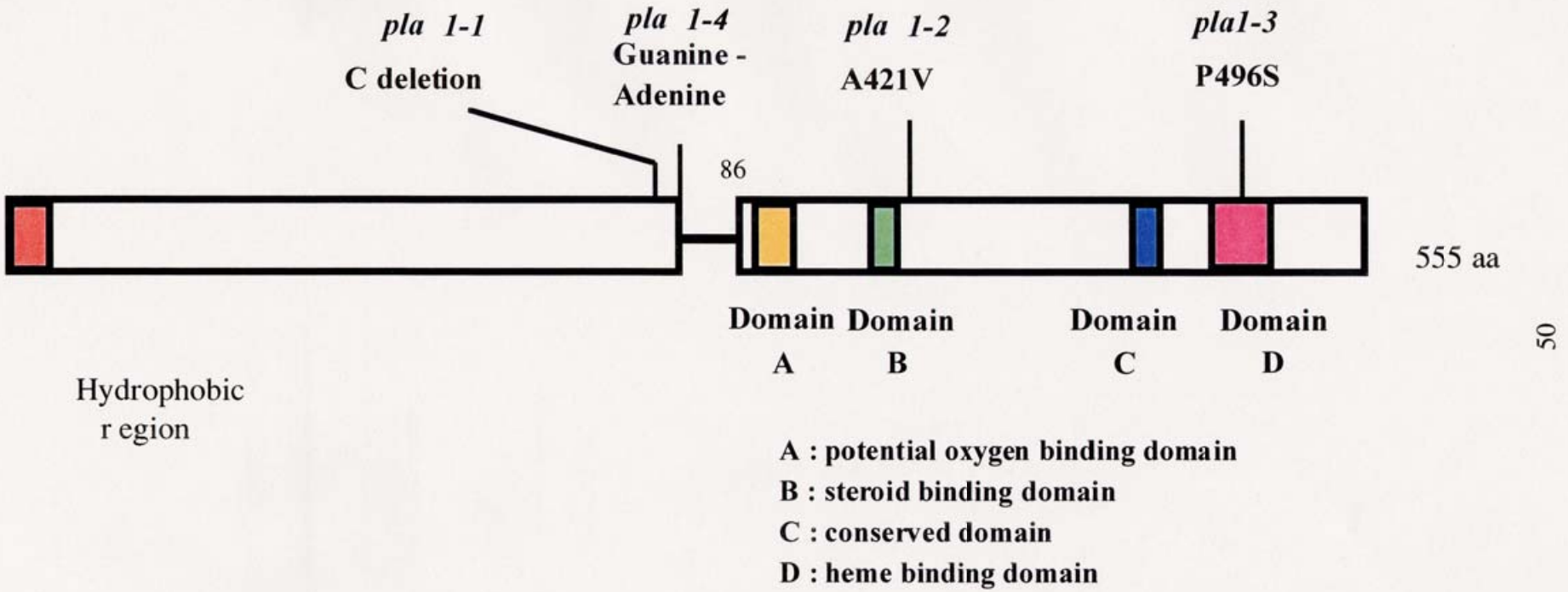

Fig. 15. Structure of the cytochrome P450 (CYP78A11) gene and mutationin four alleles of *pla1* mutants.

Molecular complementation experiment

To confirm whether the P450 gene rescues the *pla1* phenotype, a molecular complementation experiment was conducted (Appendix 5). A 6Kb genomic DNA fragment encompassing the P450 coding sequence was cloned into a binary vector pBGH1 which contains a hygromycin resistance gene (*htp*) as a selection marker, and named pBGH1/*PLA1*. The pBGH1/*PLA1* construct was introduced into *pla1-2* homozygous calli through the *Agrobacterium* - mediated transformation method. As a control, the *Agrobacterium* carrying pBGH1 vector, which lacks the P450 coding sequence, was also introduced into *pla1*-2 homozygous calli.

Ten hygromycin-resistant plants were obtained by transformation with the pBGH1/*PLA1*. As control lines, fourteen hygromycin-resistant plants transformed with pBGH1 were also obtained. Southern blot analysis using the *htp* gene as a probe detected one to five bands in all individual transgenic lines, indicating that all transformants carried at least one or more copies of the trans-gene (Fig. 17A). These transgenic lines were subjected to phenotypic observation through vegetative to reproductive development.

In vegetative phase, *pla1-2* mutant exhibits rapid emergence of leaves and size of leaves was significantly reduced (Fig. 1). All transgenic plants transformed with the control vector pBGH1 displayed dwarfism with small leaves, which were indistinguishable from the *pla1-2* homozygous plants. In contrast, all of pBGH1/*PLA1*-transformed lines apparently developed normally (Fig. 16, with a comparison to Fig. 1). Both plant height and leaf size in pBGH1/*PLA1* lines were comparable to those of wild type plants.

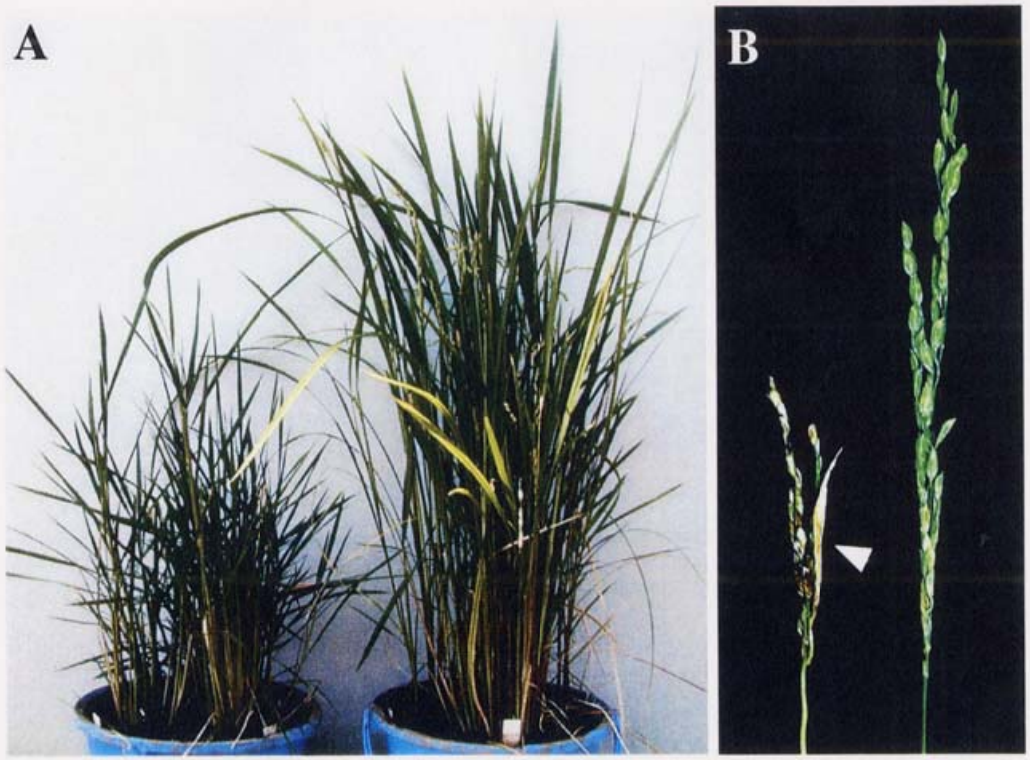

Fig. 16. Phenotypes of transgenic plants. A: Transgenic plants of *pla1-2* mutants in vegetative phase. Left; Transgenic plants with pBGH1. Right; Transgenic plants with pBGH1/*PLA1*. B: Panicles of transgenic plants. Left; A panicle of the transgenic plant with pBGH1. Enlarged bract is indicated by arrowhead. Right; A panicle of transgenic plant with pBGH1/*PLA1*.

Thus, vegetative characters of *pla1-2* mutant were well suppressed in pBGH1/*PLA1* transgenic lines compared to control lines.

In reproductive phase, *pla1-2* mutant develops truncated panicle with abnormally elongated bract. The panicles of vector-control lines completely resembled that of *pla1-2* mutants, however, in pBGH1/*PLA1* lines, such deformed panicles were never observed. Panicles of the pBGH1/*PLA1* lines displayed apparently normal phenotype (Fig. 16, with the comparison to Fig. 1). Therefore, aberrant panicle development in *pla1-2* mutant was also fully rescued in pBGH1/*PLA1* lines but not in control lines.

Finally, exact genotype of these transgenic lines was reconfirmed. CAPS analysis (Appendix 6) revealed that all plants of pBGH1/*PLA1* lines that exhibited wild type phenotype, possessed both wild type and mutant alleles for the P450 gene, whereas all control lines showing mutant phenotype, held mutant alleles only (Fig. 17B). Accordingly, introduction of the wild-type P450 gene into *pla1-2* mutant is sufficient to restore the wild phenotype through vegetative to reproductive development.

Based on these results, together with the sequencing analysis of the P450 gene, I concluded that the *PLA1* gene encodes cytochrome P450 protein.

Structure of the *PLA1* gene

To elucidate the structure of the *PLA1* gene, two cDNA libraries made from shoot (two - week after sowing) and panicle of rice were screened with 1.8Kb genomic fragment covering a complete coding region. However, attempts to clone cDNA of the *PLA1* gene were not successful. First, the P450 gene has high GC content of 64% mean (an intron is

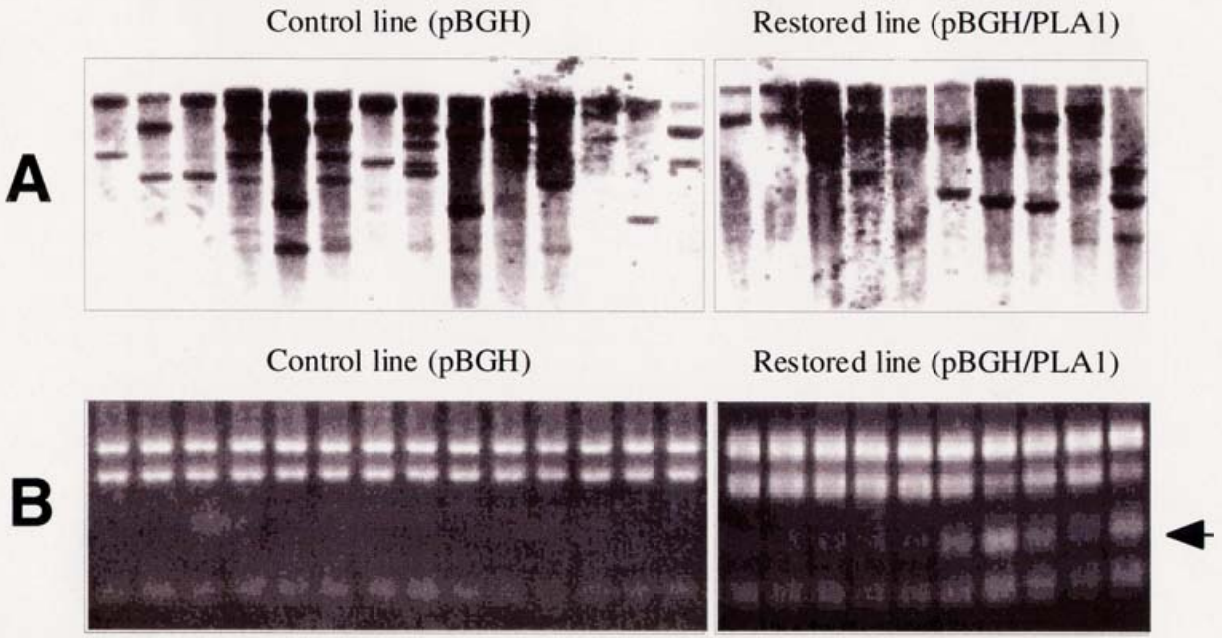

Fig. 17. Detection of the trans-genes in regenerated transgenic plants. A: Southern blot analysis of hygromycin-resistant plants. DNAs were digested by *EcoR*I and hybridized with *hpt* gene sequence. B: CAPS analysis for genotyping of P450 gene in transgenic plants (see appendix 6).

83% AT content) and it may cause high background by non-specific binding to other genomic DNAs. Second, it has been known that mRNA accumulation of P450 genes is low in plant cells (Chapple, 1998).

In the second attempt, therefore, RT-PCR was performed to clarify the structure of the P450 transcript. The P450 gene predicted by gene annotation program comprised two exons separated by a short intron (Fig. 14, Fig. 15). Total RNA was extracted from shoot apices at two weeks after germination and first strand cDNA was synthesized by reverse transcription. PCR was carried out using the cDNA and primers 2F and 21R that flank the intron, and an amplified fragment was directly sequenced. Sequencing analysis showed that mRNA of the P450 gene was exactly spliced at the site predicted by the gene annotation program.

To clarify the 5'untranslated region (UTR) of the *PLA1* gene, RT-PCR was carried out with combinations of one of the four primers 5'UTR-1F to 5'UTR-4F and a primer 14R located downstream beyond the intron (Fig. 14). RT-PCR with the primer combinations of one of 5'UTR-2F to 5'UTR-4F and 14R amplified a fragment with an expected size, whereas the combination of 5'UTR-1F and 14R showed no amplification of the fragment (Fig. 18). There is no consensus sequence or related sequences of an intron acceptor between 5'UTR-1F and 5'UTR-2F. The gene annotation program predicted a TATA box of a core promotor of *PLA1* within the primer sequence of 5'UTR-1F. These results suggest that a transcription start site is located between 5'UTR-1F and 5'UTR-2F (Fig. 14 and Fig. 18).

