Characterization, Inheritance, and Molecular Study of Opaque Leaf Mutant in Mungbean

INTRODUCTION

Mungbean (*Vigna radiata* (L.) Wilczek), a diploid (2n=22), is a widely cultivated and economically important pulse crop in Thailand and many tropical countries. The production of mungbaen is affected by many factors such as low genetic potential of the current varieties, environmental stress, disease and insect pests, and poor cultural practices (Poehlman, 1991; Chaitieng *et al*., 2002), causing the world average yield of mungbean is less than 500 kg/ha (Shanmugasundaram and Kim, 1996). Although a large number of high yielding cultivars have been release but the average yield of mungbean remains low, due to their low yielding potential and susceptibility to diseases (Lakhanpaul *et al*., 2000).

Generally, plant improvement is based on creating variation, selection, evaluation and multiplication of the desired genotypes (Ahloowalia and Maluszynski, 2001). Deficiency in a particular agronomic trait can be induced and corrected in a cultivar by using chemical or physical mutagens (Maluszynski *et al*., 1995). The mutagenic agents used most frequently have been gamma radiation, but X-rays, neutrons and chemical mutagens have also been used (Shanmugasudaram and Kim, 1996). Mutation techniques offer to the plant breeder and geneticist for improvement of specific traits in crop plants. Many mutants can be used directly as new cultivars, or as parents in crosses in a breeding program. Moreover, the mutants can also be used for developing molecular maps, understanding of gene expression and plant development, and studying heterosis and gene tagging (Maluszynski *et al*., 1995).

A large number of mutant germplasm collections have been established and used for genetic and plant breeding. Srinives *et al*. (2000) induced mutant lines by treating F_2 seeds derived from a cross between *V. radiata* x *V. radiata* (wild), *V. mungo* x *V. mungo* (wild), and their reciprocal crosses with gamma rays irradiation.

Several mutant lines were obtained including opaque leaf, dark green leaf, multiple leaflets, lobed leaf, large pod, top podding, and semi-dwarf plants.

The opaque leaf line was used in this study to investigate its properties and its gene that cause the opaque leaf appearance.

The objectives of this study are:

1. To determine the chlorophyll content and chlorophyll fluorescence of opaque leaf and normal leaf traits.

2. To determine the pattern of seed growth and development of opaque leaf and normal leaf traits.

3. To characterize the seed cell morphology of wrinkled seed of opaque leaf plant.

4. To localize the gene controlling the opaque leaf trait by molecular markers.

LITERATURE REVIEW

Chlorophyll mutation

Alterations in pigmentation, especially the chlorophyll pigments, are most common induced mutations found in crop plants (Nelson, 1967). Deficiency in chlorophyll content can lead to abnormal leaf coloration (Nair and Tomar, 2001). Ronnenkamp *et al*. (1975) classified leaf chlorophyll mutant of sweetclover (*Melilotus alba*) into yellow-green, light green, and dark-green (normal) and found that all of them are chlorophyll deficient mutants. In mungbean, several chlorophyll mutations can be classified into albina, xantha, viridis, maculate, chlorina, tigrina, lutercent, striata and virescent (Dahiya, 1973; Subramanian, 1980; Bahl and Gupta, 1982; Gupta, 1996). Recently, Sangsiri *et al*. (2005) reported chlorophyll mutations in mungbean found from their study as albina, coppery leaf, light-green leaf, variegated leaf, waxy leaf, white streak leaf, and xantha leaf.

Many chlorophyll deficient mutants in different crops have been studied, and most cases they behaved as single recessive traits (Wettstein, 1961). Hermsen *et al*. (1973) reported that light green mutant of potato (*Solanum tuberosum*) was controlled by monogenic recessive gene. Similarly, variegated, multifoliata, xantha, chlorina, albino, and unifoliata mutants of mungbean were also controlled by recessive gene (Bahl and Gupta, 1982). In wheat, each chlorina mutants conditioned by a single recessive nuclear gene and these genes appeared to segregate independently. These mutants show reduced vigor but produced enough seed for propagating in the future use (Williams *et al*., 1985). Similar observation have been made by Ronnenkamp *et al*. (1975) as they found that chlorophyll deficient mutants of sweetclover behaved as monogenic recessive and each mutant lines was sufficiently vigorous to grow in the field and the seed can be maintained and used for further investigations of the photosynthesis. However, most of chlorophyll-deficient mutants are lethal or grow very slowly, a few are able to photosynthesize and grow normally (Keck *et al*., 1970). Saunders (1960) reported that chlorophyll-deficiency of cowpea (*Vigna sinensis*) mutants are reduced in growth and productivity.

Plant characterization from chlorophyll

Chlorophyll a and b are essential pigments as they are responsible for the capture and conversion of light energy to store chemical energy for photosynthesis. The amount of light absorbed by a leaf is a function of the photosynthetic pigment content. Thus quantifying chlorophyll content can directly determine photosynthetic potential and primary production (Curran *et al*, 1990; Filella *et al*., 1995). Moreover it provides valuable information which can be used to determine the physiological status of plants and to discriminate plant species by monitoring phenological characteristics (Blackburn, 1998; Gitelson *et al*., 2003)

There are several characters to classify chlorophyll-deficient mutants such as chlorophyll a, chlorophyll b, total chlorophyll (a+b) contents, and chlorophyll a:b ratio (Kirchhoff *et al*., 1989). These pigments can be measure by various techniques such as spectrophotometry, fluormetry, and high performance liquid chromatography. Spectrophotometry is a classical method which can be utilized to determine quantitatively chlorophyll a, b, and total chlorophyll. The measurement of chlorophyll content by this method is using the organic solvent for extracting the chlorophyll. Moran (1982) developed an efficient method for extracting chlorophyll with *N,N*-dimethylformamide. The extract is then analyzed by spectrophotometric using the know optical wavelengths in the red region at the visible spectrum of 663 and 645 nm, where chlorophyll are first absorbed species and subsequently measured the concentration of chlorophyll using appropriate simultaneous equations (Markwell *et al*., 1995).

Plant characterization from chlorophyll fluorescence

During recent years, the measurement of chlorophyll fluorescence has been widely used to determine light absorption in leaves and to indicate photosynthetic potential of crop plants (Balachandran and Osmond, 1994; Rascher *et al*., 2000; Vogelmann and Evans, 2002). The principle underlying chlorophyll fluorescence is relatively straightforward. Light energy that is absorbed by the chlorophyll molecules

in the leaf can undergo following three fates; i) it can be used to drive photosynthesis (photochemistry) providing the chemical energy (in the form of ATP and NADPH) for CO_2 fixation in the Calvin cycle and photosystem II (PSII) extracts electrons from water releases oxygen, ii) the excess energy can be dissipated by a variety of non-photochemical processes (principally as heat) or, iii) it can be re-emitted as light-chlorophyll fluorescence (red fluorescence). These three processes occur in competition with each other, such that any increase in the efficiency of one process will be associated with a corresponding decrease of the other processes. Therefore, determining the yield of chlorophyll fluorescence can give information about changes in the efficiency of photochemistry and heat dissipation (Maxwell and Johnson, 2000).

The absorption of light energy by a chlorophyll molecule raises an electron from the ground state to an excited state. In photosynthetic system, these processes can be characterized into two groups; photochemical and non-photochemical processes. Photochemical process utilizes absorbed energy for photochemistry, the chemical energy involved in photosynthesis referred to as 'photochemical quenching'. However, excess excitation of energy can be converted into heat which is referred as 'non-photochemical quenching (NPQ)' (Hurry *et al*., 1997; Maxwell and Johnson, 2000). Although chlorophyll a and b are major photosynthetically active pigments in most higher plants, chlorophyll b molecule functions as an accessory pigment for harvesting light energy not playing a direct role in photochemical energy transduction. Thus, the measurement of chlorophyll fluorescence is made directly on chlorophyll a molecule (Markwell *et al*., 1985). More than half of chlorophyll a is associated with photosystem I (PSI) but about 95% of chlorophyll a fluorescence signal is derived from chlorophyll molecules associated with PSII. Therefore detection of the fluorescence signal reflected changes in efficiency is used for PSII photochemistry (Oxborough, 2004).

Chlorophyll a fluorescence has long been an important tool for estimation of a range of photosynthetic parameters in leaves (Baker *et al*., 2001). From these parameters, numerous aspects of photosynthesis process can be analyzed. The pulsed amplitude modulation (PAM) fluorometer is one of the instruments available for use

in measuring chlorophyll fluorescence as an indicator of primary productivity. The best known and probably best established miniaturized yield analyzers are PAM-2000 and the Mini-PAM (Bilger *et al*., 1995). The Mini-PAM is the photosynthetic yield analyzer which has been developed as fast and reliable assessment of the effective quantum yield of photochemical energy conversion in photosynthesis (Heinz, 1999). This analyzer generated a pulse of light and delivers to the leaf chlorophyll molecule via a fiber optic probe. This pulse of light initially induces a very low level of chlorophyll fluorescence. Since most of photons are being used to drive photochemistry, they are not re-released as fluorescence. However, as the pulse of light continues, the leaf became light saturated and none of the additional photons applied can be used photochemically. Instead, many photons are re-released from the leaf as chlorophyll fluorescence.

The ratio of variable fluorescence to maximum fluorescence or the maximum quantum efficiency of PSII photochemistry (F_v/F_m) is commonly used to assess the relative state of PSII (Genty *et al*., 1989; Griffin *et al*., 2004). F_v/F_m ratio is equivalent to the fraction of absorbed photons that are used to drive the light reactions of photosynthesis. This ratio is obtained by the measurement of dark-adapted leaf in order to open all the reaction centers before applying actinic light. Therefore dark-adapted leaf measurement representing the maximum possible efficiency at which light absorbed by light-harvesting antennae of PSII is converted to chemical energy (Baker and Rosenqvist, 2004).

Another parameter commonly used is the effectiveness of quantum yield or the PSII maximum efficiency (F'_v/F'_m). It is theoretically proportional to operating quantum efficiency of photochemistry which indicate the efficiency of excitation capture of open PSII reaction center (Qiu *et al*., 2003). This value is similar to F_v/F_m but obtained from an illuminated reading which is possible to determine the actual efficiency of light reaction under natural condition during the day, providing an estimation of the maximum efficiency of PSII photochemistry at a given light intensity as opposed to the maximum possible efficiency in a dark-adapted leaf (Baker and Rosenqvist, 2004).

Non-photochemical quenching (NPQ) is also very useful for chlorophyll fluorescence determination. NPQ obtained from estimation of the non-photochemical quenching from maximal fluorescence from dark-adapted leaf to maximal fluorescence from light-adapted leaf. This value is used for monitoring the apparent rate constant from non-radiative decay (heat loss) from PSII and its antennae (Baker and Rosenqvist, 2004).

Since NPQ indicates the de-exitation of light-generated excited state in the chlorophyll associated with PSII, the decrease of PSII can be associated with the changing of NPQ value (Genty *et al*., 1989). Two hypotheses have been proposed to explain the decrease of PSII quantum efficiency that associated with thermal deactivation of PSII excitation (Genty *et al*., 1990). The first hypothesis was proposed by Weis and Berry (1987) that some PSII reaction centers can be converted to an energisted state which is able to dissipate excitation energy and subsequently give low fluorescence yield and low efficient photochemistry. The second hypothesis has been proposed by Genty *et al*. (1989; 1990) that dissipation of excitation of energy is increased by non-radiation processes associated with NPQ processes. These processes occur within the antennae pigment molecules that associated with PSII reaction centers.

Seed growth and development

Seed is the primary sink for photosynthate during seed development. This accumulation is an important process in yield production and permitting seeds to survive in various environmental conditions (Egli and Leggett, 1976; Gallardo *et al*., 2003). Seed development is a series of differentiation events which can be proceed through histodifferentiation, seed filling and determination with a desiccation phase. In pea (*Pisum sativum*), seed development can be divided into three distinct phases. The first is called the cell division phase, indicated by the cotyledon cells actively divide. The second phase is maturation, the cotyledon cell expands and reserve compounds are stored. The third phase is related to seed desiccation (Hedley and Ambrose, 1980; Smith, 1993). Later, Ney and Turc (1993) described seed

development of pea that is divided into three phases; flowering, seed filling, and physiological maturity. Borisjuk *et al*. (1995) studied seed development of *V. faba* cotyledons and classified into seven stages. Stage I-III described organogenesis and morphogenesis while stage IV-VII described early to late stage of cotyledon development.

In legumes such as faba bean or pea the cotyledons differentiate into highly specialized storage organs, which is initiated at stage IV (early stage) and reach physiological maturity at stage VII (late stage) (Borisjuk *et al*., 1995). Starting from stage IV, young cotyledons are highly mitotic active and there was no detectable of starch accumulation. The accumulation of starch is initiated and rapidly accumulated during stage VI. At stage VII the starch content approached its maximum and reached physiological maturity later on (Borisjuk *et al*., 1995; 1998; 2002).

The histodifferentiation of cotyledons occurs gradually during stage V. The outer epidermal cells of cotyledon differentiated into transfer cells at the abaxial surface faced to the seed coat of pea and *V. faba* (Offler *et al*., 1997; Weber *et al*., 1997a). The epidermal transfer cell is related to seed development since they can facilitate an uptake of assimilates into cotyledons during storage phase (Pate and Gunning, 1972; Weber *et al*., 1998). The pathway of photoassimilate uptake, mostly sucrose, involves apoplasmic movement into the epidermal transfer cells via transporter and subsequent symplasmic transfer to storage parenchyma cells. Thus they are essential for further embryo growth. (McDonald *et al*., 1995; Tegeder *et al*., 1999; Borisjuk *et al*., 2002). These cells are characterized by thick wall layer and finger-like ingrowths of the cell wall at the outer layer of epidermal cotyledon cells where its first attach to seed coat. The cell wall ingrowths increase the surface of the plasma membrane and make them more suitable to mediate effective short-distance transfer of photoassimilates (Weber *et al*., 1998). This specific function of epidermal cells is acquired at the onset of embryo maturation. The mutation that inhibited epidermal differentiation into transfer cells will consequently result in the loss of epidermal cell function that causes the seed grow slowly and abort before complete maturation (Borisjuk *et al*., 1998; 2002).