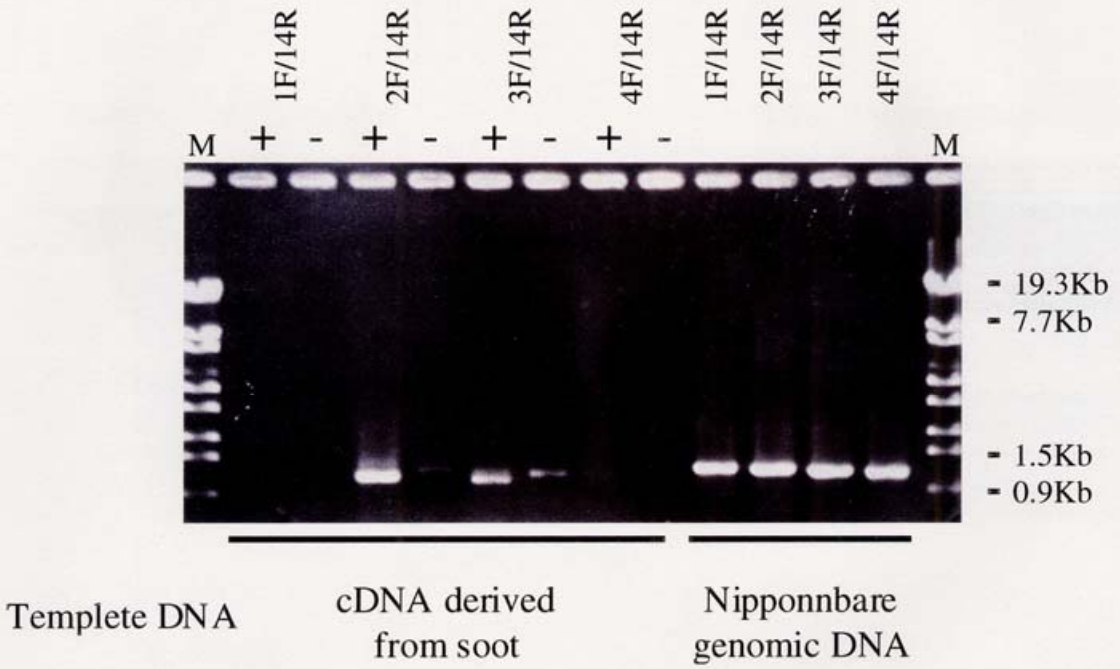

Fig. 18. RT-PCR experiment to detect 5'UTR. Primer combinations between one of the primers from 5'UTR-2F to 5'UTR-4F and 14R amplified a fragment with the expected size, whereas the combination of 5'UTR- 1F and 14R showed no amplification of the fragment. Primer sequences are shown in Fig. 14.

The predicted *PLA1* protein consists of 555 amino acids with a calculated molecular mass of 59KD. Homology search showed that the predicted *PLA1* protein has significant sequence similarity to the cytochrome P450 superfamily. The cytochrome P450 superfamily has four conserved domains, an oxygen binding domain, a steroid binding domain, a domain with unknown function and a heme binding domain, from N-terminus to C-terminus (Fig. 14 and Fig. 15). P450s are heme thiolate proteins and the sequence surrounding heme binding domain is conserved in most P450 proteins at a location about >50 amino acid sequence from the C terminus (Nelson *et al.*, 1996). The sequence of *PLA1* protein was also had a heme binding domain at about 55 amino acids from the C-terminus (Fig. 14). This gene had a highly conserved cytochrome P450 signature sequence (FxxGxxxCxG) as a heme binding domain and the cysteine of the signature sequence is involved in the binding site of heme Fe (Nebert and Gonzalez, 1987; Porter and Coon, 1991). Another signature sequence (MxYLxVxxETLR) was also conserved at 398 amino acid residues from N-terminus that is considered to be associated with steroid metabolism. A domain for potential oxygen binding includes conserved threonine in helix I preceded by an acidic residue (glutamic acid) at 352 amino acid from N-terminus (Fig. 14). In addition, similarity was conserved at 460 amino acid residues from N-terminus. Hydrophobicity plotting of *PLA1* indicated that an N-terminal amino acid sequence was a hydrophobic region, presumed to be an anchor to membrane (Fig.14, by http://www.bmb.psu.edu/). These high similarity in the amino acid sequence strongly suggested that *PLA1* encode a member of the P450 superfamily.

Potential oxygen binding domain	VLWEMIFRGTDTTA	*PLA1*(CYP78A11)
	ILWEMVFRGTDTTA	Maize(CYP78A1)
	VLWEMIFRGTDTTA	Pinus(CYP78A4)
	VLWEMIFRGTDTTA	Arabidopsis(CYP78A9)
	LIITILYSGYETVS	Dwarf(CYP85)
Steroid binding domain	MPYLQAVVKETLRA	*PLA1*(CYP78A11)
	MPYLQAVVKETLRA	Maize(CYP78A1)
	LPYIQAVVKEALRM	Pinus(CYP78A4)
	LPYLNAVVKETLRL	Arabidopsis(CYP78A9)
	MRFTRAVILETSRL	Dwarf(CYP85)
Conserved domain	ITHDGEVWADPEAFAPERF	*PLA1*(CYP78A11)
	ITHDAAVWADPDAFAPERF	Maize(CYP78A1)
	ITHDPNIWESPYEFRPERF	Pinus(CYP78A4)
	ITHDQTVWSDPLKFDPERF	Arabidopsis(CYP78A9)
	LNYDPRLYPDPYSFNPWRW	Dwarf(CYP85)
Heme binding domain	LRLAPFGAGRRVCPGKNLG	*PLA1*(CYP78A11)
	LRLAPFGAGRRVCPGKNLG	Maize(CYP78A1)
	LRLAPFGAGRRVCPGKALG	Pinus(CYP78A4)
	LRLAPFGAGRRVCPGKNLG	Arabidopsis(CYP78A9)
	NSFLVFGGGTRQCPGKELG	Dwarf(CYP85)

Fig. 19 Alignments of the conserved domains among CYP 78A subfamily.

Table 2. Similarity with other members of CYP 78A subfamily (as on 2002)

CYP78A Subfamily	Plant	Clone	Identity with CYP 78A11 (%)	Function	Accession Number
CYP78A11	Rice	Genomic sequence	100	Unknown	B44A10
78A1	Maize	CDNA	71	Lauric acid Monooxygenase	L23209-1
78A7	*Arabidopsis*	Genomic sequence	61	Unknown	AB016893
78A4	*Pinus radiata*	cDNA	59	Unknown	AF049067
78A2	*Phalaenopsis*	cDNA	51	Unknown	U34744
78A9	*Arabidopsis*	cDNA	49	Unknown	AB036059
78A5	*Arabidopsis*	cDNA	-	Unknown	T45256
78A6	*Arabidopsis*	Genomic sequence	49	Unknown	AC005819
78A3	Soybean	cDNA	48	Unknown	AF022463
78A8	*Arabidopsis*	Genomic sequence	47	Unknown	AC007323
78A10	*Arabidopsis*	Genomic sequence	-	Unknown	AC016662

PLA1 was classified into a new subfamily of P450 and named as CYP78A11 in accordance with the guideline suggested for Cytochrome P450 by the nomenclature committee (Dr. D. Nelson, dnelson@utmem.edu, USA). This is the first CYP78A11 subfamily identified in plants. P450s belonging to other subfamilies have less than 40% amino acid identity with the *PLA1* (Nelson *et al.*, 1996).

Members of the CYP78A subfamily reported in several plants and alignment of domains in P450 amino acid sequence among some members were established (Table 2 and Fig. 19).

Discussion

Among three candidate genes in 74Kb region of B44A10 expressed in shoot apex, only the P450 gene had mutation in all four alleles of *pla1* mutants (Fig. 14). The *pla1-1*, which showed very severe phenotype, had 1 bp deletion in the coding region and resulted in frameshift at 343 amino acid residue. The *pla1-4,* which also showed severe phenotype, had mutation at the donor site of the intron and it seems to induce pre-mature termination of translation. These two mutant alleles potentially encode proteins lacking all four domains conserved in the cytochrome P450 family. The other alleles, *pla1-2* and *pla1-3,* showing weaker phenotype, had mutations of amino acid substitution. These mutations of the gene were well reflected to the severity of the phenotype.

As *pla1-1* carry severe phenotyoe causing the sterility, *pla1-2* homozygous seeds were used for complementation test to identify whether P450 encodes *PLA1* or not. To select *pla1-2* homozygous calli from the mixed progenies, the mutated *HhaI* restrict site at 1462 amino acid residue in the *pla1-2* allele was utilized. The calli carrying homologous *HhaI* mutation sites were chosen from total of 800 *pla1-2* heterozygous seeds and were used for gene complementation experiment (Appendix 6).

All transgenic plants transformed with pBGH1-*PLA1* recovered to wild type phenotype. Ectopic shoot in *pla1-2* homozygotes were completely suppressed and normal panicles and normal seeds were produced in reproductive phase. The bract leaves were not developed as those of wild type (Fig. 16). Southern hybridization using *hpt* gene revealed that transgenic plants had variable copy number of pBGH1-*PLA1* transgene in the genome and they showed a specific phenotype on seed volume but not in other developmental

stages from vegetative to reproductive phase. These results together implies that mutation in CYP78A11 gene cause modification of significant phenotype during the life cycle. Analysis of phenotypes of P450 over-expression transgenic rice is now in progress.

PLA1 gene was designated as CYP78A11 by the cytochrome P450 nomenclature committee. This is the first protein belongs to the CYP 78A subfamily in plants. Database search identified another member of the cytochrome P450 family, CYP71A1, in rice. Although *PLA1* is similar to CYP71A1, it belongs to a different subfamily of cytochrome P450 and has amino acid identity of only 31% with CYP71A1 (DDBJ, Accession No. AP003434). P450 genes seem to form gene clusters at some specific regions of the genome. For example, *Arabidopsis* has over 270 genes, and members of each subfamily locate in diminutive regions of the genome (http://ag.arizona.edu/P450/). In *C. elegans,* eight cytochrome P450 genes and one pseudogene was found in a single cosmid clone of 35Kb (T10B9, Genbank Z48717) (Nelson *et al.*, 1996). However, it is not clear whether the rice cytochrome genes also exist in clusters of cytochrome P450. P450s form the largest family of plant enzymes, hence it is difficult to understand function of each member due to their low level abundance, a large number of homologs, relatively unstable and membrane-bound nature (Feldmann, 2001). Among 11 CYP78A subfamily identified so far, only one CYP78A1 was elucidated as encoding Lauric acid monooxygenase (Imaishi *et al.*, 2000).

To elucidate the function of CYP78A11 gene, it is important to search for temporal and spatial expression pattern of the *PLA1* gene and pehnotypes of over-expression transgenic plants.

Chapter 4

Spatial and temporal expression pattern of the *PLA1* gene

Introduction

In higher plants, the shoot apical meristem (SAM) is the source of all aboveground organs and necessarily governed by key processes that elaborate shoot architecture and then plant morphology. An aggregate of small cells located in the distal portion of the shoot, the SAM supplies cells that divide and differentiate to form the elements of the shoot (Medford, 1992). Through the vegetative and reproductive growth, lateral organs are formed on flanks of the SAM in regular order (phyllotaxy) and regular timing (plastochron). Patterns of lateral organ initiation directly determine plant morphology and thus the variations of spatial and temporal regularity in lateral organ initiation confer the diversity of shoot architectural characteristic in each species. Therefore, studies on regulatory mechanism for patterning of the lateral organ initiation are of primary importance for understanding plant development and evolution.

Many studies have focused on elucidating the mechanism that regulates phyllotaxy (reviewed in Steeves and Sussex, 1989; Callos and Medford, 1994). Based on the results of surgical experiments, an inhibitory field model was proposed (Snow and Snow, 1931; Wardlaw, 1949). The model predicted that a diffusible substance emanating from the center of the SAM and the preexisting leaf primordia inhibited the initiation of new leaf primordium. Alternatively, a biophysical model has suggested that the phyllotactic pattern is generated spontaneously as a result of physical force in the SAM (Selker *et al.*, 1992; Green, 1994; Green *et al.*, 1996). This model is supported by studies of expansin

application that promoted cell wall extensibility and modified phyllotaxy in tomato (Fleming *et al.*, 1997; 1999).

Genetic and molecular analyses have identified several genes affect the lateral organ patterning. In maize, the *terminal ear1* (*te1*) mutant shows an increased rate of leaf production, aberrant phyllotaxy, and abnormal phytomers (Veit *et al.*, 1998). The *TE1* gene contains several RNA binding protein motifs and appears to inhibit phytomer/leaf initiation in the SAM. Another Arabidopsis mutant, *amp1*, was also reported to have altered plastochron and phyllotaxy. The *AMP1* gene encodes putative glutamate carboxypeptidases. Potential substrates of *AMP1* are small acidic peptides and folate polyglutamate suggesting that the *AMP1* gene product modulates the level of a small signaling molecule (Chaudhury *et al.*, 1993; Helliwell *et al.*, 2001). These genes and the mutants gave us opportunity to explore the underlying mechanism. However, pleiotropic defects exhibited by these mutants wherein both plastochron and phyllotaxy are affected make it difficult to discern the mechanism concerning to individual feature. For comprehensive understanding of the regulatory network, identification of genes and mutations specifically affect the plastochron or the phyllotaxy is more favorable.

To date, only a few mutants have been identified that specifically affect the plastochron but not the phyllotaxy. The rice *pla1* mutant is one example. A better understanding of the *pla1* mutants would help to clarify the underlying mechanism for plastochron.