Growth and development of pods and seeds can be described by several parameters including fresh and dry weight, length, ratio of fresh weight to length, seed water content, and nitrogen content (Oliker *et al*., 1978; Ney *et al*., 1993). Gallardo *et al*. (2003) characterized seed development of *Medicago truncatua* into three phases according to seed dry weight and moisture content. The first phase corresponded to stages preceding 12 days after pollination. The seed is high in moisture content of about 90% of the seed fresh weight. The second phase was associated with a large increase in the seed dry weight from 12 to 36 days after pollination. The terminal phase was characterized by a decrease in fresh weight and a dramatic loss of moisture content. The transition from the cell division phase to the maturation phase in cotyledon is accompanied by an increase in fresh weight (Weber *et al*., 1998). Borisjuk *et al*. (1995) and Weber *et al*. (1997b) reported that, at filling phase, the seed synthesizing both starch and protein associated with a rapid increase in fresh weight. Ney *et al*. (1993) suggested that seed moisture content (relative to fresh weight) can be used to determine the stage of seed growth and development of pea. Similar to Munier-Jolain *et al*. (1993) who reported that seed moisture content and seed dry weight can be used to determine seed growth of soybean and concluded that seed moisture content is a convenient criterion to characterize the stage of seed development in non-stressed conditions.

Seed desiccation

The relationship between the metabolic and structural changes during seed development, especially at seed-filling period, is important for yield-related processes (Egli, 1994; Johnson *et al*., 1994). The maintenance of a steady sink activity throughout the seed-filling period is necessary for high yield (Hanson, 1991). Reduced seed maturation was associated with a reduction of dry matter accumulation and seed density (Johnson *et al*., 1994).

Accumulation of storage products are essential for seed desiccation tolerance (Borisjuk *et al*., 1995). Desiccation tolerance in seed is initiated when maturation

occurs (Sanhewe and Ellis, 1996) however, it also depends on the species, the rate of water loss, and the final water content (Hong and Ellis, 1992; Ellis and Hong, 1994).

Seed desiccation tolerance is related to soluble carbohydrates, cytoplasmic vitrification, and stability of membrane upon drying (Sun *et al*., 1994). Sucrose is a product of photosynthesis and is almost universally present in seeds (Amuti and Pollard, 1977). This sugar can markedly alter the physical characteristics of membrane phospholipids, causing it to retain characteristics of hydrated lipid even when water is absent (Caffrey *et al*., 1988). The membrane is the structural component that most sensitive to damage upon withdrawal of water which can result in irreversible loss of membrane function (Blackman et al., 1992). Thus, the ability of seeds to survive from the withdrawal of water must involve a mechanism for protecting the integrity of cellular membrane (Caffrey *et al*., 1988).

Crowe *et al*. (1984; 1988) reported that soluble sugars play an important role in desiccation tolerance and able to protect the structural integrity of membranes during dehydration by preventing membrane fusion, phase transition, and phase separation. Thus the preservation of cellular membrane integrity or membrane stability is essential for desiccation tolerance of developing seeds (Le Page-Degivry and Garello, 1991). In soybean seeds, sucrose, oligosaccharides, and proteins are most important factors in the acquisition of desiccation tolerance (Blackman et al., 1992). Since sucrose is one of an effective molecules to protect membrane integrity. Thus, the change in soluble sugar contents in seed can be correlated to the loss of desiccation tolerance. The loss of desiccation tolerance is corresponds to the loss of oligosaccharides (Koster and Leopold, 1988). If the seed dried before reaching the desiccation tolerance stage of maturity, they will not germinate (Adams *et al*., 1983; Senaratna and McKersie, 1983).

DNA markers

Different types of DNA markers are currently available for genetic analysis and new marker types are continuously developed. DNA markers can be broadly

divided into two groups. The first group is hybridization based markers and the second group is the polymerase chain reaction (PCR) based markers.

DNA markers based on hybridization

This method is called restriction fragment length polymorphism (RFLP). This technique is based on the use of DNA restriction enzymes to cleave (digest) genomic DNA, yielding fragments of specific lengths. The DNA fragments are hybridized with labeled DNA probe that are purified and isolated from the same or related species. The labeled probes can be hybridized with the homology sequence of the DNA fragment of interest. The fragment length polymorphism subsequently investigated through gel electrophoresis and may involve in the membrane transfer method referred as the Southern transfer (Southern, 1975) and subsequent autoradiography. The different in length of a particular restriction fragment or restriction patterns may be caused by mutations that lead to a loss or gain of a restriction site or by insertions/deletions that alter fragment sizes between genotypes (Gebhardt *et al*., 1989). RFLP assay is usually practiced with species-specific probe, either from genomic or cDNA library which is required as a source of single or low copy probes. Probes usually detect one or more genetic loci that share sequence homology. More than one allele can be detected at a locus so that the RFLP marker can be used to identify co-dominant genotypes from which homozygote can be distinguished from the heterozygote (Smith and Smith, 1991; Bernardo, 1992). However, RFLP has several limitations as this method requires a large amount of good quality DNA, use of radioactivity, expensive, and time consuming (Malyshev and Kartel, 1997).

DNA markers based on PCR

PCR is a method for DNA fragment multiplication via repeated cycles of denature of the double stranded DNA as template for amplification at high temperature, then decrease to optimum temperature for annealing of primers to the template. The last step is primer extension where the sequence between the primer

sites is replicated. Several types of PCR based marker have been developed as followed:

Random amplified polymorphic DNA (RAPD)

RAPD is a technique for amplifying DNA fragments with PCR reaction of a mixture containing genomic DNA and an arbitrary primer. The primer is nine to ten base pairs in length with a G+C content of at least 40 % (Williams *et al*., 1990). Amplification occurs randomly at multiple binding sites along the strands of the genomic DNA within approximately 3 kb which resulted in several DNA fragment products obtained (Waugh and Powell, 1992). The present of each amplification product identify complete or partial nucleotide sequence homology between the genomic DNA and the oligonucleotide primer at each end of amplified product. Polymorphism of a locus is obtained from mis-match of the primer due to genomic difference between individuals or insertion/deletion at a given binding site of primer and DNA fragment resulting in size polymorphism.

Microsatellite or simple sequence repeats (SSRs)

Microsatellite or simple sequence repeats (SSRs) are also called variable numbers of tandem repeat (VNTRs) (Rallo *et al*., 2000). They are DNA sequences of 1-6 base pairs that tandemly repeated from two to thousands of times in the genome. The repetitive DNA fragments that display high variability in a locus can distinguish closely related individuals. SSRs markers are considered a single locus profile that generates co-dominant markers. They are abundant throughout all the eukaryotic genomes and have a high level of allelic diversity (Morgante *et al*., 2000). Consequently, they are frequently used as genetic markers in plant genetic studies (Powell *et al*., 1996). The advantage of SSR markers are they generate multi-allele co-dominant marker, transferable, easily reproducible, easily scorable, randomly and widely distributed along the genome, and give high information per locus. However, developing of these markers is laborious, expensive, and difficult to produce because they require prior DNA sequence information for primer design (Li *et al*., 2000; Rallo

et al., 2000). Moreover, they are conserved among genotypes of the same species (Cregan *et al*., 1999).

Inter simple sequence repeat (ISSR)

ISSR marker is an alternative technique of SSR marker. This technique involves amplification of genomic segments that flanked by inversely oriented closely spaced microsatellite sequences by a 2-4 purine (R) or pyrimidine (Y) residues (Zietkiewicz *et al*., 1994; Ammiraju *et al*., 2001). These primer comprise a microsatellite sequence and a short arbitrary sequencer (anchor) which target to a subset of SSRs and amplifies the region between two closely spaced and oppositely oriented simple sequence repeats (Li *et al*., 2000). The ISSR motif appears between two core sequences that are tandemly repeated from two to many thousand of times in a genome. Therefore, they can detect more DNA bands than SSR because they can detect polymorphism in microsatellite and inter-microsatellite loci without previous knowledge of DNA sequences (Martin and Sanchez-Yelamo, 2000). The advantages of using ISSR primers that they require low DNA quantity, generate high number of markers, are highly reproducible, inexpensive, and do not require prior primer sequence. However, they may require the use of radioactivity for clear distinction of close bands (Godwin et al., 1997). The ISSR markers have been used for tagging gene in many crop plants, such as rice (Akagi *et al*., 1996), chickpea (Ratnaparke *et al*., 1998), and wheat (Ammiraju *et al*., 2001).

Amplified fragment length polymorphism (AFLP)

The AFLP technique is based on amplification of restriction fragments by specific restriction enzymes and oligonucleotide adapters of few nucleotide bases (Vos *et al*., 1995). The number of DNA fragments which are amplified can be controlled by choosing the different base number and composition of nucleotides in adapters (Mohan *et al*., 1997). With the large number of amplified restriction fragment bands, typically 50 to 100 restriction fragments, polymorphisms among the DNA fragments can easily be detected, thus makes them highly informative (Vos *et al*.,

1995). High reproducibility, rapid generation and high frequency of identifiable polymorphic (the average number polymorphic band is over eight per gel) make AFLP analysis an attractive technique for determining linkage by analyzing individuals from segregating population (Mackill *et al*., 1996; Hartl *et al*., 1999). It has been extensively used for developing polymorphic markers linked to important agronomic traits. It is an efficient method for the identification of molecular markers, useful in the improvement of crop species, and a very powerful tool for gene tagging (Cervera *et al*., 1996; Negi *et al*., 2000).

Bulked segregant analysis for mapping or tagging gene

Identification of markers tightly linked to a particular gene (tagging gene) in crop plants can be applied in marker-assisted selection (MAS) and map-based gene cloning. Mapping a gene to a certain location on the chromosome demands a linkage map of the whole genome, but genes can also be tagged with molecular markers without any previous information of the map locations of markers used. The molecular techniques used for identification of genes and evaluation the inheritance of interested traits are bulked segregant analysis (BSA), near isogenic lines (NILs), and selective genotyping.

BSA is a rapid procedure for identifying markers linked to a trait of interest without the need of a genetic linkage map. The method is based on selecting two bulks of 5 to 10 individuals in such a way that they are genetically homogeneous at all genomic regions, except at the target locus. The two bulks could be easily selected using individuals with contrasting phenotypes for a trait of interest and screened with markers. The bulks of DNA are then screened with markers in search of polymorphism. The markers that show clear polymorphism between the bulks will be genetically linked to the loci determining the trait used to construct the bulks. (Michelmore *et al*., 1991).

NILs differ only by the presence or absence of the target gene and a small region of flanking DNA. Hundreds of markers can easily be screened to identify the

difference between isogenic lines and these differences are likely to link with the target gene (Muehlbauer *et al*., 1988; Martin *et al*., 1991).

In selective genotyping, the entire mapping population is phenotyped for a particular trait. However, only the individuals representing the phenotypic extremes are chosen for marker genotyping, and subsequent linkage and QTL analysis.

Linkage map

Construction of genetic linkage map is based on observed recombination frequency between marker loci in the segregating population such as F_2 or recombinant inbred lines. Genetic map distances are based on recombination fractions between loci by converting the recombination fractions to map units or centi Morgans (cM) using Haldane (1919) or Kosambi (1944) mapping functions. The Haldane mapping function takes into account the occurrence of multiple crossovers. While the Kosambi mapping function accounts for inheritance which one crossing-over inhibit the formation of another in its neighborhood (Ott, 1985). A computer program Mapmaker/Exp can perform full multipoint linkage analysis from a giving set of data (Lander *et al*., 1987).

A mungbean genetic map was constructed by Menancio-Hautea *et al*. (1993) comprised of 171 RFLP markers combined into 14 linkage groups spanning a total of 1570 cM. Lambrides *et al*. (2000) constructed the F_2 and recombinant inbred line (RI) maps of mungbean. The F_2 map consisted of 52 RFLP and 56 RAPD markers, while the RI map consisted of 115 RAPD markers. Both maps were assigned into 12 linkage groups. The linked markers of F_2 map spanned a total distance of 758.3 cM, while RI map spanned a total distance of 691.7 cM. Recently, Humphry *et al*. (2002) developed an RI genetic map of mungbean consisting of 255 RFLP markers. The total length of the map spanned 737.9 cM in 13 linkage groups.

MATERIALS AND METHODS

Plant materials

An opaque leaf line was obtained from gamma radiating F_2 seeds derived from a cross between the cultivated mungbean *V. radiata* var. *radiata* 'Chai Nat 36 (CN36)' and the wild type *V. radiata* var. *sublobata* 'TC1966'. The mutant was purified for 8 generations until uniform with green petiole and indeterminate growth habit. The line was late maturing (1 to 2 weeks alter than Thai cultivars), set small and wrinkled seed (approximately 2.18 g/100 seed weight) with finally resulted in low yield. It was a new character ever reported in mungbean (Srinives *et al*., 2000).

The opaque leaf line was crossed with Berken, a popular Australian cultivar with normal leaf, purple petiole, and determinate growth habit. The parents, F_1 and F_2 seeds were grown and compared in plastic pots at the University of Queensland, Australia, during the spring and summer of 2003. Two plants were grown in a pot of 25 cm in diameter. Each plant was scored for leaf appearance, petiole color, and growth habit. These F_2 plants were evaluated for inheritance study and DNA extraction.

During 2004-2005, the F_2 plants were subsequently grown and selfed until F_5 seeds were obtained. The location in Thailand is the field at Asian Vegetable Research and Development Center (AVRDC) through its Asian Regional Center located at Kasetsart University, Kamphaeng Saen, Nakhon Pathom, Thailand. Four F_5 lines (N531, N541, OP531 and OP541) were selected and used for studying chlorophyll content, chlorophyll fluorescence, and seed growth and development.

Determination of chlorophyll content

Five leaf discs (0.82 cm^2) were punched from the laminar of a trifoliate leaf from 10 individual plants at the flowering stage of plant growth. Five leaf discs from each parents and progenies were placed into 4 ml of *N*,*N*-dimethylformamide (DMF)

and incubated in the dark for 48 hr at room temperature. The content of chlorophyll a, b, and total chlorophyll (a+b) were determined using spectrophotometer (DU® 530, Life Science, Becman Coulter). The absorbance density of the chlorophyll solution was determined at 664 nm for chlorophyll a and 647 nm for chlorophyll b. The chlorophyll a, b, total chlorophyll, and a/b ratio was calculated from the equations of Moran (1982) as follow:

$$Chl_a = (-2.99\ A_{647} + 12.64\ A_{664}) * Vol / (X*Area*100)$$

$$Chl_b = (23.26\ A_{647} - 5.60\ A_{664}) * Vol / (X*Area*100)$$

$$Chl_{total} = (20.27\ A_{647} + 7.04\ A_{664}) * Vol / (X*Area*100)$$

Where Chl_a , Chl_b , and Chl_{total} are chlorophyll a, b, and total chlorophyll content (g/ m^2), respectively. A_{647} and A_{664} are light absorbance at 647 and 644 nm, respectively. Vol is the DMF volume (ml) that used for extracting chlorophyll. X is a diluted volume, in the case that the first extracted chlorophyll give higher reading concentration than 0.8. Area is the leaf area used for chlorophyll extraction (cm^2).

Determination of chlorophyll fluorescence

Chlorophyll fluorescence was measured on fully expanded leaves from 10 individual plants of each parents and progenies using a Mini-PAM fluorometer (Heinz Walz, Germany) at 10.00 am, 12.00 am, 14.00 pm and 20.00 pm with a leaf clip holder (Model 2030-B). The following parameters were recorded and calculated by the flourometer's on-board program using mode 1 for day time and mode 25 for night time measurements.

F_0 = fluorescence yield of dark adapted leaf

F_m = maximum fluorescence yield of dark adapted leaf

F = fluorescence yield of light adapted leaf

F'_m = maximum fluorescence yield of light adapted leaf

F_v/F_m = the maximum quantum efficiency of PSII photochemistry

F'_v/F'_m = PSII maximum efficiency

NPQ = non-photochemical quenching

Seed weight and seed moisture content

Twenty to 50 flowers opened on the same day were labeled with tags. The marked pods (pod age was expressed as days after flowering) were harvested from each plant at 3 day intervals and kept at 4ºC to prevent dehydration. Pod, pod wall, seed, seed coat, and cotyledons fresh weight (FW) were determined from 10 pods of 10 random plants of each line. Dry weight (DW) was measured after drying pod parts at 80ºC for 48 hr.

The seed moisture content relative to fresh weight was calculated as (FW-DW)/FW.

Data analysis

The mungbean parents, F_1 hybrid and 85 F_2 plants were scored for leaf characteristic (opaque vs. normal), petiole color (green vs. purple), and growth habit (determinate vs. indeterminate). Segregation in number of these traits in the F_2 was evaluated by Chi-square test for goodness-of-fit against the 3:1 Mendelain ratio. Linkages between leaf characteristics and the other two traits were also tested according to Mather (1951).

Data from the measurement of chlorophyll content, chlorophyll fluorescence, and seed weight were subjected to ANOVA and, where appropriate, means were separated with a protected DMRT at $P \leq .05$ (SAS Institute Inc., N.C.).

Seed cell morphology

Pods and seeds were cut into small pieces (5x5 mm) and fixed in 50% formalin-aceto-alcohol (FAA) solution for killing and fixing cells under light vacuum for 3 hr at room temperature and let stand overnight. Dehydration was done with a tertiary butyl alcohol (TBA) series 50, 70, 85, 95, and 100%, respectively. Each series was incubated for 12 hr. The tissues were then incubated in 1:1 of TBA:paraffin oil at 60 ºC for 12 hr, then removed into pure paraffin at the same period of time and temperature. This step was repeated three times. The tissue was finally embedded in a plastic block filled with paraffin and left them until became solid.

The embedded explants were cut into 7-10 µm thick on a Microtome, transferred on glass slides and dried overnight at 45ºC. Staining was performed with Toluidine blue and Fast green according to Brooks *et al*. (1950), then visualized with microscopes (OLYMPUS BX41 and OLYMPUS SZX 12) and photographed with a microscope digital camera system (OLYMPUS DP12).

DNA extraction

Young expanded leaves were collected from parental plants and 80 individual F_2 for DNA extraction. The extraction was performed following Lambrides *et al*. (2000) which was developed from Dellaporta *et al*. (1983). The quality of DNA was evaluated on agarose gel electrophoresis using standard DNA. DNA concentration was determined by fluorometry using a Hoeffer TKO 106 Fluorometer. A portion of the concentrated stocks was used to prepare 20 ng/µl polymerase chain reaction (PCR) stocks. Two DNA bulks, one for normal leaf and the other for opaque leaf were obtained by mixing equal volumes of DNA solution from each of 10

respective progenies. DNA from the two parents, two bulks and 80 F_2 individuals were used for SSR, ISSR and AFLP analyses.

SSR analysis

Twenty-seven of SSR primer pairs from cowpea [*Vigna unguiculata* (L.) Walp] were used in this study. Their names, repeat types and PCR conditions were modified from Li *et al*. (2001). The PCR reaction was carried out in 1x reaction buffer, 3 mM $MgCl_2$, 0.25 mM dNTP, 0.3 µM reverse primer, 0.3 µM forward primer, 50 ng genomic DNA, and 0.5 unit *Taq* DNA polymerase (Fermentas) per 25 µl reaction volume.

The PCR amplification was performed in a PTC 100 Programmable Thermal Controller (MJ Research Inc.) with initial denaturation at 94ºC for 2 min, then 94ºC for 1 min, annealing at 60ºC for 1 min, extension at 72ºC for 2 min for 34 cycles**,** and final extension at 72ºC for 7 min. Some primers were performed for 38 cycles as followed: 18 cycles at 94ºC for 1 min, annealing at 64ºC that was subsequently reduced by 0.5 ºC for cycle 2 through 18, and extension at 72ºC for 1 min, then remained at 55 ºC for the remaining 30 cycles, and the final extension at 72ºC for 10 min.

ISSR analysis

Twenty-eight ISSR primers were used in this study. The PCR reaction mixture consisted of lx Mg free buffer, 2.5 mM $MgCl_2$, 0.1mM dNTP, 0.2 mM primer, 20 ng genomic DNA, and 0.5 unit *Taq* DNA polymerase (Fermentas). All reaction volumes were executed at 25 µl.

The PCR amplification was performed in a PTC 100 Programmable Thermal Controller with initial denaturation at 94ºC for 2 min, then 94ºC for 15 sec, annealing at 55ºC for 15 sec, extension at 72ºC for 1 min for 34 cycles**,** and final extension at 72ºC for 5 min.

AFLP analysis

Template preparation

A total of 120 ng of genomic DNA from each of the parents, opaque leaf bulk, normal leaf bulk, and each of the 80 F_2 individuals were digested with 12 units *Eco*RI, 8 units *Mse*I, and 1x restriction-ligation buffer in a 10 µl reaction volume adjusted with distilled water. The product was incubated for 3 hr at 37 ºC. The digested DNA fragments were ligated with 0.5 µl *Eco*RI adapter (5 pmol/µl), 0.5 µl *Mse*I adapter (50 pmol/µl), 0.5 µl ATP (10 mM), 0.5 µl 10x restriction-ligation buffer, 2.5 units T4 DNA ligase, and distilled water. The product was incubated for 3 hr at 37 ºC and terminated by warming at 65 ºC for 5 min. The restriction-ligation products were diluted for 10 times, then used as the template DNA for pre-selective amplification reaction.

Pre-selective amplification step (PCR I)

Pre-amplification of the mixture was prepared from 1 µl of ligated DNA, 2 µl of 1mM dNTPs (Promega), 0.6 µl of 25 mM $MgCl_2$, 1 µl 10x buffer, 0.5 µl *Eco*RI+1 primer (5 µM/ µl), 0.5 µl *Mse*I+1 primer (5 µM/ µl), 0. 75 unit *Taq* DNA polymerase (Fermentas) and distilled water to the volume of 10 µl. The PCR reaction was carried out in a PTC 100 Programmable Thermal Controller consisting of steps from initial denaturation at 94 ºC for 30 sec, followed by 28 cycles of 94 ºC for 15 sec (denaturation), 60 ºC for 30 sec (annealing), 72 ºC for 1 min (extension), and 72 ºC for 2 min in the final extension. The pre-selective amplification products were diluted for 10 times and used as templates for selective amplification.

Selective amplification step (PCR II)

Selective amplification reaction was conducted by adding two or three selective nucleotides into the reaction. The components for each 10 µl reaction comprised 1 µl of pre-amplified DNA, 2 µl of 1 mM dNTPs (Promega), 0.6 µl of 25

mM $MgCl_2$, 1 µl 10x buffer, 0.5 µl *Eco*RI+2-3 primer (5 µM/µl), 0.5 µl *Mse*I+2-3 primer (5 µM/µl), 0. 75 unit *Taq* DNA polymerase (Fermentas) and distilled water. The PCR program for selective amplification was performed with a touch-down PCR with the same PTC 100 Programmable Thermal Controller. The PCR was performed from an initial denaturation of 30 sec at 94 ºC, 30 sec for an annealing temperature at 65ºC that was subsequently reduced by 0.7ºC from cycle two through thirteen, and 2 min at 72ºC, then retained at 56ºC for the remaining 25 cycles.

Gel electrophoresis and detection

SSR and some ISSR products were separated on 1.5 % agarose gel and run at 100 Volt for 1 hr. The DNA bands were stained with ethidium bromide, visualized with ultraviolet light and photographed.

AFLP and some ISSR (in the case that the DNA bands were very close and difficult to score) products were separated on 6% (w/v) polyacrylamide using the following steps. The PCR products were mixed with 10 µl sequencing dye (95% formamide, 10mM NaOH, 0.05% bromophenol blue, and 0.05 xylene cyanol FF). The 5 µl of samples were loaded on 6% sequencing polyacrylamide gel and 1X TBE running buffer. Electrophoresis was performed at the constant temperature of 55ºC and electrical current of 60 Watt for about 2 hr. Upon electrophoresis, the gel was fixed for 30 min in 10% acetic acid solution, rinsed 3 times with de-ionized H_2O for 2 min each, then stained with staining solution (1% silver nitrate, 0.6% formaldehyde) for 30 min, rinsed thoroughly and briefly (less than 10 sec) with de-ionized H_2O and detected the signal in chilled developer solution (30% sodium carbonate, 0.1 sodium thiosulphate, 0.56% formaldehyde) until the signal was detected. Then the reaction was stopped by adding the stop solution (10% acetic acid solution) for 1 min, and washed 5 min with de-ionized H_2O. The gel was left at room temperature until completely dried and the DNA band pattern was visualized as presence (1) or absence (0) according to the primer.

Analysis of genetic distance

The F_2 plants segregating in opaque leaf vs. normal leaf for each of AFLP marker were evaluated by the presence or absence of DNA amplification bands, and were subjected to Chi-square analysis to test the goodness-of-fit of the observed and expected ratio (1:2:1 for co-dominant or 3:1 for dominant markers). Only those loci fitting the Chi-square test (P>.05) were used in mapping procedure. The genetic distance between the markers and the gene controlling opaque leaf trait were calculated using MAPMAKER/EXP 3.0 program (Lander *et al*., 1987). The relationship between markers and gene was evaluated using STATGRAPHICS PLUS 3.0 program. Genetic distance was described by centi-Morgan (cM) using Kosambi (1944) mapping function.

RESULTS

Determination of chlorophyll content

The chlorophyll contents in normal and mutant leaf genotypes are shown in Figure 1. The result revealed that all opaque leaf mutants (Opaque, OP531 and OP541) contained less chlorophyll a, b, and total (a+b) contents than normal leaf genotypes (Berken, N531, and N541). The opaque parent gave the lowest values of 0.089, 0.027, and 0.118 g/m^2 for chlorophyll a, b, and total chlorophyll, respectively. Whereas normal leaf genotypes gave the values of 0.135-0.139, 0.041-0.043, and 0.176-0.183 for chlorophyll a, b, total (a+b), respectively. The result indicated that opaque leaf was a type of chlorophyll-deficient mutant.

The study of chlorophyll a/b ratio showed that there was no significant different among parents and the sister lines. Although the mutant genotypes tended to give less a/b ratio than the normal genotypes it was not different when compared between Opaque parent and N541 sister line.

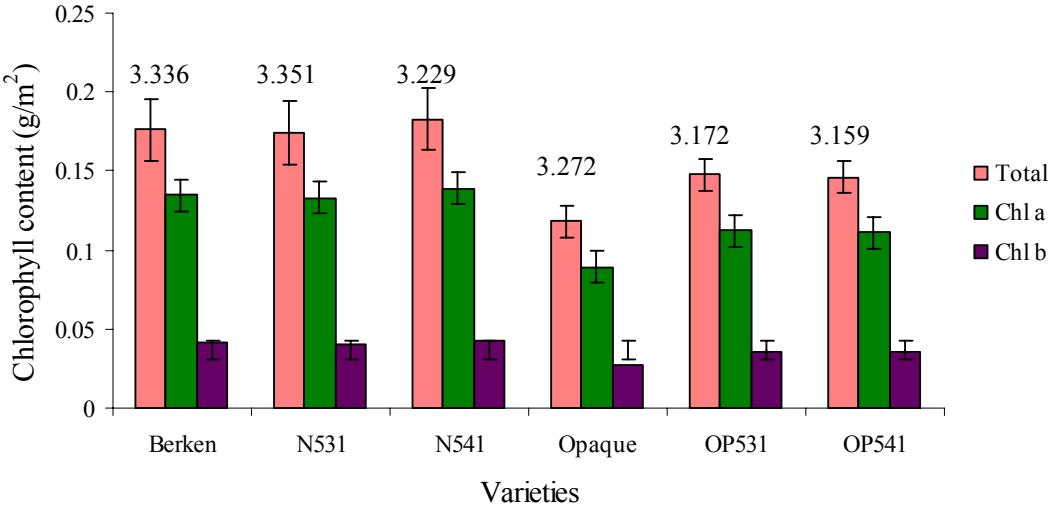

Figure 1 Chlorophyll a, b, total (a+b) contents (g/m^2), and a/b ratio in opaque leaf parent 'Opaque', opaque lines OP531, OP541, and normal leaf parent 'Berken', normal leaf lines N531, N541. The number above each bar represents the chlorophyll a/b ratio of each genotype. Vertical bars denotes standard deviations.

Determination of chlorophyll fluorescence

Table 1 summarizes the results obtained from chlorophyll fluorescence analysis in mutant and normal leaf genotypes. The chlorophyll measurement in dark-adapted leaf revealed that the mutant leaf genotypes, Opaque, OP531 and OP541, gave the ratio of variable fluorescence to maximum fluorescence or the maximum quantum efficiency of PSII photochemistry (F_v/F_m) value of 0.815±0.007 to 0.825±0.005, while the normal leaf genotypes (Berken, N531, and N541) accounted for 0.838±0.005 to 0.843±0.004, or approximately 1.55 to 2.8% higher than the mutant genotypes. Among them, Opaque gave lowest F_v/F_m value and N541 gave highest value.

The data from light-adapted leaf measurement during 10.00 am to 14.00 pm found that the ratio of F'_v/F'_m was not much different during the course of the day in all genotypes. However, all opaque leaf genotypes gave lower F'_v/F'_m value than normal leaf at any given time of measurement, approximately 7.4 to 15.2% .

The parameter non-photochemical quenching, NPQ, that used to determined the de-excitation of light generated from chlorophyll was statistical difference in throughout the course of the day. It was low in the morning and increased in the afternoon. The values were higher in opaque leaf genotypes than normal leaf genotypes up to approximately 9 to18% (Table 2).

Table 1 Chlorophyll fluorescence in F_v/F_m and F'_v/F'_m values of normal leaf and mutant leaf genotypes. Data are means of 10 measurements ±SD. Means within a row not sharing the same lowercase letter are significantly different ($P < .05$) by Duncan comparison test.