Another important aspect exhibited by *pla1* mutations is ectopic expression of vegetative program on reproductive growth. Simultaneous expression of vegetative and

reproductive program results in ectopic shoot formation on panicle branches. Thus, meristem identity as well as leaf initiation process is also affected in *pla1* mutants. Similar situations are observed in maize and tobacco. Maize *te1* mutants develop a female organ of ear on the top of the male organ, tassel branches, in certain genetic backgrounds (Matthews *et al.*, 1974). The *puzzle box* mutant of tobacco that exhibits irregular plastochron and dwarfism develop aberrant flower wherein inflorescence and floral programs are expressed together (Trull and Malmberg, 1994). These results suggest that abnormalities in temporal regulation of leaf initiation may affect meristem identity involving phase change.

The vegetative-to-reproductive phase change is known to be regulated by factors produced by leaves and then translocated to the shoot apex (Bernier 1988; Colasanti *et al.* 1998). In contrast, the source of the factors regulating vegetative phase change is very poorly understood (Hackett and Murray, 1997). Several possible mechanisms are proposed on the basis of meristem culture experiments. Irish and Karlen (1998) have suggested two possibilities; 1) the SAM is ''neutral with respect to phase'' and propose that the adult phase is initiated and maintained by signals originated from preexisting leaves, with no direct involvement of the SAM. 2) leaf primordia stabilize the developmental state of the shoot rather than specifying this state. As considering the heterochronic nature accompanied with abnormal leaf development in *pla1* mutant, these interpretations are quite attractive for further examination. Identification of functional site of the *PLA1* gene must provide useful information about plant development.

In this chapter, I have described detail analysis of the expression pattern of *PLA1* gene. The expression domain was approximately determined by RT-PCR followed by

detailed investigation through *in situ* hybridization experiment. Based on the results, I discuss about biological function of the *PLA1* gene in relation to regulatory mechanism for the plastochron and the phase change.

Materials and methods

Plant Materials

The japonica variety, Nipponbare, was used to investigate expression patterns of the *PLA1* gene.

Total RNA extraction

Several organs such as shoot (one - week after germination), shoot apices (two-week after sowing), young panicles (early differentiation stage of panicle of 1-5 mm length), meiotic stage panicles (panicles containing various stages of reproductive organs; 10 cm in length), young leaf (5-6 leaf age), expanded leaves (before flowering), callus, regenerating callus and root (one - week after germination) were collected, frozen in liquid nitrogen and stored at -80℃. About 0.3 - 0.9 grams of each sample was carefully ground in liquid nitrogen with the aid of a mortar and pestle. Total RNA was extracted using ISOGEN kit (NIPPONGENE, Japan). Ten volumes of ISOGEN solution pre-heated at 50℃ were added to the homogenized sample and incubated for 10 minutes at 50℃. After incubation the sample was placed for 5 minute at room temperature and 1/5 volume of chloroform was added and then the sample was centrifuged at 10,000 rpm for 15 minute at 4 ℃. An upper aqueous phase of about 500μl was transferred to a new 1.5ml tube and 500μl of 4M LiCl was added and stored for 1 hour at -80℃. The sample was centrifuged at 12,000 rpm for 15

minutes at 4℃. The pellet was dissolved in 400 μl DEPC (diethylpyrocarbonate)-treated water and was mixed with 400μl isopropyl alcohol and stored for 30 minutes at 4℃. The tube was centrifuged at 12,000 rpm for 15 minutes at 4℃ and the pellet was washed with 1ml 70% EtOH at 12,000 rpm for 15minutes at 4℃. The pellet was briefly dried up, dissolved with 400 μl DEPC-treated water. Forty μl of 3M sodium acetate and 1ml of EtOH were added to the RNA solution and then it was stored for 1 hour at -80℃. The tube was centrifuged at 12,000 rpm for 15 minute at 4℃ and the pellet was washed twice with 70% EtOH and dried up in air and was dissolved in 100 - 200 μl of DEPC-treated water.

Purification of messenger RNA

Poly $(A)^+$ RNA was purified from total RNA using Oligotex™-dT30 SUPER (Takara, Japan) kit. Fifty μl of 2×binding buffer and 10 μl of Oligotex™-dT30 were added to 90 μl of total RNA and mixed gently and heated for 10 minutes at 70℃ to denature the RNA. The sample was placed at room temperature for 10 minutes and centrifuged at 15,000 rpm for 5 minutes. After removing the supernatant poly $(A)^+$ RNA bound to Oligotex™-dT30 was suspended to 350 μl of wash buffer and transferred to spin column. The sample was centrifuged at 15,000 rpm for 30 seconds. This procedure was repeated once again. The poly $(A)^+$ RNA bound to Oligotex™-dT30 was suspended in 30μl of DEPC-treated water pre-heated at 70℃. This procedure was repeated twice to recover poly $(A)^+$ RNA.

RT-PCR

First strand cDNA was synthesized from poly $(A)^+$ RNA using Superscript II reverse transcriptase (GIBCO, BRL). Reverse transcription was performed at 30℃ for 10

minutes, 50℃ for 1 hours and 80℃ for 2 minutes. The reaction mixture contains 5μl of polyadenylated RNA, 4μl of 5×RT buffer, 0.2 μl of 0.1M DTT, 8μl of 2.5mM dNTP, 2μl of dT primer (10μM), 0.1μl of Superscript II, 0.7μl of SDW in a total volume of 20μl. An aliquot of the synthesized first strand cDNA was used for amplification by PCR with oligonucleotide primers 5'-2F(TCCCACCCCACRTCATCACA) and 3'-21R(GAGAGCTT-GAGGACCTCGTC), which flank an intron of *PLA1*. After pre-heating at 94℃for 1 min, PCR was carried out with 40 cycles of denaturation at 98℃ for 10 sec, annealing at 58℃ for 1 minute and extension at 72 ℃ for 1 minutes. The RT-PCR product was electrophoresed on a 0.7% agarose gel, stained with ethidium bromide and transferred onto a nylon membrane. Southern hybridization was performed to detect a signal of *PLA1* expression by using ECL system as described in chapter 1. Actin was used as an internal control. Primers used for RT-PCR of actin were 5'- (AACTGGGATGATATGGAGAA) and 3'- (TACAGTGTCTGGATTGGAGG).

***In situ* hybridization**

Samples including shoot apices were fixed in 4% paraformaldehyde and 0.25% glutaraldehyde in 0.1M sodium phosphate buffer for over 20 hours at 4℃. They were dehydrated in graded ethanol series, replaced in xylene, and embedded in Paraplast Plus (Oxford Labware). Microtome sections were applied to slide glass coated with poly - L - lysine with 7μm thick. Templates for in situ probes were generated from the pBluescript vector containing the CYP78A11 genomic fragment of 1.8Kb. Digoxigenin (DIG)-labeled UTP (Boehringer Mannheim) probes were generated using T3 (antisense) or T7 (sense)

RNA polymerase. *In situ* hybridization, hybridization and detection steps were performed as described (Sato *et al.*, 1996) except that hybridization was performed at 55℃ for the high GC content of the *PLA1* gene.

Results

Tissue specificity of *PLA1* expression

Rapid emergence of leaves and transformation of panicle into shoots suggest that *PLA1* play a role in shoot apical meristem. To determine organs and stages where *PLA1* gene is expressed, RT-PCR experiment was conducted. cDNAs synthesized with purified RNAs form several organs such as shoot apices, young panicles, young leaves (5-6 leaf age), expanded leaves (before flowering), proliferating calli and roots were examined for RT-PCR.

PLA1 mRNA was detected in seedling, shoot apex and young panicle, but not in young leaves, expanded leaves, callus and root as shown in Fig.20. This suggested that *PLA1* was expressed at shoot apices that differentiate leaf, leaf primordium, rachis primordium and SAM. No RT-PCR product of *PLA1* amplified with a given volume of cDNA template was seen on an agarose gel stained with ethidium bromide, whereas a large amount of actin products could be detected directly on the same gel. *PLA1* RT-PCR products could be detected only by the Southern hybridization analysis. These results indicated that *PLA1* mRNA was synthesized at very low level or in small restricted regions of organs used in this experiment. Spatial and temporal expression patterns of *PLA1* mRNA were further examined by *in situ* hybridization.

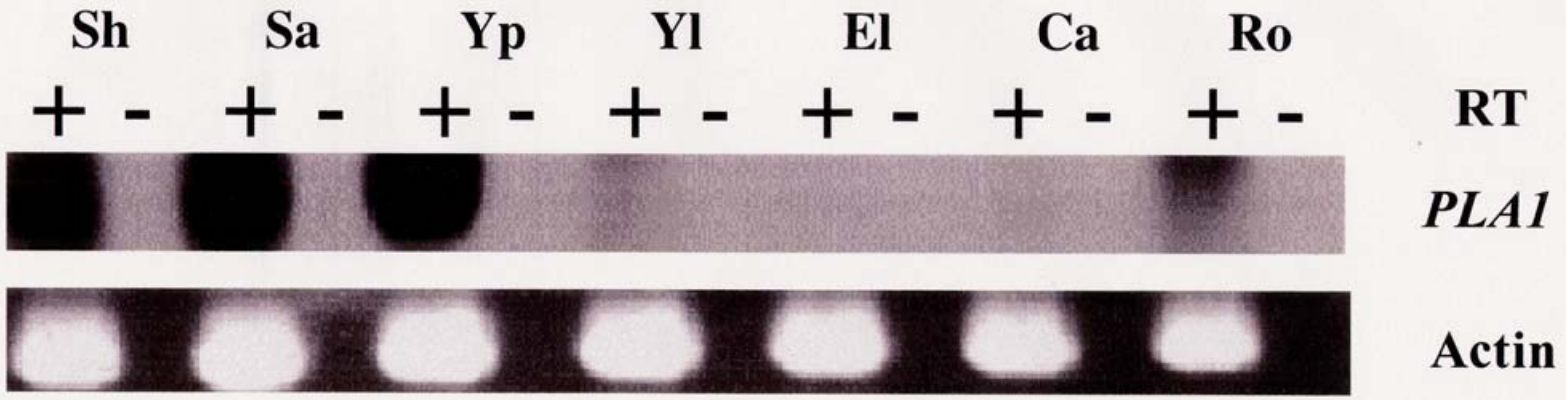

Fig. 20. Tisssue specific expression of *PLA1*. Five μl of PCR products were applied on a gel. Southern hybridization analysis was carried out with ECL system to detect *PLA1* product. Actin bands of internal control was detected by ethidium bromide on a gel. Sh: shoot, Sa: Shoot apex, Yp: young panicle, Yl: young leaf, El: expanded leaf, Ca: calli, Ro: root.

Expression of the *PLA1* gene during vegetative phase

Successive rice leaves are initiated on the flanks of the vegetative SAM in a distichous pattern. The unit of time between initiation of successive leaves from the meristem is termed a plastochron (Sharman, 1942). As shown in Figs. 21 and 22 the preprimordial founder cell population within the meristem is termed plastochron 0 and the next youngest leaf primordia is termed P1 leaf. Then successive leaf primordiums are described P2, P3 and P4 leaves. A mature rice leaf consists of a blade separated from a sheath by a ligular region, which consists of a ligule that rises from the adaxial surface at the proximal part of the sheath, and a pair of auricle projects as horn-like tissues from both sides of the lamina joint. These basic parts of leaf are gradually specified in the course of development.

The expression of the *PLA1* gene was detected at differentiating leaf and leaf primodium but not at SAM (Figs. 21 and 22). The *PLA1* expression was first observed at the P0 leaf (Fig. 21). In the P1 leaf, *PLA1* expression is restricted to leaf margin, and abaxial side at the lower part of the primordia that will differentiate into the sheath (Fig. 21). In P2 leaf *PLA1* mRNA was localized at leaf margin and abaxial side at the sheath excluding the differentiating vascular tissue (Fig. 21). The abaxially localized expression was also observed in the P3 leaf (Fig. 21). In this leaf, the expression was not spread along whole abaxial side, but localized at the central region in the proximal portion of the sheath. In addition, the expression was observed at the lamina joint (Fig. 22). In the P4 leaf, *PLA1* expression was not observed (date not shown). These results indicate that the *PLA1* is specifically expressed at developing young leaf, but not in SAM during vegetative phase.

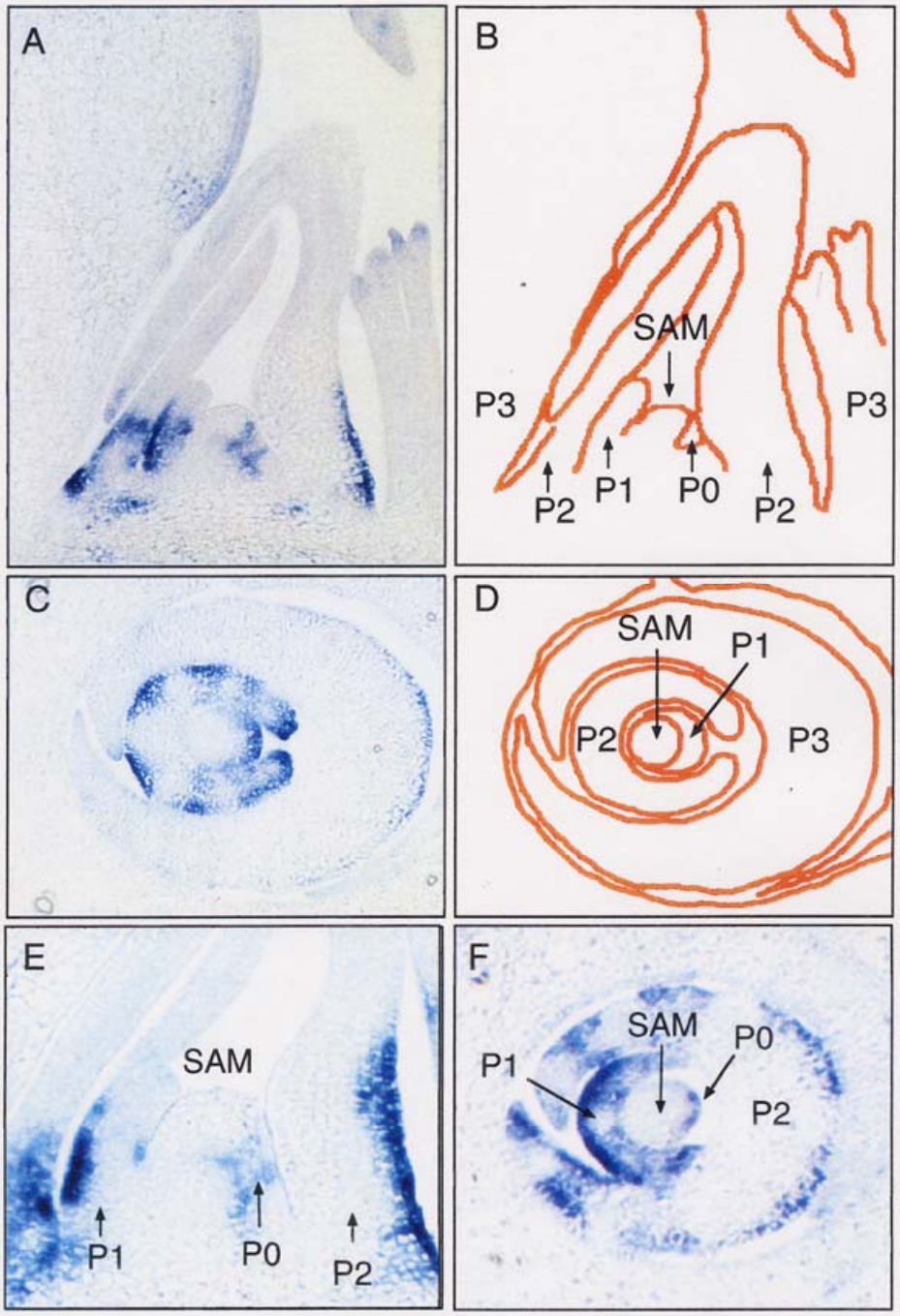

Fig. 21. *In situ* expression pattern of the *PLA1*gene in vegetative phase. A: Median longitudinal section of shoot apex at 1 month after germination. B: Schematic representation of shoot apex in A. C: Cross-section of shoot apex at 1 month after germination. D: Schematic representation of shoot apex in C. E: Median longitudinal section of shoot apex at slightly later stages than shoot in A. F: Cross-section of shoot apex at slightly later stages than shoot in C. SAM; Shoot apical meristem, P0; Incipient leaf (Plastochron 0), P1; Leaf primordia 1 (Plastochron 1), P2; Leaf primordium 2 (Plastochron 2), P3; Leaf primordium 3 (Plastochron 3).

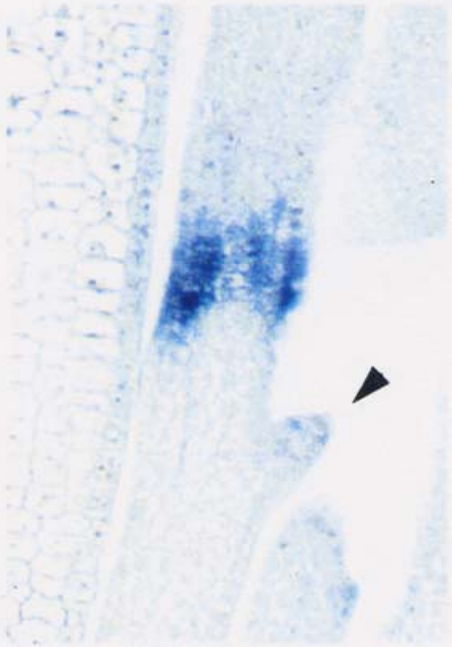

Fig. 22. *In situ* expression pattern of the *PLA1*gene in developing leaf. Longitudinal section of legular region of plastochron 3 leaf. Arrowhead indicates differentiating ligule. *PLA1* mRNA was detected at lammina-joint which is the most proximal region of leaf blade just above the blade-sheath boundary.

Expression of *PLA1* gene during reproductive phase

The leaf specific expression was also observed in reproductive phase. After the transition to reproductive growth, a young inflorescence meristem produces several bracts of the panicle in a helical pattern. Primary rachis branches grow out from the axil of each bract. Secondary branches are produced from the primary branches in a distichous pattern. These branches terminate in a single-flowered spikelet (Greyson, *et al.* 1994). In the inflorescence apices, *PLA1* expression was observed in developing bract leaves and their incipient primodium prior to their emergence, but not in SAM and primary rachis primordia (Fig. 23). Therefore, *PLA1* mRNA is localized at early stages of leaves in both vegetative and reproductive development. However, the expression pattern in bract leaf was different from those of vegetative leaves. Expression in bract leaves in inflorescence meristem showed uniform pattern without differences between top and base and between abaxial and adaxial sides. In contrast, the expression in vegetative leaf was more specified in abaxial cells. The change in expression pattern of vegetative leaf seemed to be rapidly progressed in early developmental stage. The abaxially localized expression was not observed even in well-elongated bract in which *PLA1* expression was detected in whole leaf. After the onset of secondary rachis differentiation, *PLA1* expression was diminished from the inflorescence apices (date not shown).

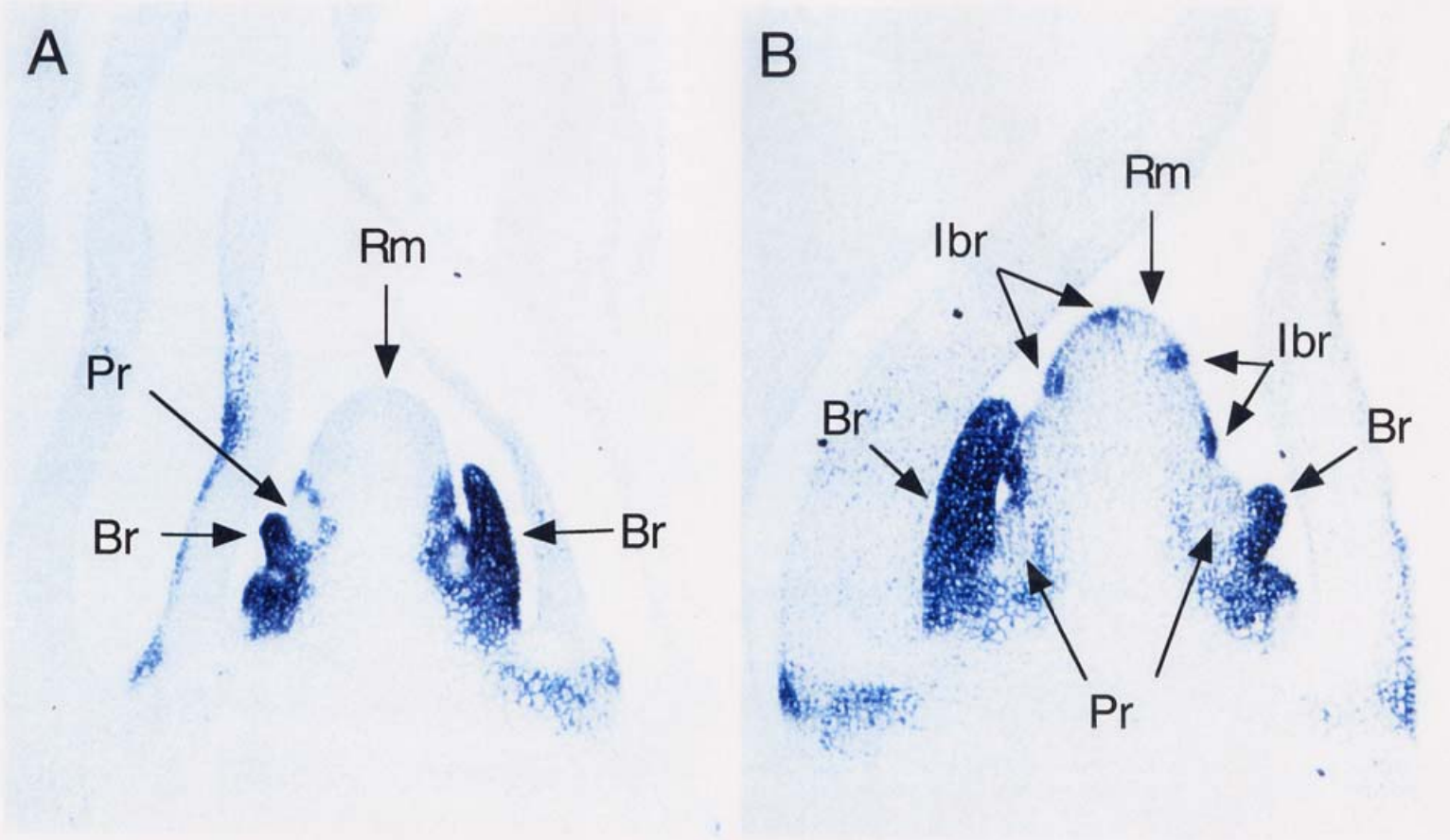

Fig. 23. *In situ* expression pattern of the *PLA1* gene in early reproductive phase. A: Longitudinal section of young panicle just after transition to reproductive phase. B: Longitudinal section of young panicle at slightly later stage than the panicle in A. Br: Bract, Ibr: Incipient bract, Rm: Rachis meristem, Pr: Primary branch primordium.

Discussion

Patterns in lateral organ initiation called phyllotaxy and plastochron are important determinants to define the shoot architecture. Many studies have focused on elucidating the mechanism that regulates leaf initiation pattern (reviewed in Steeves and Sussex, 1989; Callos and Medford, 1994). To date, two plausible models have been proposed, the inhibitory field model and the biophysical model (Snow and Snow, 1931; Wardlaw, 1949; Selker *et al.*, 1992; Green, 1994; Green *et al.*, 1996). Both models have been supported by several experiments through surgical or physiological approaches. Genetic and molecular approaches, however, are relatively few, mainly because of shortage of the mutants and the genes available. Accordingly, the regulatory mechanisms for phyllotaxy or plastochron is still unclear. Molecular cloning of the *PLA1* gene would advance the understanding of regulatory mechanism for plastochron.

Non cell-autonomous action of the *PLA1* gene

In situ hybridization analysis revealed several characteristics of *PLA1* gene expression. *PLA1* mRNA is expressed at leaf but not at SAM through vegetative and reproductive phase. The mRNA localization is gradually changed during leaf development. Although *pla1* mutants exhibit several defects in leaf and SAM, for example short and narrow shape of leaves and larger size of SAM compared to those of wild type plants, the specific expression in the restricted area of very young leaves suggests that the *PLA1* gene functions in non cell-autonomous manner but affects leaf development and SAM activity.

It is known that the vegetative to reproductive phase change is regulated by factors

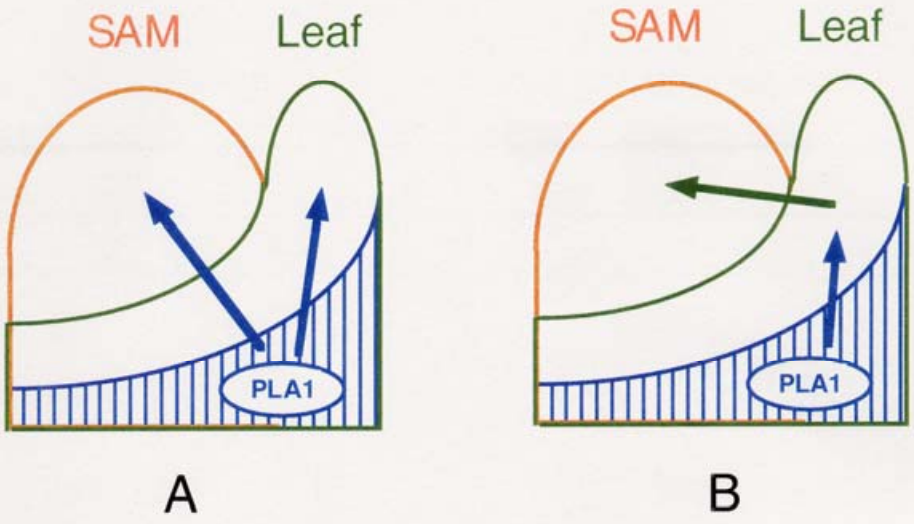

Fig. 24. Possible mechanism for non cell-autonomous action of the *PLA1* gene
(→) Primary signal, (→) Secondary signal ▭ *PLA1* expression site

Model A : *PLA1* protein and/or the target substrate is provided to SAM and leaf and then acts independently in each site. Model B : *PLA1* regulates leaf development. Developing leaf, in tern provides secondary signal that are required for SAM activity.

produced by leaves and translocated to the shoot apex (Bernier 1988; Colasanti *et al.* 1998). However, the source of the factors regulating vegetative program is almost unknown (Hackett and Murray, 1997). The fact that *PLA1* gene encodes the CYP78A11 protein indicates one possibility that *PLA1* produce some diffusible factor acting in leaf and SAM as presented in Fig. 24A, though target substrate awaits for identification. Alternatively, *PLA1* functions specifically in developing leaf, and in turn provides some signals required for normal SAM activity as shown in Fig. 24B. These two possibilities however, do not exclude each other. Both the pathways may act in concert for normal shoot development. Possible interpretations for functional role and mechanism of *PLA1* will be discussed further in the following sections.