Genotypes	F_v/F_m	F'_v/F'_m		
	20.00 pm.	10.00 am	12.00 pm	14.00 pm
Berken	0.839±0.006 b	0.583±0.034 a	0.560±0.022 a	0.546±0.051 a
N531	0.837±0.005 b	0.580±0.373 a	0.572±0.056 a	0.548±0.053 a
N541	0.846±0.004 a	0.604±0.036 a	0.527±0.028 b	0.552±0.063 a
Opaque	0.815±0.007 d	0.515±0.042 b	0.511±0.030 bc	0.492±0.020 b
OP531	0.825±0.004 c	0.518±0.049 b	0.514±0.038 bc	0.507±0.050 ab
OP541	0.825±0.005 c	0.512±0.049 b	0.488±0.031 c	0.471±0.069 b

Table 2 Chlorophyll fluorescence in NPQ value of normal leaf and mutant leaf genotypes. Data are means of 10 measurements ±SD. Means within a row not sharing the same lowercase letter are significantly different ($P < .05$) by Duncan comparison test.

Genotypes	NPQ		
	10.00 am	12.00 pm	14.00 pm
Berken	2.314±0.415 ab	2.832±0.307 ab	2.862±0.326 b
N531	2.265±0.429 b	2.659±0.541 b	2.937±0.426 b
N541	2.158±0.405 b	2.812±0.575 ab	3.097±0.291 ab
Opaque	2.777±0.450 a	3.494±0.655 a	3.416±0.338 a
OP531	2.513±0.699 ab	2.925±0.541 ab	3.249±0.374 ab
OP541	2.560±0.527 ab	3.183±0.572 a	3.488±0.453 a

Seed growth and development

Pod weight

Pod fresh and dry weight accumulation pattern of opaque and normal leaf plants from six genotypes were initiated from 3 days until complete seed desiccation at 18 days after flowering. The result revealed that from 3 up to 9 days after flowering, pod fresh and dry weight were similar in both genotypes. The difference was noticeable at 12 days after flowering in which both fresh and dry weights of normal genotypes increased linearly at 3 to 15 days after flowering and slightly decreased afterward, whereas pod fresh and dry weights of mutant genotypes were increased from 3 to 12 days, then decreased after 12 days as pod became yellow and wilted at 13 days and completely dried at 14 to 15 days after flowering (Figure 2).

Pod wall weight

The pattern of pod wall fresh weight was slightly different from dry weight. Pod wall fresh weight varies within the genotype but not between mutant and normal leaf plants during 3 to 12 days after flowering. During these period, pod wall fresh weight from all six genotypes increased rapidly up to 12 days for mutant genotypes and 15 days for normal genotypes, then decreased. The pod wall dry weight of mutant genotypes increased linearly from 3 to 9 days before decreasing whereas pod wall of normal genotypes still increased until 15 days after flowering and then decreased (Figure 3).

Seed weight

The accumulation pattern of seed dry weight of both normal and mutant seeds began at approximately 3 days after flowering and increased very slowly over the first 3 to 6 days. However, there was no different between mutant and normal genotypes in seed dry weight during this period. The difference became more evident in the later stages. The increase in seed fresh and dry weight of normal genotypes

occurs rapidly after 6 days to 15 days and then slowly until seed reached desiccation at 18 days with dry matter content 0.776, 0.744, and 0.673 g/pod for Berken, N531, and N541, respectively. While the increase in seed fresh and dry weight of mutant seed genotypes occurs slowly from 6 to 12 days and ceased at 12 days after flowering. After this stage, the seed rapidly reduced in size and became wrinkled at 13 days, then dried at 14 to 15 days after flowering. The final accumulation of seed dry weights at 15 days after flowering were only 0.072, 0.075, and 0.081g/pod for Opaque, OP531 and OP541, respectively, as compared to 0.776, 0.744, and 0.673 g/pod for Berken, N531 and N541, respectively, or approximately 9.76 to 12.27% of normal seed at desiccation period (Figure 4).

Seed coat weight

The accumulation pattern of seed coat fresh weight in opaque and normal leaf genotypes increased rapidly from 6 to 12 days after flowering. Then the accumulation in the opaque leaf genotypes decreased after 12 days. Whereas in the normal leaf genotypes, it was stable or slightly reduced during 12 to 15 days after flowering and rapidly decreased later on. The seed coat dry weight accumulation in the opaque leaf genotypes increased very slowly from 6 to 12 days and slowly declined while in the normal leaf genotypes, it gradually increased up to 15 days after flowering and slowly declined (Figure 5).

Cotyledon weight

The pattern of cotyledon growth and development was similar to seed growth in which fresh weight increased rapidly from 6 to 12 days in opaque leaf and 6 to 15 days after flowering in normal leaf genotypes and suddenly declined at 15 days in opaque leaf and 18 day after flowering in normal leaf genotypes. However, cotyledons dry weight increased linearly from 6 to 15 days and slightly increased until they were completed mature at 18 days. Whereas in the opaque leaf genotype the cotyledon weight at 15 days after flowering was only 5 to 8% of the final weight of the normal genotype (Figure 6).

Seed moisture content

Seed moisture contents of all six genotypes were similar as they remained constant approximately 75 to 88 % from 3 to 12 days after flowering. The differences became more evidence after 12 days of seed development with dramatically loss of moisture content to about 8 to 10% at 15 days after flowering in mutant genotypes. While, it remained approximately 60% at 15 days followed by rapidly decreased to 13 to 18 % when seed reached desiccation at 18 days after flowering (Figure 7).

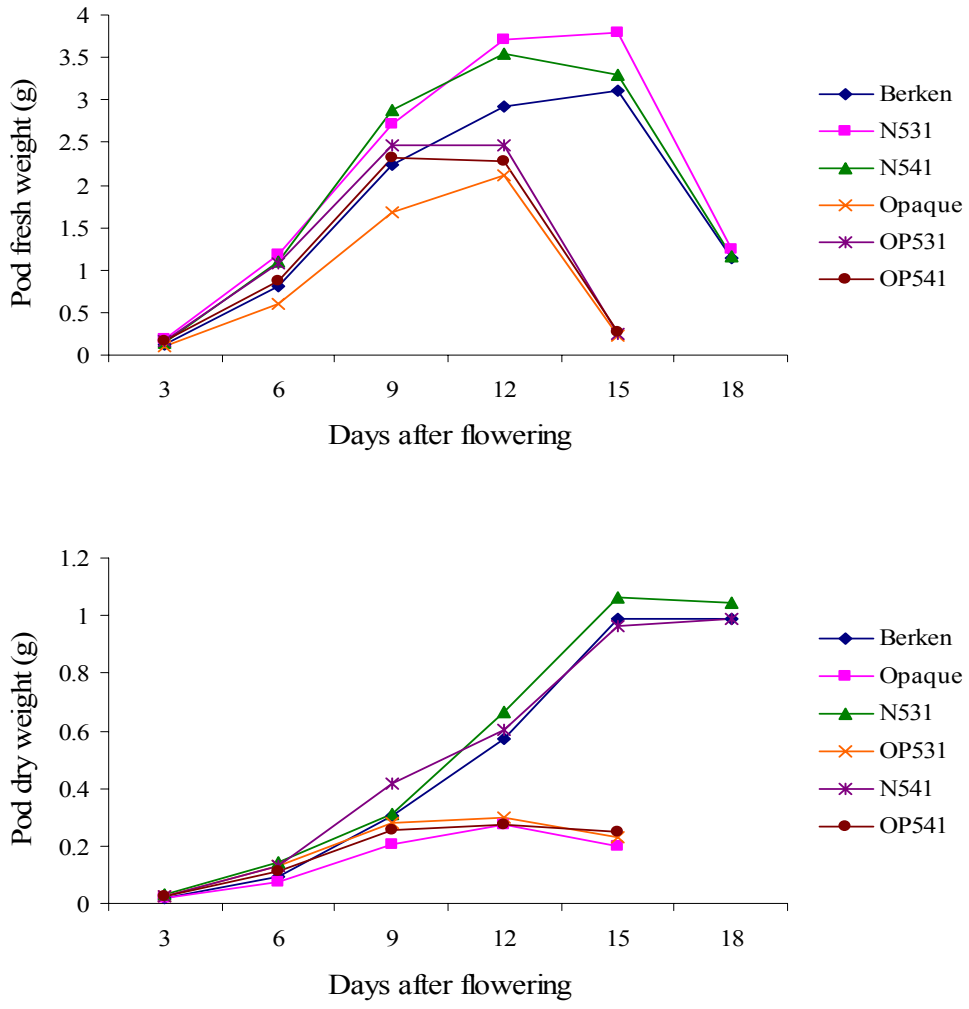

Figure 2 Accumulation of pod fresh weight (a) and dry weight (b) of opaque vs. normal leaf genotypes at 3 to 18 days after flowering.

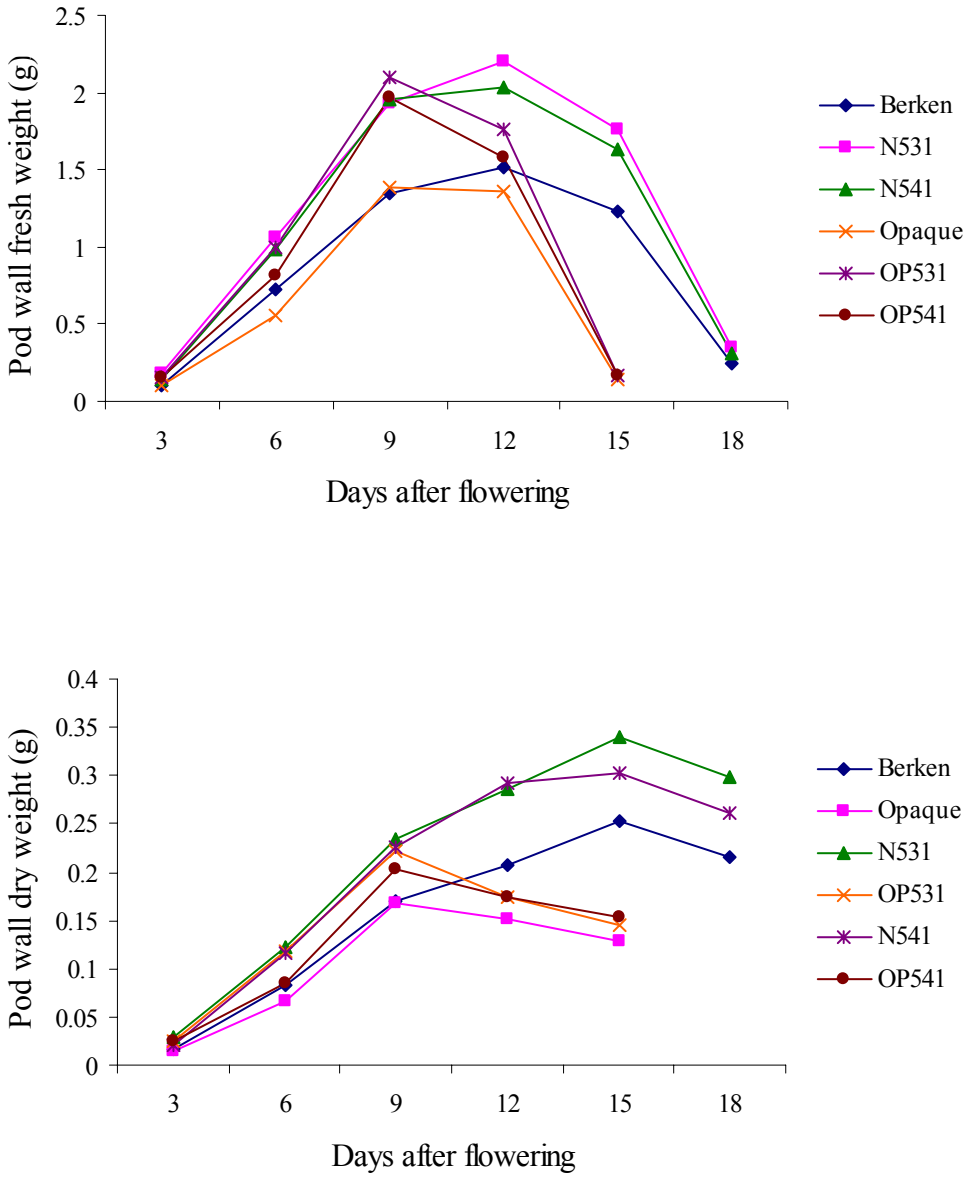

Figure 3 Accumulation of pod wall fresh weight (a) and dry weight (b) of opaque vs. normal leaf genotypes at 3 to 18 days after flowering.

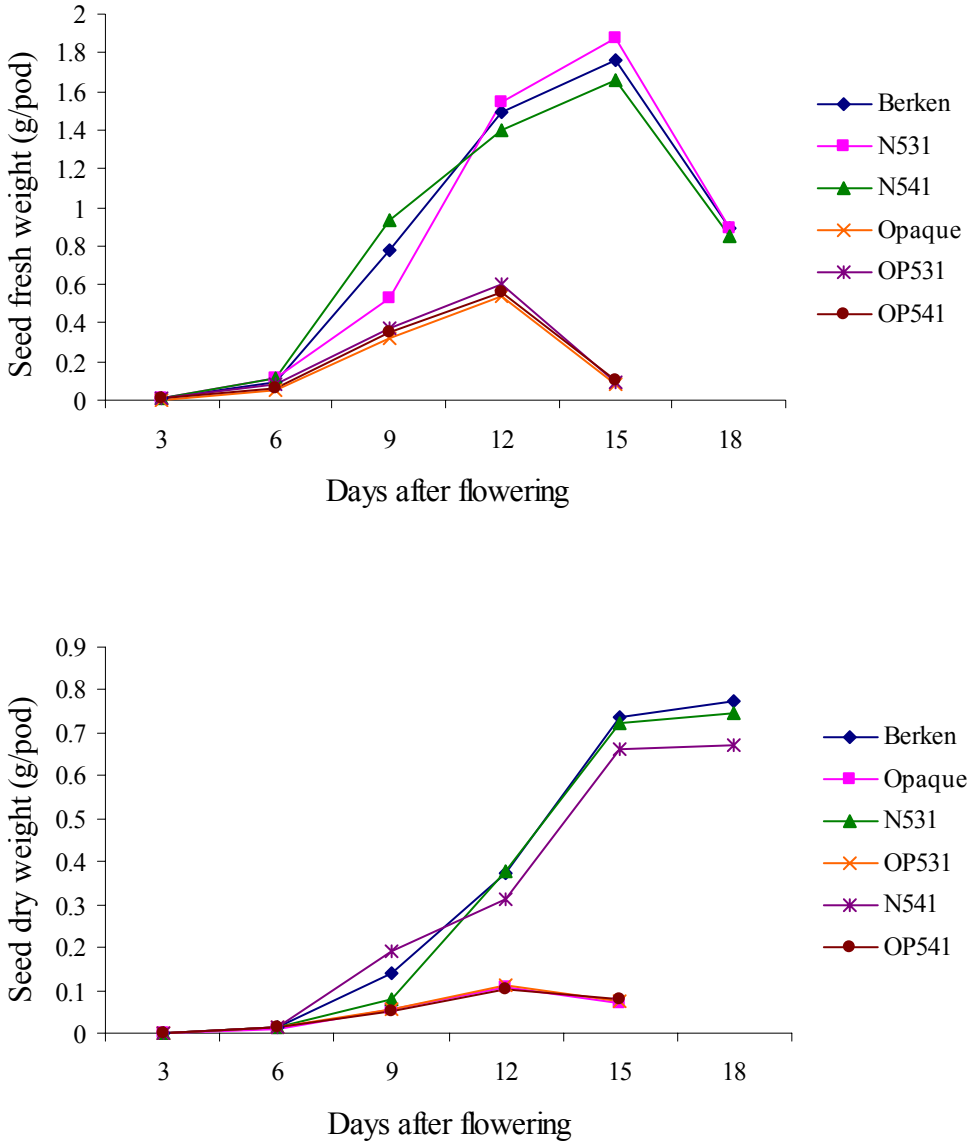

Figure 4 Accumulation of seed fresh weight per pod (a) and dry weight per pod (b) of opaque vs. normal leaf genotypes at 3 to 18 days after flowering.

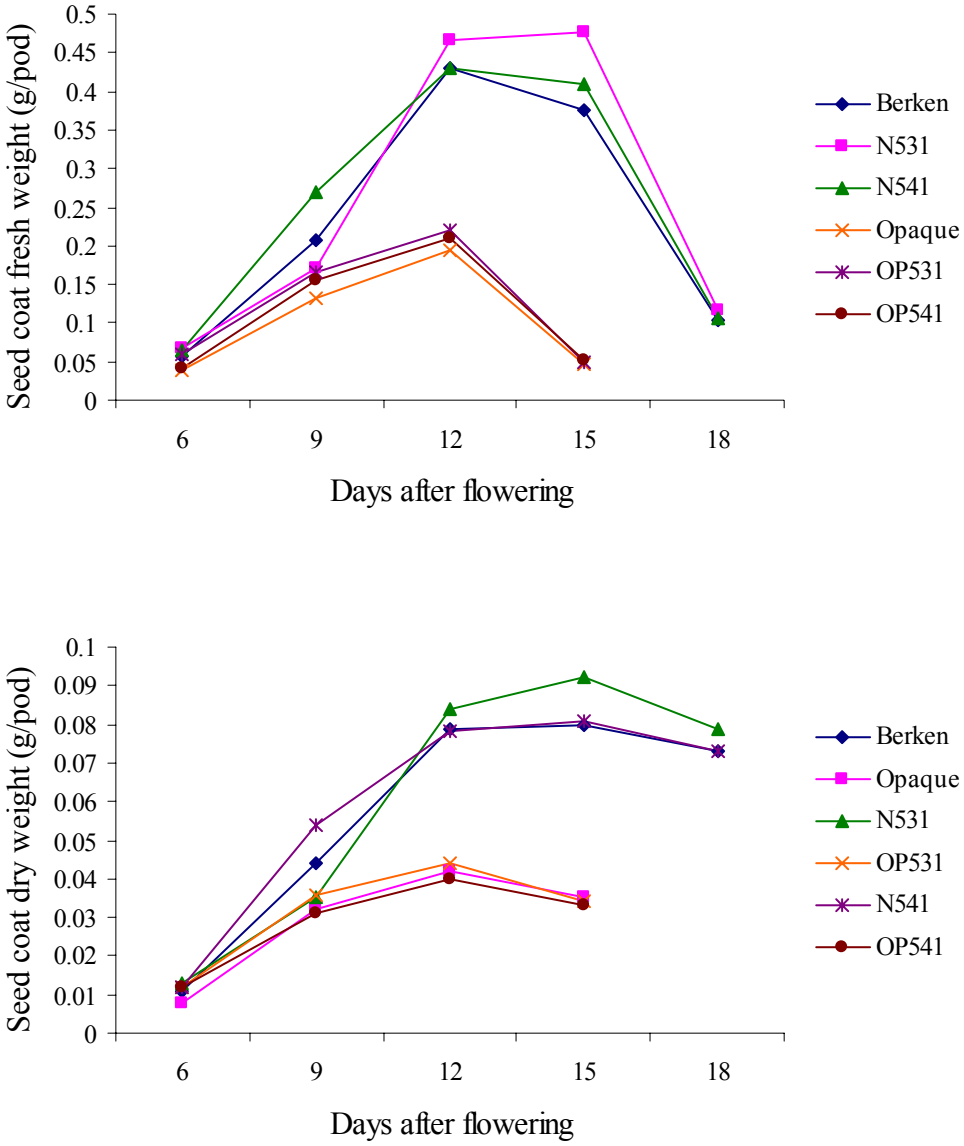

Figure 5 Accumulation of seed coat fresh weight per pod (a) and dry weight per pod (b) of opaque vs. normal leaf genotypes at 3 to 18 days after flowering.

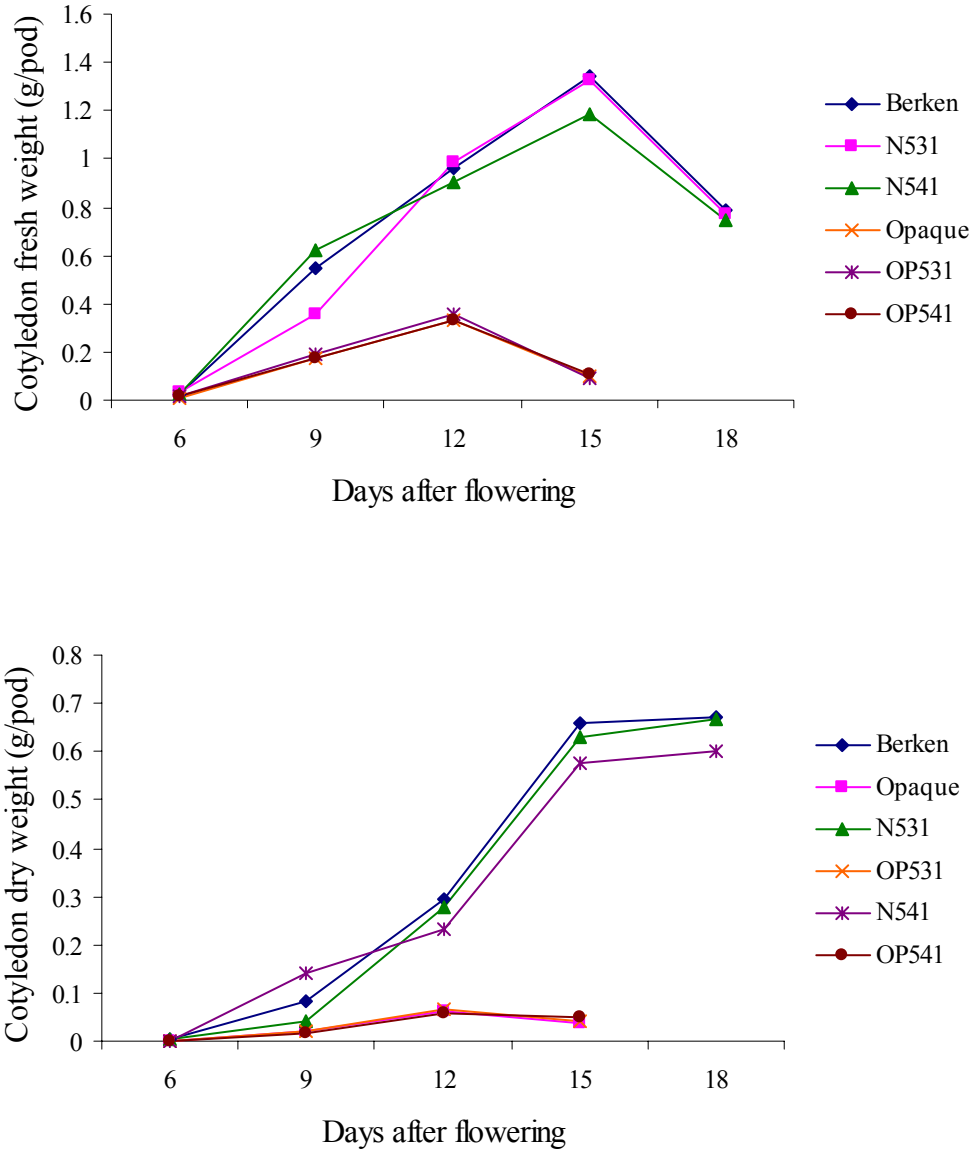

Figure 6 Accumulation of cotyledons fresh weight per pod (a) and dry weight per pod (b) of opaque vs. normal leaf genotypes at 3 to 18 days after flowering.

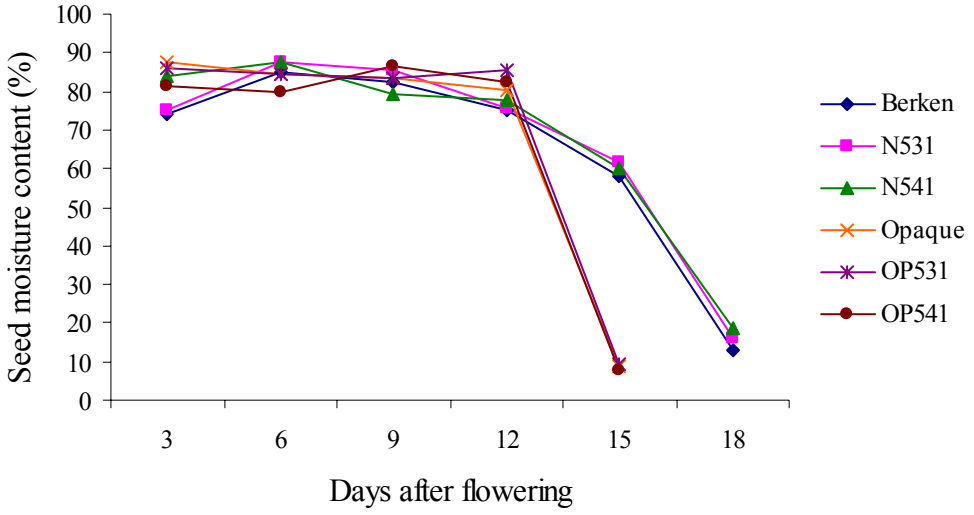

Figure 7 Seed moisture content per pod of opaque vs. normal leaf genotypes at 3 to 18 days after flowering.

Seed cell morphology

Light microscopic study of seed morphology revealed that seed initiation began at about 3 days after flowering in both opaque and normal leaf genotypes (Figure 8a and 9a) and they were different in growth at day 6 of seed development (Figure 8b and 9b). Difference in shape and size began at day 9 of seed development as the mutant seed became smaller than the normal one (Figure 8c and 9c). At day 12 after flowering, the normal seed grew while the seed coat remained attach to the pod wall, whereas the mutant seed shrank and separated from the pod wall tissues (Figure 8d and 9d). This might be the reason that the seed stopped growing beyond this date. The normal seed kept growing up to 15 days after flowering (Figure 9e) while the mutant seed was smaller at about day 13 of seed development (Figure 9e) and became wrinkled one day later. Parts of cotyledons were tightly adhered to seed coat (Figure 8f). During seed maturity the normal seed was also slightly reduced in size but the cotyledons were fully filled inside the seed coat (Figure 9f).

A histological study of the outer epidermal cotyledonary cells revealed that these cells were able to differentiate into transfer cells of both mutant and normal seeds. At 9 days of seed development, both types of seed were able to induce transfer cells from the outermost cotyledonary epidermal cells (Figure 10a and b). However, they were different in shape and size. Epidermal transfer cells of normal seeds were more uniform in size with rectangular shape of approximately 1000 to 1300 μm long and 100 to 200 μm thick. While the transfer cells of mutant seed varied in shape and sizes with approximately 400 to 1000 μm long and 300 to 600 μm thick. The epidermal transfer cells of normal seed were longer and thinner than those of the mutant.

During 12 to 15 days of seed development in normal leaf genotypes, the transfer cells were stable in shape and size while the parenchyma cells underneath the transfer cell layer were filled with starch granules (Figure 10c and e). However, transfer cells of the cotyledons from the mutant seeds malformed at 12 days of seed development. Some cells were vacuolated, some became round (Figure 10d) and finally deteriorated at 14 days of seed development (Figure 10f). Parenchyma cells were vacuolated with very few starch granules. The starch grains of opaque leaf genotype were also smaller and varied in size than those from the normal one (Figure 11).