How does *PLA1* regulate plastochron and leaf size?

The *pla1* mutants exhibit rapid emergence of vegetative leaves. The shoot apical meristems of *pla1* plants are always enlarged compared to wild type ones via activated cell divisions (Itoh *et al.* 1998). Genetic and molecular studies of several genes associated with the SAM indicated that the number and initiation pattern of lateral organs are closely associated with the size of the SAM (Sinha *et al.*, 1993; Barton and Poethig, 1993; Long *et al.*, 1996; Souer *et al.*, 1996; Clark *et al.*, 1993;1997; Laux *et al.*, 1996; Mayer *et al.*, 1998; Moussian *et al.*, 1998; Lynn *et al.*, 1999; Callos *et al.*, 1994; Jackson and Hake, 1999). Thus, simple explanation for *pla1* is that the increase of leaf initiation rate is caused by the activated cell divisions in SAM. However, a dominant negative mutation of the cell cycle Cdc2 kinase showing reduced cell division rate did not affect the rate of leaf

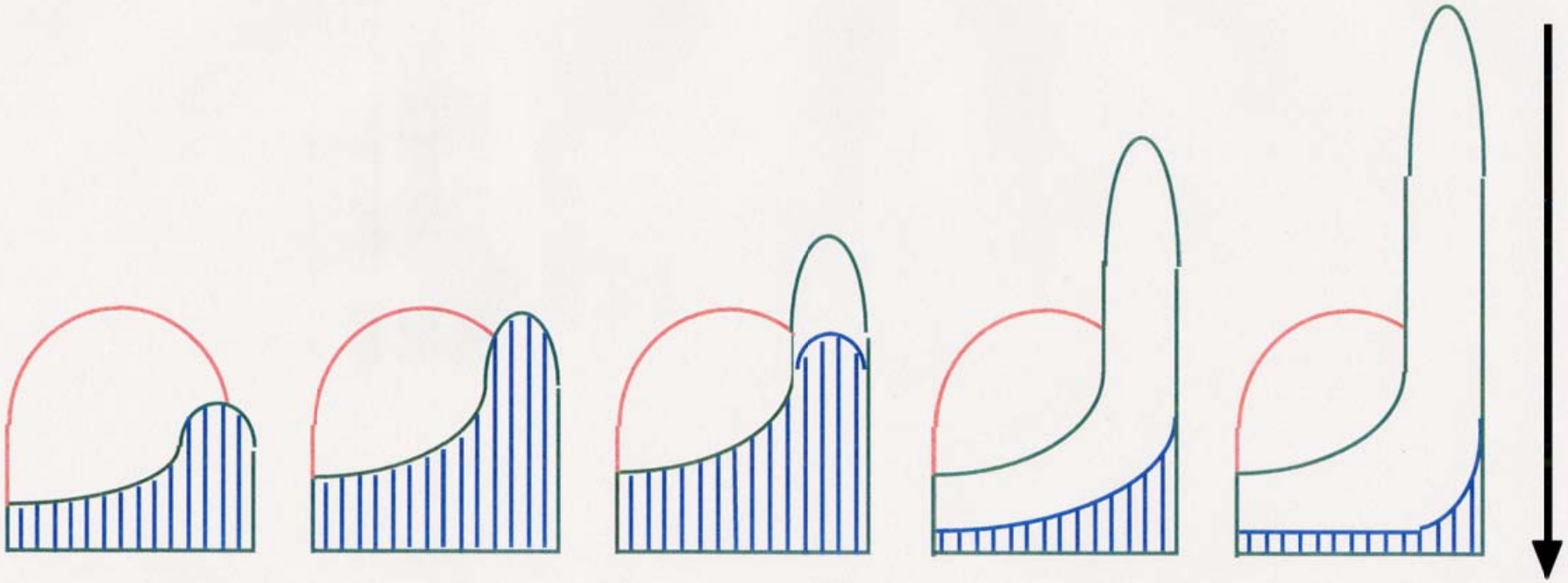

Fig. 25 Schematic representation of *PLA1* expression in developing leaf. *PLA1* expression is basipetaly down regulated during leaf development. SAM Leaf *PLA1* expression site.

production (Hemerly *et al.*, 1995). The *perianthia* (*pan*) mutation exhibits aberrant patterning of floral organs without affecting the size and shape of the meristem (Running and Meyerowitz, 1996). Therefore, the cell division rate in the SAM or the size of SAM should not be a sole determinant for lateral organ patterning. In addition, rapid leaf initiation in *pla1* is always accompanied by the reduction of leaf size. Multiple effects on SAM and leaf of *pla1* together with leaf specific regional and temporal expression of *PLA1* gene indicate that leaf initiation pattern might be controlled through communication between leaf and SAM.

The expression pattern suggests the possible role of *PLA1* gene in leaf development. In developing leaf, *PLA1* expression was down regulated in a basipetal (top to bottom) manner (Fig. 25). The changing pattern of *PLA1* expression shows some correlation to developmental pattern of leaf in monocot plants (Sharman, 1942, Kaufman, 1959, Kemp, 1980, Davidson and Milthorpe, 1966). In monocot developing leaf, growth and maturation are progressed in a basipetal manner; i.e. in developing leaf, basal portion of leaf is younger than extended apical portion (Fig. 26). The *PLA1* expression seemed to be restricted to leaf cells at immature state rather than at mature state. This expression pattern suggests the possibility that *PLA1* functions to maintain leaf cells at immature state. In this case, loss-of-function of *PLA1* gene would accelerate leaf maturation process, and might cause early cessation of leaf growth. Thus, the size reduction of *pla1* leaves may be the consequence of the precocious cessation of leaf growth caused by accelerated maturation in the absence of *PLA1* product (Fig. 27). Furthermore, the increase of leaf initiation rate in *pla1* mutants can be explained by 'accelerated maturation of leaf' in

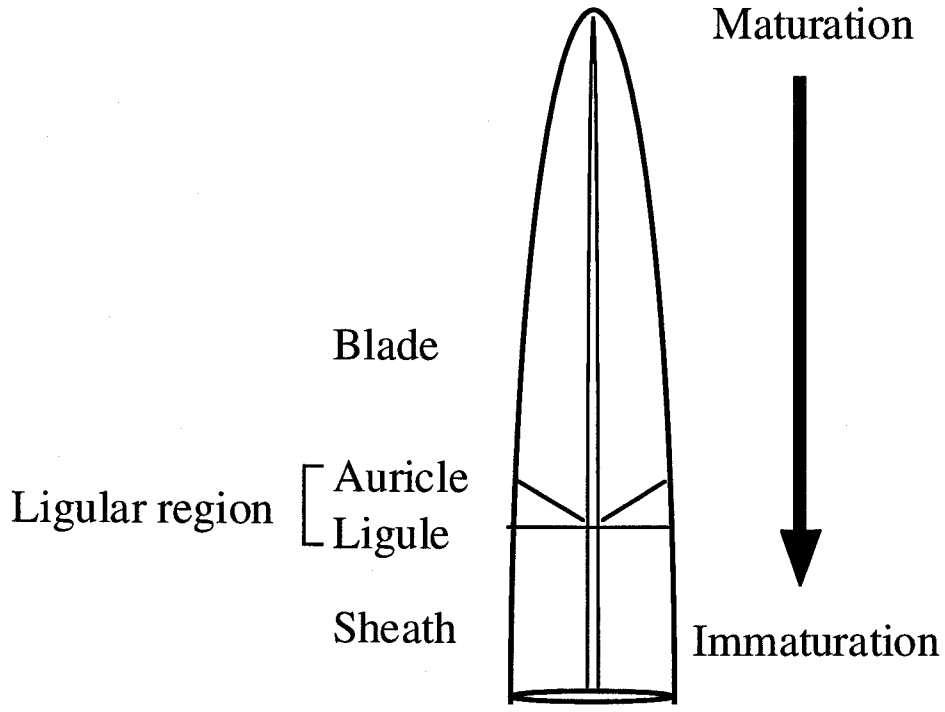

Fig. 26. Schematic representation of leaf development in rice. A mature rice leaf consists of a blade separated from a sheath by a ligular region These basic parts of leaf are gradually specified in a basipetal manner. Basipetally maturation is a common feaure in monocot leaf development

accordance with the inhibitory field model (Snow and Snow, 1931; Wardlaw, 1949). In this model, leaf-generating inhibitor is interpreted as acts in leaf age dependent manner, i.e. inhibitory effect raised from leaf primordium decrease gradually as leaf develops. The 'accelerated maturation of leaf' could also hasten the reduction of inhibitory effect, leading to early initiation of successive leaf (Fig. 27). Accordingly, alteration of leaf maturation schedule seemed to be sufficient to cause the reduction of leaf size as well as the increment of leaf initiation rate in *pla1* mutants. Detailed analysis of developmental schedule of *pla1* leaf would clarify this possibility.

How does *PLA1* regulate the duration of vegetative phase?

The *pla1* mutation causes prolonged expression of vegetative program without affecting the initiation of reproductive phase. This results in ectopic shoot formation on primary branch primordium, indicating that meristem identity of the primary rachis primordium is affected. Nevertheless, *PLA1* was not expressed at rachis primordium but only expressed at developing bracts. This suggests the *PLA1* regulates meristem identity as well as the leaf initiation rate in non cell-autonomous manner.

Leaf is considered as possible source of developmental signals that regulate phase change and meristem identity. Irish and co-workers studied the effects of physical separation of the SAM from the leaves (Irish and Karlen, 1998; Irish and Nelson, 1991). Maize plants from a particular inbred line form a fixed number of leaves and then develop flower. By surgically removing and re-rooting the SAMs of growing plants, they showed that the cells of the apex were not committed to produce a particular number of leaves:

SAMs that were excised from preexisting leaves produced more leaves before flowering than SAMs left connected to leaves. Based on the results, they suggested that shoot maturation (progression of developmental phase) depends on factors produced by preexisting leaves. They also suggested an alternative interpretation that leaves stabilize the developmental state of the SAM. Molecular and genetic studies of maize *id1* plant support these interpretations (Colasanti, *et al.* 1998). The *id1* mutant exhibits late flowering as well as abnormal tassel and ear wherein vegetative and reproductive characters are co-expressed. The *ID1* gene is expressed at immature leaf but not at SAM. Thus, *ID1* is considered to function to generate 'leave-derived signal(s)' that transmitted to SAM for promotion and maintenance of the reproductive program.

Just after transition to the reproductive phase, rice leaves became severely suppressed to emerge although the primordia were formed. In the *pla1* mutants, however, leaves do not cease to grow out even after the entry to reproductive phase. Thus, the SAM at reproductive phase in *pla1* mutant is surrounded by leaves, which is much larger than that of wild type siblings. If the rice leaves as well as maize act as source of signals that regulate the developmental state of the SAM, the size of leaves would affect the amount or intensity of the signals and the enormous bract leaves of *pla1* mutant might produce the excess amount of signals. The ectopic shoot formation on the panicle in *pla1* therefore may be the consequence derived from enhanced signals that maintain or stabilize the vegetative program (Fig. 28).