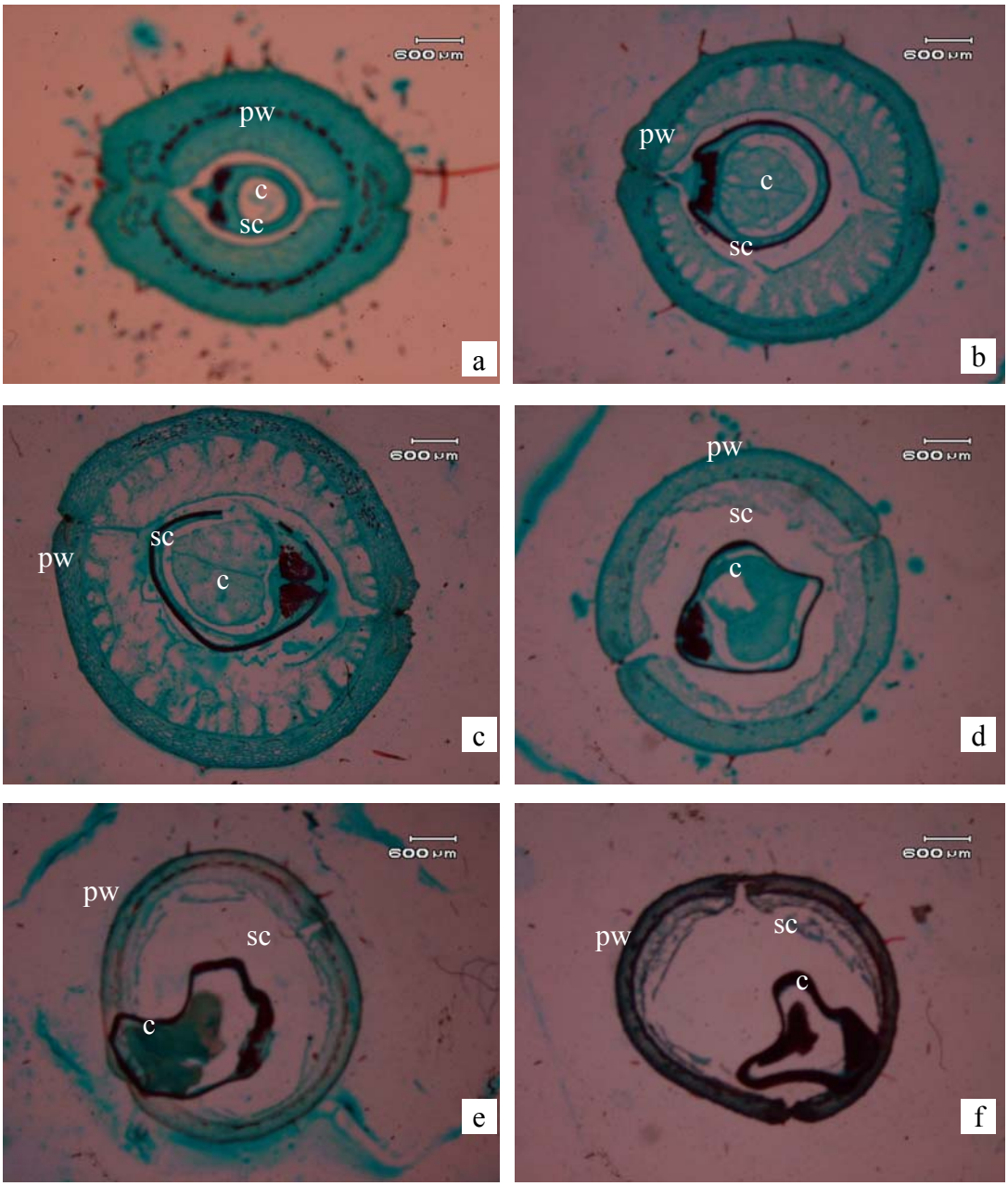

Figure 8 Photographs from light microscope of pod and seed cross sections from opaque leaf plant at 3 (a), 6 (b), 9 (c), 12 (d), 13 (e), and 14 (f) days after flowering. c: cotyledons, sc: seed coat, pw: pod wall.

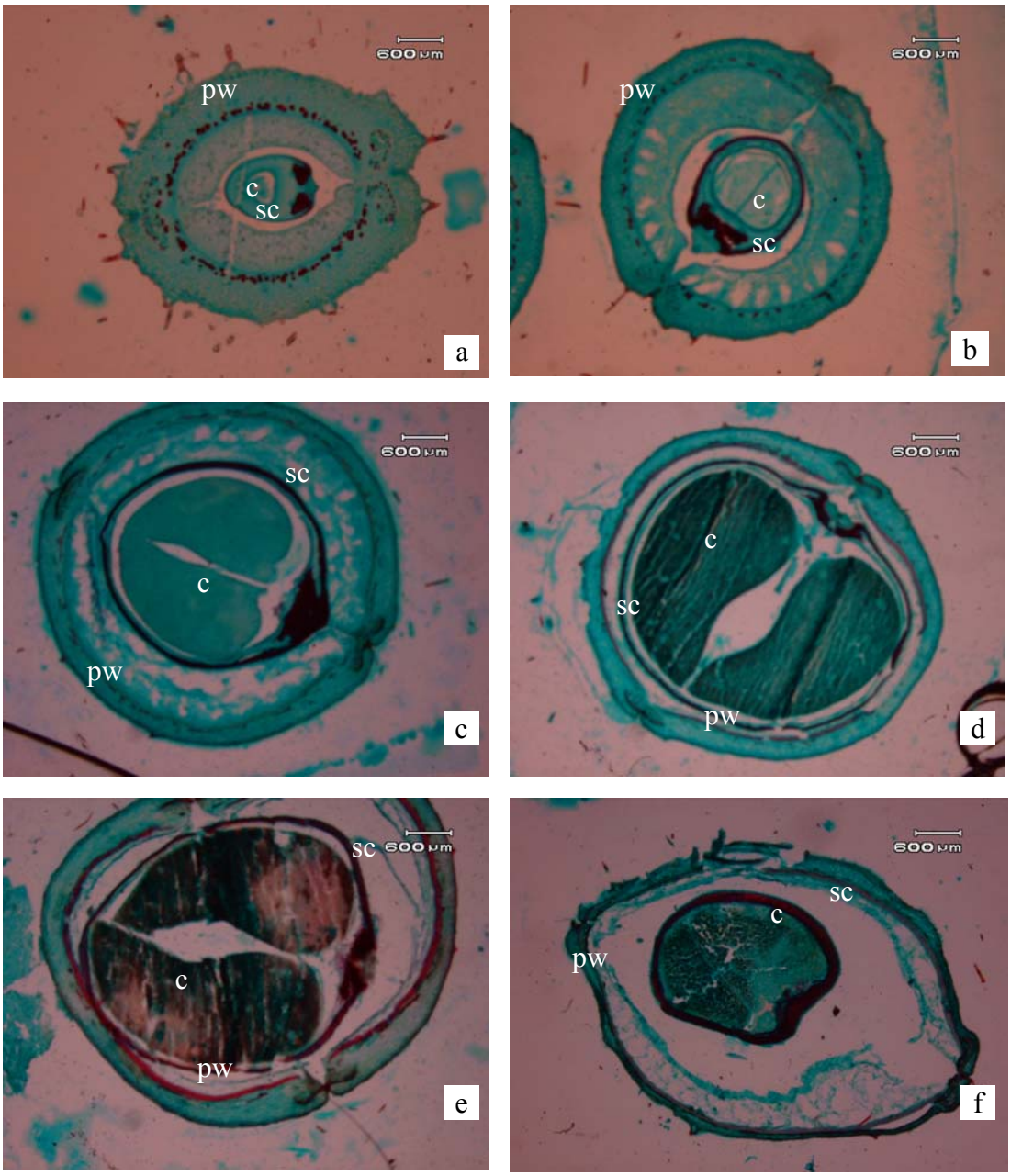

Figure 9 Photographs from light microscope of pod and seed cross sections from normal leaf plant at 3 (a), 6 (b), 9 (c), 12 (d), 15 (e), and 17 (f) days after flowering. c: cotyledons, sc: seed coat, pw: pod wall.

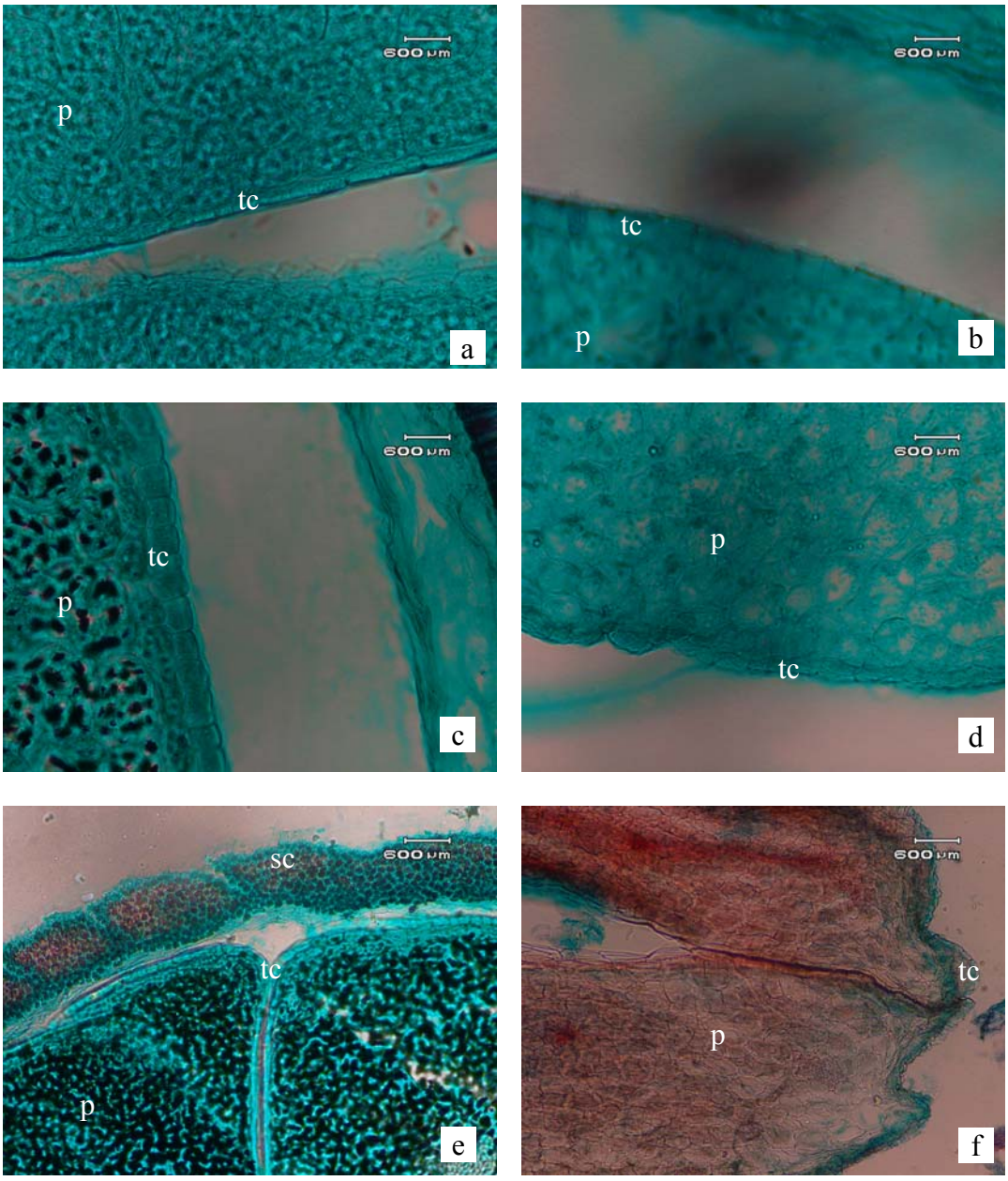

Figure 10 Photographs from light microscope of pod and seed cross sections from opaque at 9 (b), 12 (d), and 14 (f) and normal leaf plant at 9 (a), 12 (c), and 15 (e) days after flowering. p: parenchyma cell, sc: seed coat, tc: transfer cell.

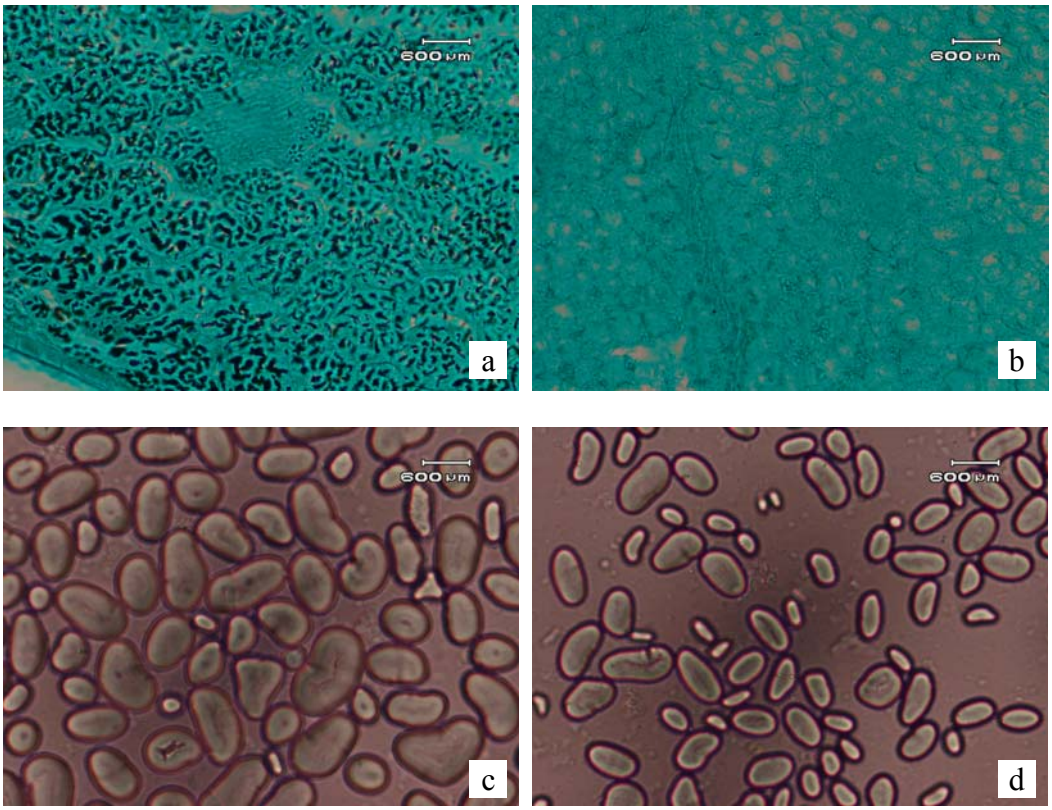

Figure 11 Photographs from light microscope of starch content in parenchyma cells of seed from normal (a) and opaque leaf plant (b), and starch grains of seed from normal (c) and opaque leaf plant (d) at 15 days after flowering.

Inheritance of opaque leaf and linkage test

The appearance of mungbean opaque leaf line used in this study was compared with the normal one in Figure 12. All F_1 plants obtained from Berken x Opaque leaf line showed normal green leaf, purple petiole and determinate growth habit. This result indicated that green leaf, purple petiole and determinate growth are dominant over opaque leaf, green petiole and indeterminate growth habit, respectively. The dominant nature of these traits was further confirmed in the F_2 generation. The observation made on the F_2 plants indicated that 70 plants had normal leaf while 15 plants had opaque leaf. A Chi-square test for goodness-of-fit to a 3:1 segregation ratio showed that the normal leaf was completely dominant to opaque leaf and the trait is controlled by a single gene.

The number of F_2 plants showing purple and green petiole fitted well with a 3:1 ratio indicated that purple petiole was dominant to the green one. Likewise, determinate growth habit was dominant to the indeterminate one (Table 3). The expected ratio of 3:1 revealed that these traits were also controlled by a single gene.