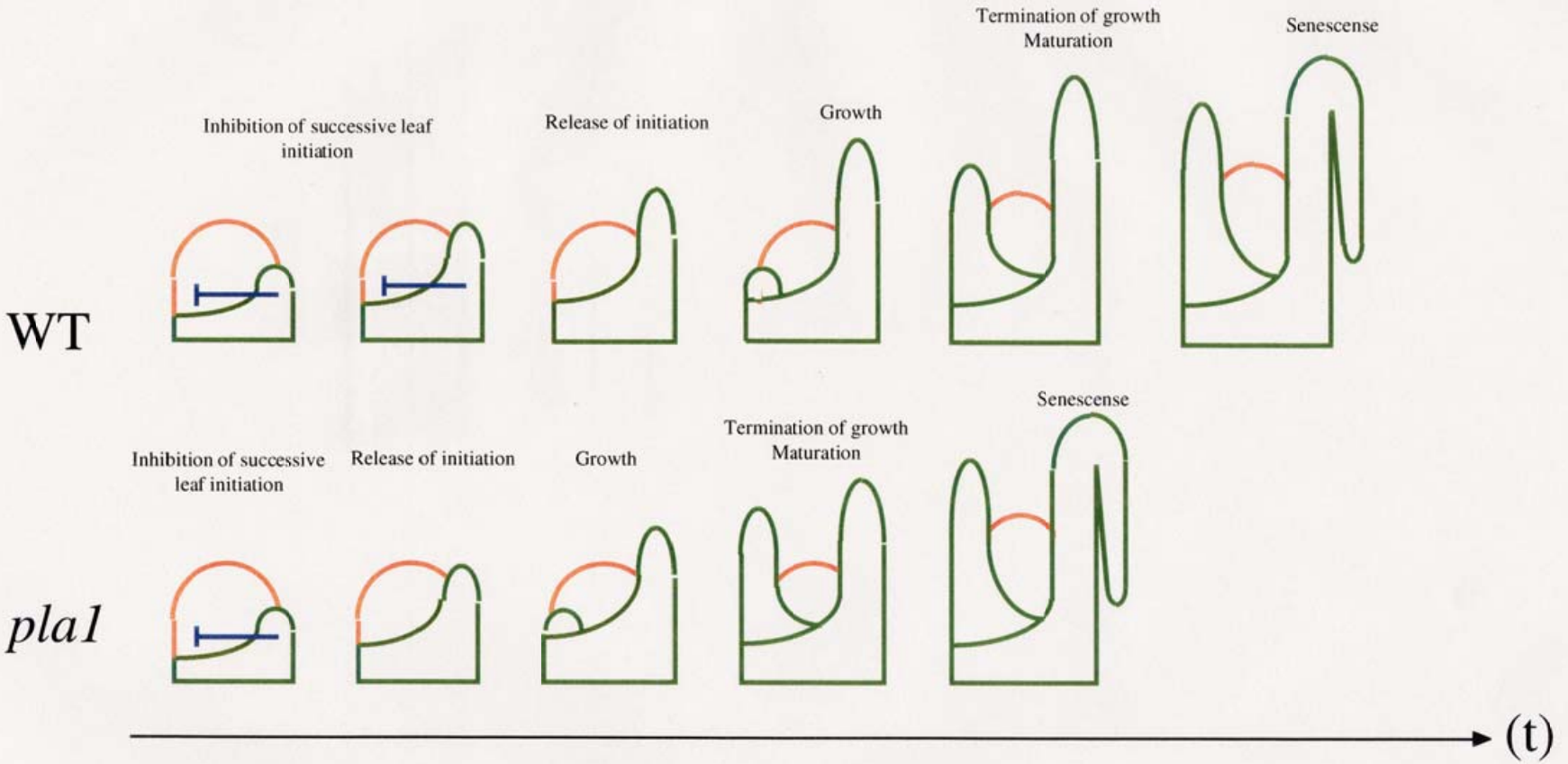

Fig. 27. A possible mechanism for vegetative phenotype of *pla1* mutant. 'Accelaretad maturation of leave' could affect both the size andthe initiation rate of leaves. Precocious reduction of inhibitory effect raised from preexisting leaf (blue bars) premits early initiation of sucessive leaf. Early cessation of leaf growth leads to size reduction of leaves.

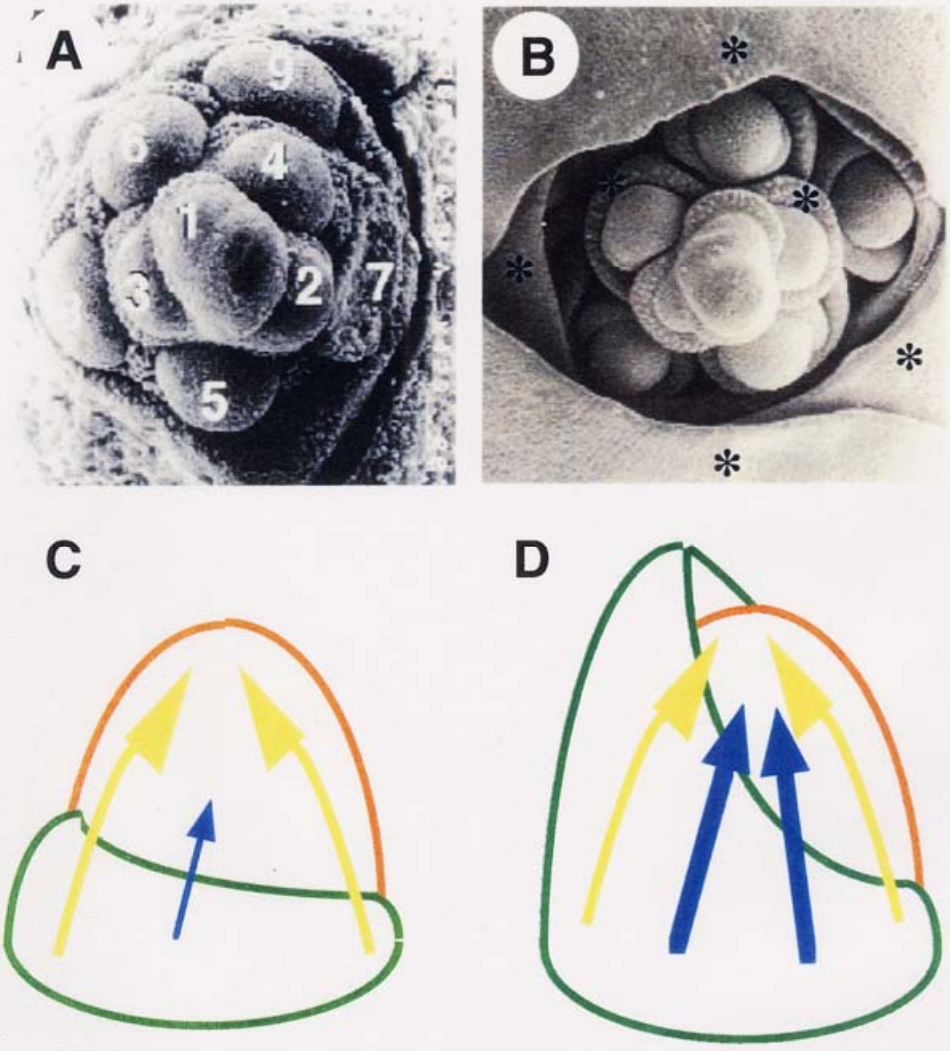

Fig 28. A possible mechanism for reproductive phenotype of *pla1* mutant. A: Wild-type primary rachis primordia. B: *pla1* primary rachis primordia. Note the abnormaly elongated bract leaves (indicated by asterisks) C: Schematic representation of single primary rachis primordia of wild-type. D: Schematic representation of single primary rachis primordia of *pla1*. Bract leaves produce signals that stabilize vegetative program (blue arrows in C and D). (C) In wild-type plants, the stabilizing signal was overcome by another signals that prpmote reproductive development (yellow arrows). (D) In *pla1* mutant, abnormaly elongating bract generates the excess amount of stabilizing signal (blue arrows) resulting prolonged expression of vegetative program.

Summary

The rice PLASTOCHRON1 locus is considered to regulate the leaf initiation rate (plastochron) and the duration of vegetative phase without affecting reproductive phase (Itoh *et al.*, 1998). To elucidate regulatory mechanism for the plastochron and the duration of vegetative phase, I have isolated the *PLASTOCHRON1* gene by map-based cloning and investigated the *in situ* expression pattern during rice development. Molecular cloning of *PLA1* gene gives a clue to understand the mechanism for the temporal regulation of developmental program in rice.

A genetic and physical map was constructed to isolate the causal gene for *pla1* syndrome. Small-scale mapping was carried out to determine approximate map position of the *pla1* locus and then a high-resolution genetic mapping was performed for *pla1-2*, one of the *pla1* alleles, using an F_2 population comprising 578 *pla1-2* homozygous plants. In a high-resolution genetic map, the *pla1* locus was found to be mapped between RFLP markers C961 and R1738A on chromosome 10, within a 3.6 cM genetic distance.

A physical map encompassing the *pla1* 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 (167Kb), contained the *pla1* locus within 74Kb corresponding to a 0.52cM genetic distance. Gene prediction of 74Kb region carrying the *pla1* locus was performed by using gene prediction program and designated several candidate genes for *pla1* gene including cytochrome P450, putative GTPase regulator protein and transposable like element.

Nucleotide comparison analysis between four alleles of *pla1* mutants, *pla1-1, pla1-2, pla1-3* and *pla1-4*, and wild type revealed that all alleles of *pla1* mutant had mutations only in P450 gene among several candidate genes. Genetic complementation experiment confirmed that all transgenic plants transformed with binary vector pBGH1-*PLA1* (6Kb), which contain P450 gene, to *pla1-2* homozygous plants completely recovered to wild type phenotype, in contrast to no recovery by transformation with pBGH1 vector only.

The *PLA1* gene of P450 was designated as CYP78A11 in accordance with the guidelines for Cytochrome P450 by the nomenclature committee. This is the first protein belong to the CYP78A subfamily in plants. The predicted CYP78A11 was 1.8kb in size and consists of two exons and one short intron. The CYP78A11 codes 555 amino acid with a calculated molecular mass of 59KD. Similarity search showed that the PLA1 protein conserved significant sequences such as heme binding domain near C-terminus, potential oxygen binding domain and steroid binding domain, which were conserved in all cytochrome P450 superfamily.

In situ hybridization experiments elucidated the spatial and temporal expression pattern of the *PLA1* gene. *PLA1* mRNA was localized in leaves but not at SAM through vegetative and reproductive phase. The mRNA localization was gradually changed during leaf development. Although *pla1* mutants exhibited several defect in leaf and SAM, the leaf specific expression suggested that *PLA1* gene functions in non cell-autonomous manner and affects leaf development and SAM activity. The decay pattern of *PLA1* expression in leaves shows a correlation to the pattern of leaf development in monocotyledous plants, suggesting the possible role of *PLA1* gene for regulating maturation schedule of leaf.

In the future works, transgenic plants overexpressing the CYP78A11 gene may provide an additional clue to elucidate the functional role of the *PLA1*. The P450s, however, form the largest family of plant enzymes and have wide diversity of substrates and high degree of amino acid variation in the superfamily. Though the CYP78A11 seems to be functional in a given step of plant hormone biosynthesis or degradation pathway, analysis of changes of limited amount of plant hormones in the restricted regions would be very difficult. Even taking a time, biochemical analysis of CYP78A11 including a search of the substrates will give a clue to unravel the molecular mechanism of the phase change in plant growth and development controlled by *PLA1*.

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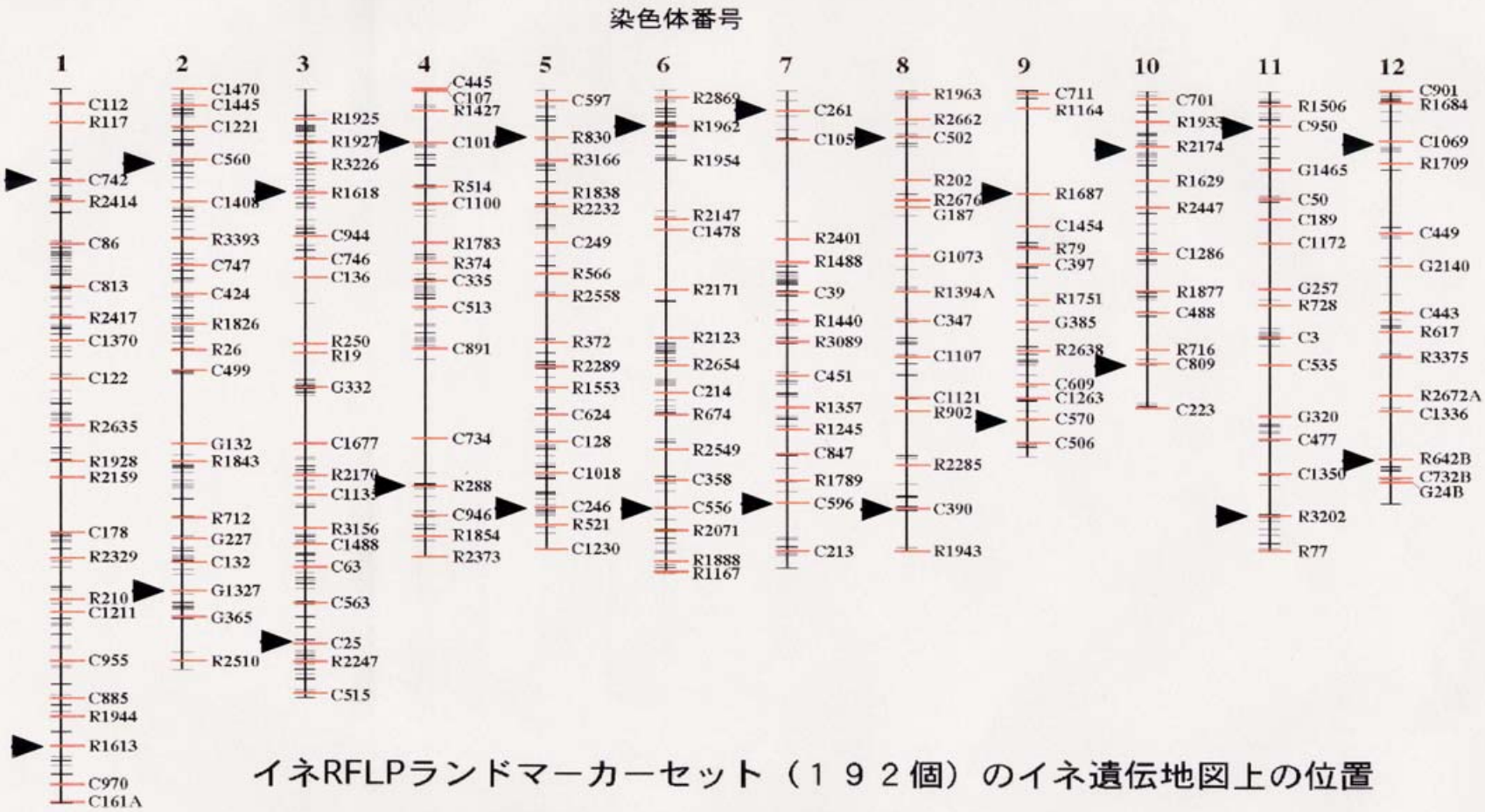


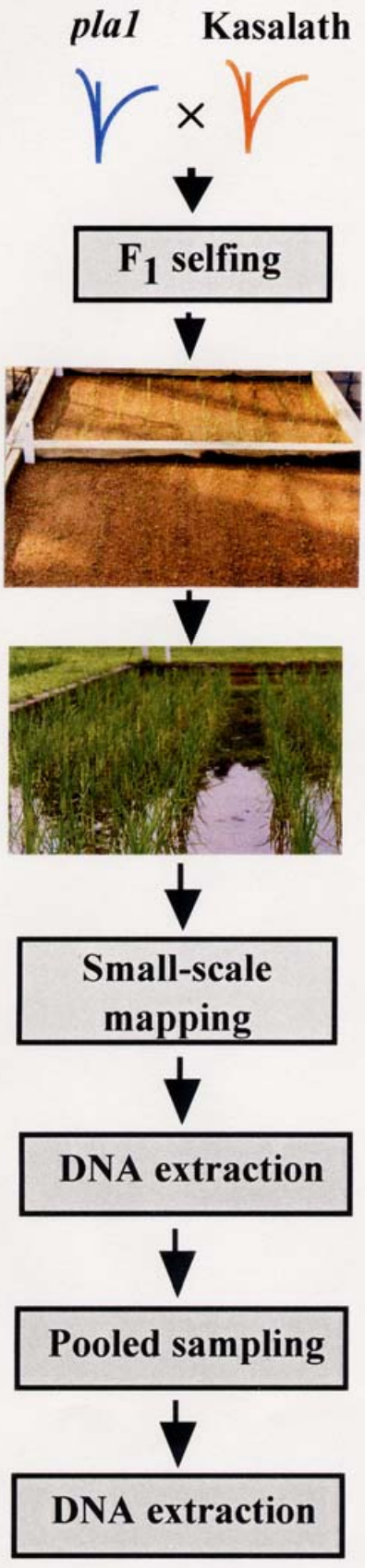
イネRFLPランドマーカーセット（１９２個）のイネ遺伝地図上の位置

各RFLPマーカーのイネ日本晴（日本型イネ）／カサラス（インド型イネ）間におけるサザンハイブリダイゼーション（多型の写真）結果については、URL http://www.dna.affrc.go.jp:84/index.htmlを御覧下さい。

Appendix. 1. RFLP land markers used for small-scale mapping in this study.
Arrowheads indicate markers used for small-scale mapping of *pla1* locus.

Appendix 2. DNA extraction method

Two grams of rice leaves was grounded until to be very fine podwer in liquid nitrogen with hiscotron homogenizer. The powder was transferred into a 15ml polypropylene centrifuge tube. At once, 4ml of extraction buffer (1.5×CTAB, heated to 56℃) was added to it and mixed well. After incubated at 56℃for 20 minutes, 4ml CIAA (Chloroform and Isoamyl alcohol ; 24 : 1) was added to it and was rotated slowly for 20 minutes at room temperature. The sample was centrifuged at 3000 rpm for 20 minutes at room temperature. The supernatant solution was transferred into a new 15ml polypropylene centrifuge tube and mixed well with 400 ul of 10% CTAB (heated to 56℃). The sample was added 4ml of CIAA and was slowly rotated for 20 minutes at room temperature. And then the centrifuge was done with for 20 minutes at room temperatures and the supernatant solution was transferred new 15ml centrifuge tube. An equal of precipitation solution (room temperature) was added to it and mixed gentry. The mixed sample was centrifuged for 10 minutes at 3000rpm and the solution was discard carefully. The precipitation(CTAB/nucleic acid complex) was completely resolved in 1ml of 1M sodium chloride and was incubated for over 2 hours at 56℃ with 1ul of RNase (10mg/ul). Two volumes of 100% EtOH was added and mixed by inversion gentry. The sample was centrifuged for 20 minutes at 3000rpm and the solution was discared carefully. The pellet was washed two times with 70% EtOH for 20 minutes at 3000 rpm. The pellet was dried briefly and desolved in 100 – 200 ul of TE buffer.



(1) Generation of mapping population

(2) 3,300 of F2 seeds sown in seed bed.

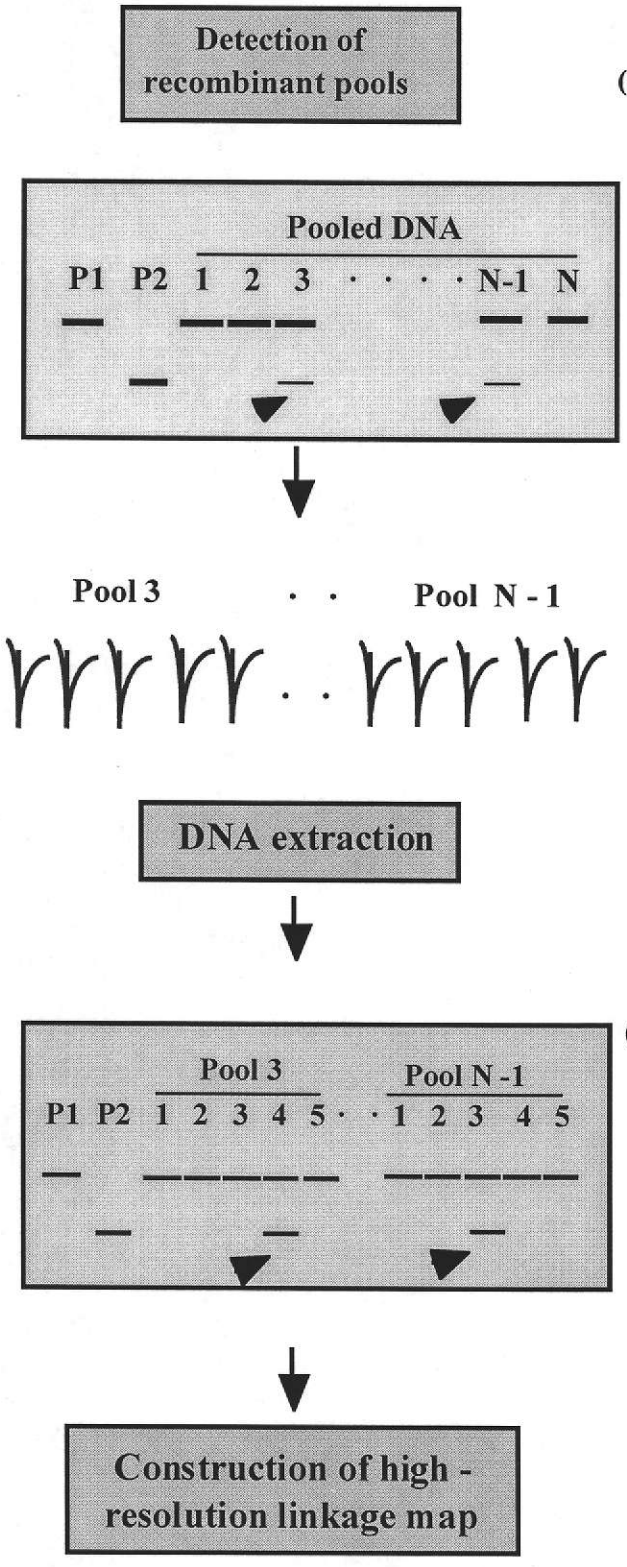
(3) 578 *pla1* homozygous plants were selected at the seedling stage and transplanted into a paddy field

(4) To determine approximately map position of *pla1*, 30 plants out of F2 population were selected and linkage analysis was done with RFLP markers

(5) Five plant leaves were bulked for one pool.

(6) Genomic DNA of N pools were extracted for linkage analysis

Appendix 3-1. Scheme of high-resolution linkage mapping



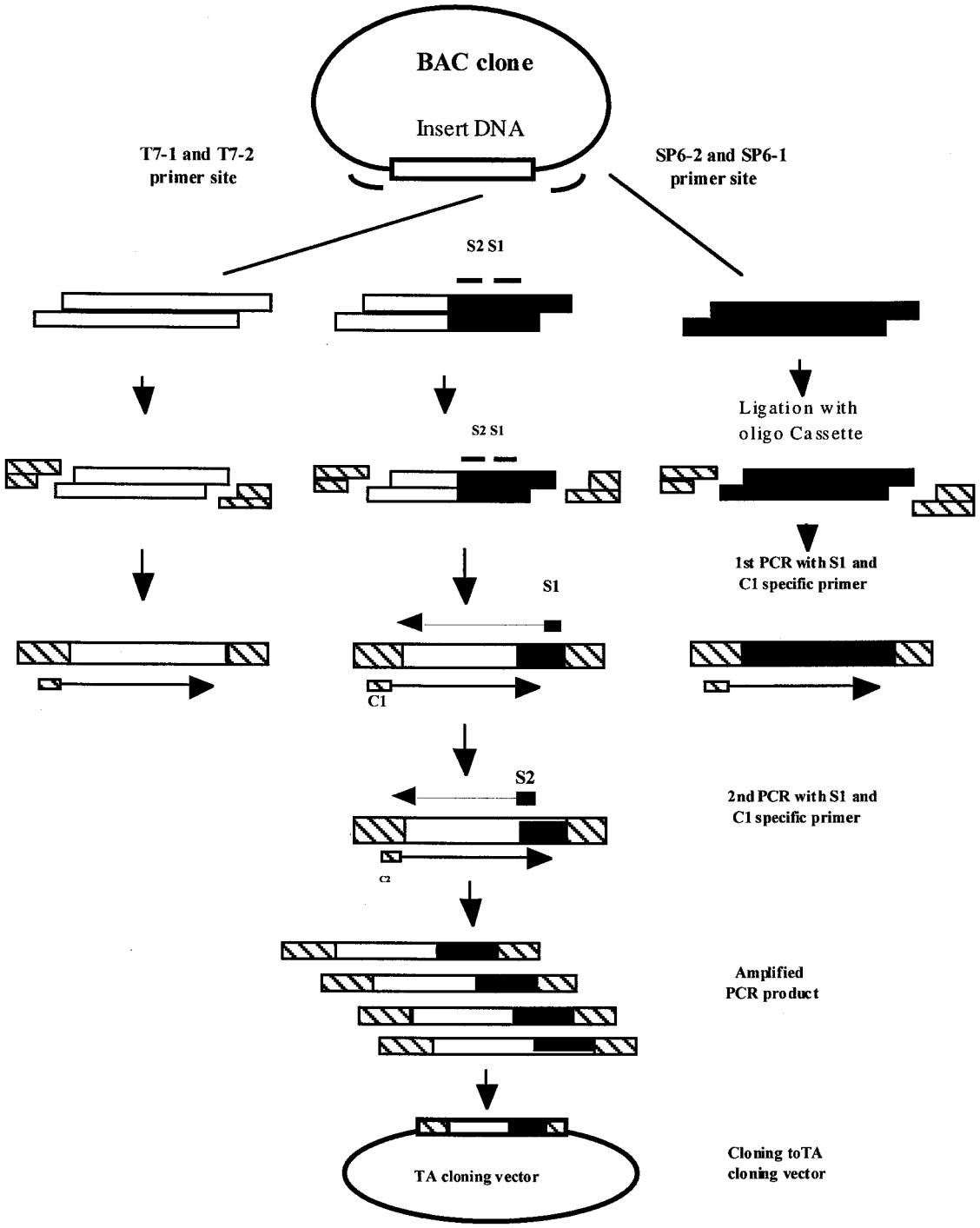
(7) Recombinant pools were detected with RFLP markers by Southern hybridization.

Arrow head indicate recombinant pools

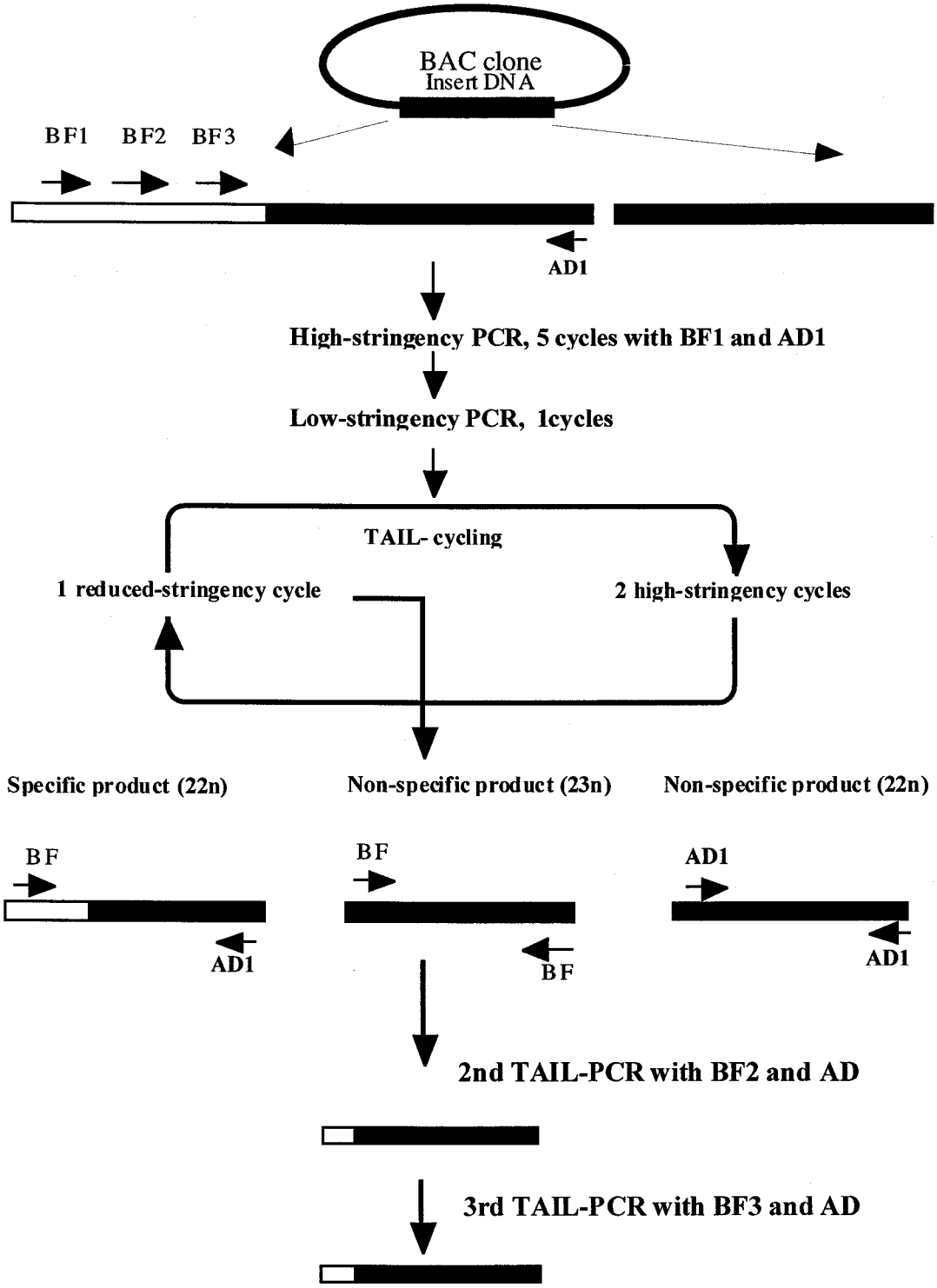
(8) Individual plants from recombinan pools were selected.