The gene controlling opaque leaf was tested whether it linked with either the petiole color or growth habit genes. Using F_2 data , co-segregation between two traits can be determined from number of plants in each phenotypic class as shown in Table 2. The number of F_2 plants co-segregating in leaf appearance and petiole color gave a good fit to 9 normal leaf - purple petiole : 3 normal leaf - green petiole : 3 opaque leaf - purple petiole : 1 opaque leaf - green petiole with a probability of 0.2 to 0.05 which indicated that the genes conditioning leaf appearance and petiole color were independent and located on different chromosomes or on the same chromosome with a distance of greater than 50 cM. The same conclusion was drawn regarding leaf appearance trait and growth habit (Table 4). Thus it can be concluded that the leaf appearance gene is independent of petiole color and growth habit.

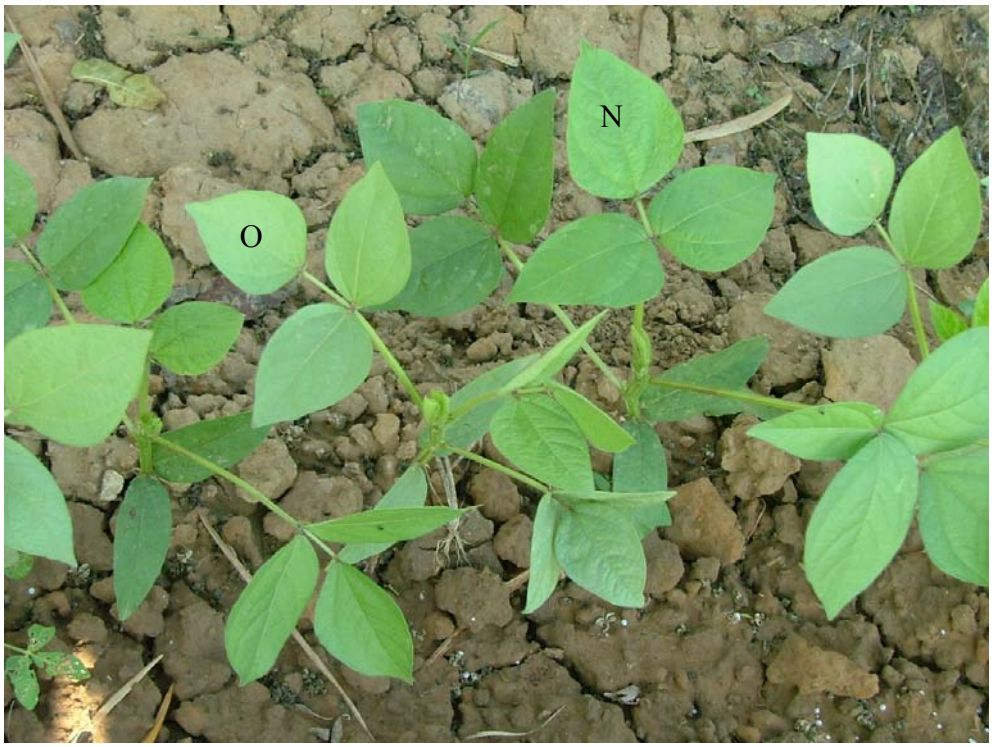

Figure 12 Photograph of plants with normal leaf (N) vs. opaque leaf (O) at seedling growth stage.

Table 3 Number of F_2 plants segregating for opaque leaf, petiole color, and growth habit.

Traits	F_1 phenotype	No. F_2 of plants	Exp. ratio	Prob. of χ^2
Normal vs. opaque leaf	Normal	70:15	3:1	0.20-0.05
Purple vs. green petiole	Purple	64:21	3:1	0.95-0.80
Determinate vs. indeterminate growth	Determinate	58:27	3:1	0.20-0.05

Table 4 Linkage test between two traits in F_2 plants.

Traits	Number. of plants [+]				Exp. ratio	Prob.
	NP	NG	OP	OG		
Leaf vs. petiole color	52	18	12	3	9:3:3:1	0.20-0.05
	ND	NI	OD	OI		
Leaf vs. growth habit	47	23	11	4	9:3:3:1	0.95-0.80

[+] N, P, G, O, D, and I stand for normal, purple, green, opaque, determinate, and indeterminate, respectively.

Molecular markers linked to opaque leaf

Only 8 primer pairs out of 27 SSR primer pairs were successfully amplified the parental DNA. Among them, 5 (VM22, VM27, VM31, VM37, and VM71) amplified a single band while the rests (VM12, VM13, and VM23) produced multiple bands and thus the later were not used for further study.

Out of the 5 primer pairs that amplified a single band, only VM27 showed polymorphism between the parents. It was used to further characterize the two bulks and 80 F_2 plants and gave a band in the normal but not in the opaque leaf bulked DNA. The segregating scores were subjected to Chi-square test for goodness-of-fit with the 1:2:1 ratio and could be concluded that it was not fitted to the expected ratio and thus was excluded from further linkage analysis.

Among 28 ISSR primers used in this study, 19 showed multiple (2 to 11) bands, while 2 primers produced a single band. Among 21 amplified primers, 12 were polymorphic between the parents. They were then used to identify the opaque and normal leaf bulks. Four primers, $(GA)_9AT$, $(TG)_8A$, $(TC)_9A$, and $(ATG)_6G$, could distinguish them. When these 4 primers were applied to individual F_2 plants, the segregation did not fit to a 3:1 ratio and thus they were excluded from the linkage analysis.

Since the SSR and ISSR techniques did not provide markers closely linked to *op* gene, were search for additional markers was undertaken using AFLP analysis. From 193 primer combinations, 96 primers (49.74%) showed putatively polymorphic bands between the parents. However, only 22 out of 96 primers (11.4%) produced polymorphic bands that distinguished the opaque leaf parent and bulk from normal leaf parent and bulk. When these primers were used to further characterize the F_2 plants, only 6 primer combinations , ACA/AGG, AGG/ATA, AAA/TGA, AA/TAC, AAA/TAC, and AC/AGG amplified bands that were consistently polymorphic between the opaque and normal leaf plants. Five primers were found segregate following a 3:1 ratio, indicated that these markers segregated as a monogenic dominant.

The most closely linked marker to *op* gene was AGG/ATA with a probability of 0.00. A single regression analysis revealed that the R^2 value (phenotypic value explained) of this marker accounted for 77.56% of the total variation in the opaque leaf trait in the F_2 progenies. Only two recombinant plants were observed between this marker and the *op* gene, thus the genetic distance between them is 3.4 cM. The other two markers that were found linking to opaque leaf gene were ACA/AGG and AAA/TGA. ACA/AGG marker was 8.0 cM apart with a probability of 0.00 and R^2 of 64.34%, while the genetic distance between AAA/TGA marker and the gene was 7.2 cM with the probability of 0.00 and R^2 of 64.25% (Figure 13).

Multiple regression analysis of the two most closely linked markers, ACA/AGG and AGG/ATA, revealed the combined effect of R^2 84.33 %. Multipoint linkage analysis suggested the most likely order of these loci are ACA/AGG, *op* gene, AGG/ATA, AAA/TGA, AAA/TAC, and ACA/GG. The linkage group spanned at a length of 60.3 cM. All five markers showed a coupling linkage with *op* gene.

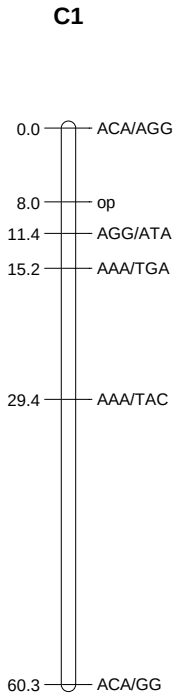

Figure 13 A partial linkage map between AFLP markers and *op* gene.

DISCUSSION

Determination of chlorophyll content

The chlorophyll content in opaque leaf plants was 16 to 34% less than normal leaf plants, indicated that the opaque leaf is a type of chlorophyll-deficient mutant. However, the chlorophyll a/b ratio of opaque and normal leaves were not different from 3:1 which is the normal ratio found in most land plants (Nobel, 1999) revealed that both chlorophyll a and b are proportionally reduced.

Among the opaque leaf genotypes, there were significantly difference in chlorophyll contents between Opaque (0.118 g/m^2) and the other two genotypes, OP531 (0.148 g/m^2) and OP541 (0.146 g/m^2), although they gave the same leaf color appearance. The result was different from Spech *et al*. (1975) who reported that chlorophyll content generally reflected the difference in color that appeared to the eyes and found that the intensity of green coloration of sweetclover ranged from pale yellow-green to dark green according to chlorophyll content from 25 to 87.5% of the wild type. The difference in chlorophyll contents among the opaque lines in this experiment is relatively less pronounced than their sweetclover.

Generally, a reduction in chlorophyll content can lead to lethality of the plants or retardance of plant growth (Keck *et al*., 1970). In this experiment, opaque leaf genotypes had less germination (~ 60%), delay in germination (7 to 10 days as compared to 3 to 4 days in normal plants), and grew very slowly. Saunder (1960) reported that a chlorophyll deficient mutant of cowpea was much weaker than the normal plant. However, some mutants such as M39 has loss approximately 35 % chlorophyll as compared to the normal plant but still showed similar photosynthetic rates and productivity.

Determination of chlorophyll fluorescence

The analysis of maximum quantum efficiency of PSII photochemistry, F_v/F_m that obtained from dark-adapted leaf was statistically differences between opaque leaf and normal leaf plants. The values of F_v/F_m were 0.815 to 0.825 in opaque leaf plants and 0.838 to 0.843 in normal plants. However, these values were not different from the values of 0.83 to 0.85 which usually found in unstressed leaves of higher plants (Björkman and Demmig, 1987). They investigated 37 species of C_3 plants and found that they had constant values of F_v/F_m at 0.832 ±0.004. Since the F_v/F_m ratio corresponds to the maximum efficiency of energy capturing (photon) by reaction centers or the proportion of light energy, thus reduction in F_v/F_m value indicated that less photon was used in driving the light reaction PSII of photosynthesis in the form of photochemistry.

The F_v/F_m ratio from dark-adapted leaf of opaque leaf mutant was not much less from the normal one, reducing approximately 1.5 to 2.8%. However, averaging across all the opaque genotypes the F'_v/F'_m ratio from light-adapted leaf showed significantly less value than the normal leaf genotypes, approximately 7.4 to 15.2% throughout the day time. The F'_v/F'_m value has a similar function as F_v/F_m but the former represent the actual efficiency of light reaction under day light or represents the quantum yield of electron transport (Genty et al., 1989; Rolfe and Scholes, 1995). This indicated that the mutant has less efficiency of excitation captured in open PSII reaction center or, in the other hand, has less electron transport through the PSII reaction center.

The NPQ value indicates the de-excitation of light-generated excited state in the chlorophyll associated with PSII (Ruban and Horton, 1995). Since all opaque leaf genotypes gave higher NPQ (10 to 20%) at any given time of measurement, the plants have higher de-excitation of chlorophyll fluorescence or have more ability to dissipate excess energy thermally. Thus they could acclimate to high irradiance than the normal plants or they possessed a mechanism to protect against excess absorption by PSII.

Seed growth and development

Pod is the primary sink of photosynthate during seed development. Accumulation of pod dry weight, pod wall and seed are important processes in yield production (Egli and Leggett, 1976). The pattern analyses of pod, pod wall, seed, seed coat and cotyledons fresh and dry weight gave similar results in which all opaque leaf genotypes gave lower fresh and dry weight than the normal leaves.

The pattern of growth and development of normal seed as indicated by fresh and dry weight parameters could be divided into 3 phases. In the first phase, dry matter accumulated slowly in the seed. This phase began from first day up to 6 days after flowering. At the end of this phase, the seed dry weight represented approximately 2% of the final seed weight. The seed moisture content remained 75 to 90% throughout this phase. In the second phase, seed accumulated dry matter rapidly from 6 to 15 days. The seed moisture content reduced from about 85% to 60% at 15 days after flowering. In the last phase, seed accumulation was stable or increased very slowly but seed water content reduced to 13 to 18%. However, seed growth and development pattern of opaque leaf mutants did not follow this pattern, but rather had shorter seed-filling period and terminated growth and development at 12 days after flowering.

At day 12 of seed development was a critical point for development of pod wall, seed, seed coat, and cotyledons of all opaque leaf genotypes. All parts were reduced in dry matter accumulation at the same time. The dry matter accumulation was suddenly reduced at 13 days which corresponded to pod wilting. Additionally, some pods fell off easily at this stage, seeds were dramatically reduced in size and became wrinkled within one or two days. Le Deunff and Rachidian (1998) concluded that 55% moisture content is the critical point for the last stage of seed filling phase which appears to be specific feature of physiological maturity in the leguminous family. We also found that seeds from opaque leaf genotypes rapidly loss their moisture content to about 8 to 10% at 13 days after flowering and dry weight accumulation in the seed was only 10 to 12% of the normal one.

The sudden reduction of seed water content at about 13 days after flowering may lead to terminating of seed growth and development of the opaque mutant. Fraser *et al*. (1982) suggested that progressive dehydration of the seed involved in termination of seed growth. Similar report by Tekrony *et al*. (1979) concluded that a rapid decrease of moisture content of seeds in response to environment occurred as the seed was no longer connected to the plants vascular system.

The risk of seed abortion decreases with its development (Ney *et al*., 1993). The final stage in seed abortion corresponded to the end of the cell division of the embryo (Duthion and Pigeaive, 1991). It was found in this study that seed younger than 15 days after flowering did not completely tolerate to the fastest drying condition. Although the dried seed of opaque leaf mutant which terminated their growth at 12 days after flowering could be generated up to about 60%, some seedlings were abnormal such as no hairy root, no shoot, no primary leaf, no trifoliate leaf, or have only one cotyledon. These seedlings were very weak and died prematurely.

Seed cell morphology

The opaque leaf plants have low chlorophyll content and the maximum quantum efficiency of PSII photochemistry that may lead to low photoassimilates, including sucrose. Weber *et al*. (1998) suggested that low concentration of sucrose caused a specific down-regulation of storage function at different levels. On a metabolic level, less sugar leads to less carbon and thus less starch. On a cellular level, it causes changes in cell structure and differentiation. Sucrose can induce the differentiation process of storage parenchyma cells which explains the change of cotyledonary transfer cells of opaque leaf genotype at 12 days of seed development as the cells lost their identity and changed from rectangular and square into round shaped. Additionally, epidermal transfer cells became vacuolated and deteriorated at 13 to 14 days of seed development.

Transfer cells are very important for seed growth and development. Borisjuk *et al*. (2002) found that seeds E2748 pea mutant had no transfer cells which made

them retard in growth and abort before maturation. The deterioration of membrane of the transfer cells at 12 days after flowering may change the membrane properties and resulted in cellular deterioration and inability to maintain physiological integrity of membrane in the seed at 12 days after flowering. Corbineau *et al*. (2000) found that young seeds induced strong deterioration of cell membrane. The deleterious effects of drying on membranes normally decreased during pea seed development. Desiccation tolerance in pea seed is associated with a decrease in sucrose content in the embryo and cotyledons and accumulation of oligosaccharides such as raffinose and stachyose in the axis. Boyer (1996) reported that wrinkled pea seed mutants resulted from altered sugar conditions by increasing sucrose to starch ratio. This may be the reason that normal mungbean seeds show more desiccation tolerance than mutant seed, since the seed from normal leaf plants have more photosynthate accumulation.