(9) To constructive high resolution linkage map of *pla1*, genomic DNA of all individual plants from recombinant pools were extracted and linkage analysis were done with RFLP markers around *pla1* locus using Southern hybridization.

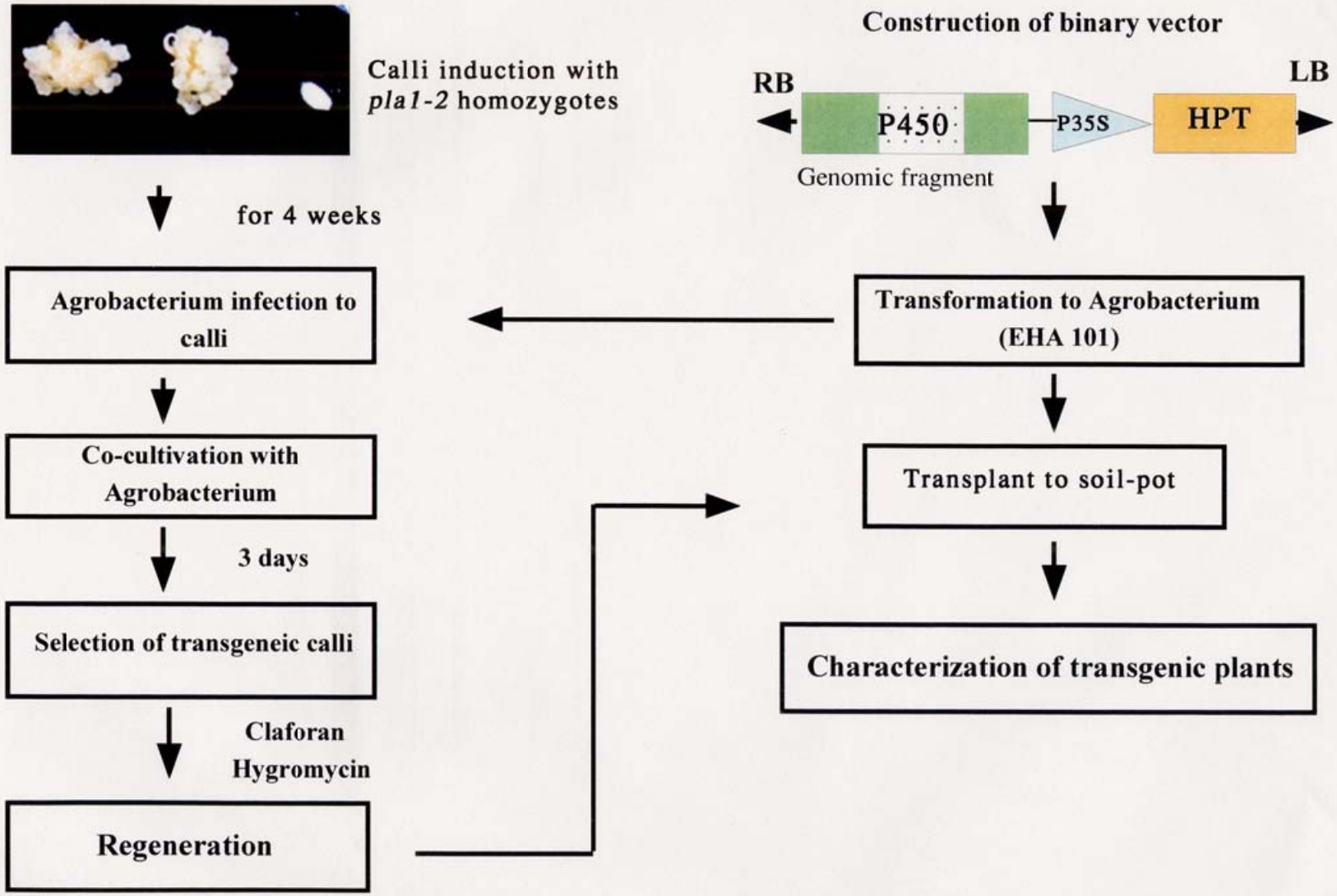
Appendix 3-2 Scheme of high-resolution linkage mapping



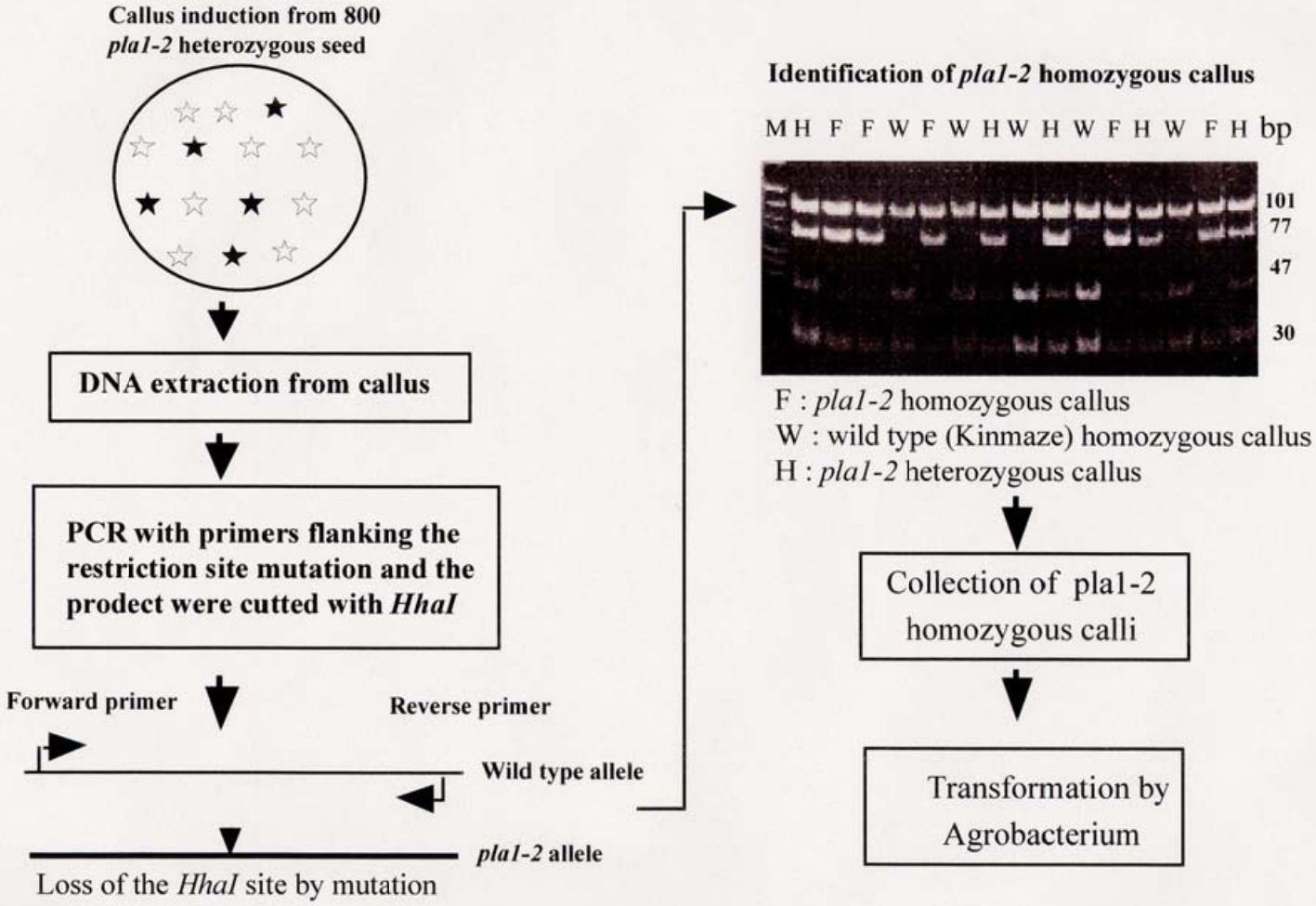
Appendix 4-1. Scheme for cloning generation of BAC end DNA fragment by Cassette PCR. C1 and C2 are specific primers to oligo Cassette. S1 (SP6-1) and S2 (SP6-2) are specific primers to BAC vector. T7-1 and T7-2 are also vector specific primers to be used in cloning of another side end clones.



Appendix 4-2. Scheme for generation of BAC end DNA fragment by TAIL PCR. BF1, BF2 and BF3 are long and specific primers to BAC vector. AD1 is a degennerate primer.



Appendix 5. Scheme of complementation test



Appendix 6. Scheme of selection of *pla1-2* homozygous calli from heterozygous seeds for utilization in the complementationtest. The *pla1-2* homozygous abolished the *HhaI* restriction site in the CYP78A11 gene. The genome DNA at around the mutation site were amplified by PCR. The amplified PCR fragments showed polymorphism between *pla1-2* and wild type after digestion with *HhaI* and *PstI*. *PstI* was used to clarify the polymorphism.

Appendix 7. Primer sequences used in this studies

No	Primer name	Sequence	Note
1	BF1	GAC GTT GTA AAA CGA CGG CCA GT	TAIL PCR
2	BF2	GTA ATA CGA CTC ACT ATA GGG CGA	TAIL PCR
3	BF3	GAG TCG ACC TGC AGG CAT GCA	TAIL PCR
4	BR1	CTT CCG GCT CGT ATG TTG TGT GG	TAIL PCR
5	BR2	GAG CGG ATA ACA ATT TCA CAC AGG	TAIL PCR
6	BR3	TAG GTG ACA CTA TAG AAT ACT CA	TAIL PCR
7	AD1	NGT CGA (G/C)(A/T)G ANA (A/T)GA A	TAIL PCR
8	AD2	GTN CGA (G/C)(A/T)C ANA (A/T)GT T	TAIL PCR
9	AD3	(A/T)GT GNA G(A/T)A NCA NAG A	TAIL PCR
10	SP6-1	GGA AAC AGC TAT GAC CAT G	Cassette-PCR
11	SP6-2	GCT ATG ACC ATG ATT ACG CCA	Cassette-PCR
12	T7-1	GTA ATA CGA CTC ACT ATA GGG	Cassette-PCR
13	T7-2	AGG GCG AAT TCG AGC TCG GTA	Cassette-PCR
14	C1	GTACA TATTG TCGTT AGAAC GCGTA - ATACG ACTCA CTATA GGGAG	Cassette-PCR
15	C2	CTCCC TATAG TGAGT CGTAT TACGC - GTTCT AACGA CAATA TGTAC	Cassette-PCR
16	SSR-3F	GCT TAC TCA CCG ATG AGT CAG	SSR marker
17	SSR-3R	AAG TCC TGT AGTAGG TCA CAC	
18	B44A10-101Kb-F	CCT CAA CAA GCT CCT GAA GC	RFLP marker
19	B44A10-101Kb-R	GTC CAA GAT CCA GTC CAC CA	
20	B44A10-89Kb-F	CAG TGA CCA GTG GTG ATG CA	RFLP marker
21	B44A10-89Kb-F	TAG GCT AGG GAC ACT CGT TG	
22	B44A10-142Kb-F	AGG GTC TGA AAG TCC CAG AG	RFLP marker
23	B44A10-142Kb-R	TCC TTC ACC ATC CT TCC GA	
24	B44A10-131Kb-F	CCG GTC ATT GTG TCA AGC TAC C	RFLP marker
25	B44A10-131Kb-R	ATC CTT AGC AGC GCC ACT CT	
26	B44A10-18Kb-F	AAC TGG CAT CTG GGC AAT GG	RFLP marker
27	B44A10-18Kb-R	AGG GTG GTG AGC TCT CCT AT	
28	B44A10-16Kb-F	GTA CGC ATT GTT GGG TCT GC	RFLP marker

29	B44A10-16Kb-R	CTG CTA GCT GGG AAC TGA GT	
30	B44A10-2Kb-F	CTA TCG ATC TCC CTG TTC AC	RFLP marker
31	B44A10-2Kb-R	TAT GAC GCC TGC TTG GAT AG	