Losing of transfer cell membrane integrity of the seed from opaque leaf plant may lead to an extreme water uptake. As water is withdrawn, the membrane structure can be damaged due to less sucrose and oligosaccharides to replace water and protect the membrane surface (Caffrey *et al*., 1988). Cellular consolidation can be initiated if water loss is not too rapid (Black *et al*., 1999). The rapid loss of water can also result in loss of membrane integrity (Caffrey *et al*., 1988). The initial loss of water leads to reduction of turgor and alteration in osmotic balance (Black *et al*., 1999). Caffrey *et al*. (1988) suggested that sugar and oligosaccharides can form a substitute for water uptake at the membrane surface and protect the damage of membrane. Since seeds from opaque mutant genotypes may have less photosynthate to substitute the water, the injury from water uptake may cause a permanent loss of membrane integrity as can be seen in Figure 10f.

Inheritance of opaque leaf and linkage test

The opaque leaf line was later in maturity, set very small and wrinkled seed. To obtained F_1 seeds, this line should be used as the male parent, just as crossing with Berken. Although, the mutant is male and female fertile but using them as male parent is more successful as these are still ample amount of fertile pollen formed by the

opaque mutant. This mutant was named “opaque leaf” since its leaves have chalky green color compared to the bright green color of normal leaves as shown in Figure 12. The opaque leaf plants can be easily distinguished from normal leaf plants from the seedling stage on. The leaf color is stable until the plant reaches maturity. Thus, the segregating progeny can be visualized at any stages of plant growth. We concluded that the opaque leaf mutant was controlled by a recessive gene and proposed the gene symbol *op*. Wettstein (1961) reported that most chlorophyll deficient mutants behaved as single recessive traits. Several chlorophyll deficient mutants of *Solanum* and wheat were also conditioned by a single recessive gene (Jones et al., 1963; Hermsen *et al*., 1973; Williams *et al*., 1985).

Molecular markers linked to opaque leaf

The use of ISSR, SSR and AFLP markers in tagging *op* gene revealed that degree of polymorphism in parental survey exhibited by AFLP markers was the highest (49.74%), followed by ISSR (42.86%), while the SSR marker gave very low polymorphism (3.7%). This is due to the fact that AFLP generates multiple bands in a single assay. Russell *et al*. (1997) compared the polymorphism in barley genome using RFLP, AFLP, SSR and RAPD markers and found that AFLP was the most efficient marker because of its capacity to reveal many polymorphic bands in a single lane. The number of bands per lane (or per PCR) for AFLP was about 50, compared to 1 band per lane for SSR (Russell *et al*., 1997). Similarly, ISSR markers which can detect polymorphism in either microsatellite or inter-microsatellite loci that tandemly repeat from two to many thousands of times in a genome, and thus can generate many alleles per locus (Brown *et al*., 1996; Li *et al*., 2000) compared to SSR. Although, SSRs are abundant, dispersed throughout the genome and have a high level of allelic diversity (Powell et al., 1996; Morgante *et al*., 2000), not many primers sequences have been publicly developed for *Vigna* species (Wang *et al*., 2004). Moreover, SSR amplification across species is limited by high variation of SSR-flanking sequences between species and frequent loss of the SSR motif where amplification occurs (Proven *et al*., 1996; Westman and Kresovich, 1998). These factors may generate low polymorphism of SSR markers in tagging *op* gene in this study.

CONCLUSION

1. Opaque leaf plants have significantly less in chlorophyll a, b, and total (a+b) contents than the normal leaf plants, whereas there were no differences in chlorophyll a/b from approximately 3:1 ratio.

2. Analysis of chlorophyll fluorescence showed that opaque leaf plants have significantly less F_v/F_m and F'_v/F'_m values than normal leaf plants, but higher in NPQ value.

3. The pattern of seed growth and development of normal leaf plants could be divided into 3 phases initiated from 3 and terminated at 18 days after flowering, whereas the pattern of seed growth of opaque leaf plants terminated at 15 days due to termination of seed growth at 12 days after flowering.

4. Both seeds from opaque leaf and normal leaf plants display cotyledonary transfer cells at 9 days after flowering, but became deteriorated at 12 days after flowering in seed from opaque leaf plant.

5. The *op* gene controlling opaque leaf characteristic is recessive to normal leaf trait and independent from the gene controlling petiole color and growth habit.

6. In this study, AFLP was the most efficient technique for tagging *op* gene and the most closely linked marker was AGG/ATA with the distance 3.4 cM apart from the *op* gene.

7. Identification of closely linked marker to *op* gene can be a valuable tool for future studies of plant chlorophyll and photosynthesis. Since opaque leaf is chlorophyll deficient mutant and the plants set small and wrinkled seeds with low yield potential. It is assumed that the *op* gene may involve in reduction of certain steps of chlorophyll biosynthesis that affect photosynthetic processes. The opaque leaf

plants grow normally under good sun light, showing that they are male and female fertile. Thus this mutant can be easily maintained in the field for further study.

LITERATURE CITED

Adam, C.A., M.C. Fjerstad and R.W. Rinne. 1983. Characteristics of soybean seed maturation : necessity for slow dehydration. **Crop Sci.** 23:265-267.

Ahloowalia, B.S. and M. Maluszynski. 2001. Induced mutations - A new paradigm in plant breeding. **Euphytica** 118: 167-173.

Akaki, H., Y. Yokozeki, A. Inagaki, A. Nakamura and T. Fujimura. 1996. A co-dominant DNA marker closely linked to the rice nuclear restorer gene, *Rf-1*, identified with inter-SSR fingerprinting. **Genome** 39:1205-1209.

Ammiraju, J.S.S., B.B. Dholakia, D.K. Santra, H. Singh, M.D. Lagu, S.A. Tamhankar, H.S. Dhaliwal, V.S. Rao, V.S. Gupta and P.K. Ranjekar. 2001. Identification of inter simple sequence repeat (ISSR) markers associated with seed size in wheat. **Theor. Appl. Genet**. 102:726-732.

Amuti, K.S. and C.J. Pollard. 1977. Soluble carbohydrates of dry and developing seeds. **Phytochemistry** 16:529-532.

Bahl, J.R. and P.K. Gupta. 1982. Chlorophyll mutations in mungbean (*Vigna radiata* (L.) Wilczek). **Theor. Appl. Genet**. 63: 23-26.

Baker, N.R., K. Oxborough, T. Lawson and J.I.L. Morison. 2001. High resolution imaging of photosynthetic activities of tissues, cells and chloroplasts in leaves. **J. Expt. Bot.** 52:615-621.

Baker, N.R. and E. Rosenqvist. 2004. Applications of chlorophyll fluorescence can improve crop production strategies: an examination of future possibilities. **J. Expt. Bot.** 55:1607-1621.

Balachandran, S. and C.B. Osmond. 1994. Susceptibility of tobacco leaves to photoinhibition following infection with two strains of tobacco mosaic virus under different light and nitrogen nutrition regimes. **Plant Physiol.** 104:1051-1057.

Bernardo, R. 1992. Relationship between single-cross performance and molecular marker heterozygosity. **Theor. Appl. Genet.** 83:628-634.

Bilger, W., U. Schreiber and M. Bock. 1995. Determination of the quenching efficiency of photosystem II and of non-photochemical quenching of chlorophyll fluorescence in the field. **Oecologia** 102:425-432.

Björkman, O. and B. Demmig. 1987. Photon yield of O_2 evolution and chlorophyll fluorescence characteristics at 77 K among vascular plants of diverse origins. **Planta** 170:489-504.

Black, M., F. Corbineau., H. Gee and D. Côme. 1999. Water content, raffinose, and dehydrins in the induction of desiccation tolerance in immature wheat embryos. **Plant Physiol.** 120:463-471.

Blackburn, G.A. 1998. Spectral indexes for estimating photosynthetic pigment concentrations: a test using senescent tree leaves. **Int. J. Remote Sensing** 19:657-675.

Blackman, S.A., R.L. Obendorf and A.C. Leopold. 1992. Maturation proteins and sugars in desiccation tolerance to developing soybean seeds. **Plant Physiol**. 100:225-230.

Borisjuk, L., H. Weber, R. Panitz, R. Manteuffel and U. Wobus. 1995. Embryogenesis of *Vicia faba* L. Histodifferentiation in relation to starch and storage protein synthesis. **J. Plant Physiol.** 147: 203-218.

Borisjuk, L., S. Walenta, H. Rolletschek, M. Mueller-Klieser, U. Wobus and H. Weber. 2002. Spatial analysis of plant metabolism : Sucose imaging within *Vicia faba* cotyledons reveals specific development patterns. **Plant J.** 29:521-530.

Borisjuk, L., S. Walenta, H. Weber, M. Mueller-Klieser and U. Wobus. 1998. High resolution histographical mapping of glucose concentrations in developing cotyledons of *V. faba* in relation to mitotic activity and starch accumulation: Glucose as a possible developmental trigger. **Plant J.** 15:583-591.

Boyer. C. 1996. Biochemical genetics of carbohydrate metabolism in source and sink tissue. pp. 341-367. *In* E. Zamski, and A.A. Schaffer, eds. **Photoassimilate Distribution in Plants and Crops, Source-Sink Relationship**. Marcel Dekker, New York.

Brooks, R.M., M.V. Bradley and T.I. Anderson. 1950. **Plant Microtechnique Manual.** University of California, California.

Brown, S.M., A.K. Szewc-Hcfadden and S. Kresovich. 1996. Development and application of simple sequence repeat (SSR) loci for genome analysis. pp.147-159. *In* P.P. Jauhar, ed. **Method of Genome Analysis in Plants.** CRC Press, New York.

Caffrey, M., V. Fonseca and A.C. Leopold. 1988. Lipid-sugar interactions. **Plant Physiol**. 86:754-758.

Cervara, M.T., J. Gusmao, M. Steenacker, J.peleman, V. Storme, A. Vanden-Broech, M. Van-Montagu and W. Borerjan. 1996. Identification of AFLP markers for resistance against *Melampsora larici*- population in Populous. **Theor. Appl. Genet.** 93:733-737.

Chaitieng, B., A. Kaga, O.K. Han, X.W. Wang, S. Wongkaew, P. Laosuwan, N. Tomooka and D.A.Vaughan. 2002. Mapping a new source of resistance to powdery mildew in mungbean. **Plant Breed.** 121: 521-525.

Corbineau, F., M.A. Picard, J.-A. Fougereux, F. Ladonne and D. Côme. 2000. Effects of dehydration conditions on desiccation tolerance of developing pea seeds as related to oligosaccharide content and cell membrane properties. **Seed Sci.** 10:329-339.

Cregan, P.B., J. Mudge, E.W. Fickus, L.F. Marek, D. Danesh, R. Denny, R. C. Shoemaker, B. F. Mathews, T. Jarvik and N.D. Young. 1999. Targeted isolation of simple sequence repeat markers through the use of bacterial artificial chromosomes. **Theor. Appl. Genet.** 98:919-928.

Crowe, J.H., L.M. Crowe and D. Chapman. 1984. Preservation of membranes in anhydrobiotic organisms: the role of trehalose. **Science** 223:701-703.

Crowe, J.H., L.M. Crowe, J.F. Carpenter, A.S. Rudolph, C.A. Wistrom, B.J. Spargo and T.J. Anchordoguy. 1988. Interactions of sugars with membranes. **Arch. Biochem. Biophys.** 947:367-384.

Curran, P.J., J.L. Dungan and H.L. Gholz. 1990. Exploring the relationship between reflectance red edge and chlorophyll content in slash pine. **Tree Physiol**. 7:33-48.

Dahiya, B.S. 1973. Improvement of mungbean through induced mutations. **Indian J. Genet. Pl. Br.** 33: 460-468.

Dellaporta, S.L., J. Wood and J.B. Hicks. 1983. A plant DNA minipreparation: version II. **Plant Mol. Biol. Rep.** 1: 19-21.

Duthion, C. and A. Pigeaive. 1991. Seed lengths corresponding to the final stage in seed abortion of three grain legumes. **Crop Sci.** 31:1579-1583.

Egli, D.B. 1994. Seed growth and development. pp. 127-147. *In* K.J. Boote, J.M. Bennet, T. Sinclair, G.M. Paulsen, eds. **Physiology and Determination of Crop Yield.** Crop Society of America, Madison, WI.

Egli, D.B. and J.E. Leggett. 1976. Rate of dry matter accumulation in soybean seeds with varying source-sink ratios. **Agron. J.** 68:371-374.

Ellis, R. and T.D. Hong. 1994. Desiccation tolerance and potential longevities of developing seeds of rice (*Oryza sativa* L.). **Ann. Bot.** 73:501-506.

Filella, I, I. Serrano, J. Serra and J. Peñuelas. 1995. Evaluating wheat nitrogen status with canopy reflectance indices and discriminant analysis. **Crop Sci**. 365:1400-1405.

Fraser, J., D.B. Egli and J.E. Leggett. 1982. Pod and seed development in soybean cultivars with differences in seed size. **Agron. J.** 74:81-85.

Gallardo, K., C. L. Signor, J. Vandekerckhove, R.D. Thompson and J. Burstin. 2003. Proteomics of *Medicago truncatula* seed development establishes the time frame of diverse, metabolic processes related to reserve accumulation. **Plant Physiol.** 133:664-682.

Gebhardt, C., E. Ritter, T. Debener, U. Schachtschabel, B. Walkemeier, H. Uhrig and F. Salamini. 1989. RFLP analysis and linkage mapping in *Solanum tuberosum*. **Theor. Appl. Genet.** 78:65-75.

Genty, B., J.-M. Briantais and N.R. Baker. 1989. The relationship between the quantum yield of photosynthetic electron transport and quenching of chlorophyll fluorescence. **Biochimica et Biophysica Acta** 990:87-92.

Genty, B., J. Wonders and N.R. Baker. 1990. Non-photochemical quenching of Fo in leaves is emission wavelength dependent: Consequences for quenching analysis and its interpretation. **Phosyn. Res.** 26:133-139.

Gitelson, A.A., Y. Gritz and M.N. Merzlyak. 2003. Relationships between leaf chlorophyll content and spectral reflectance and algorithms for non-destructive chlorophyll assessment in higher plant leaves. **J. Plant Physiol**. 160:271-282.

Godwin, I.D., E.A.B. Aitken and L.W. Smith. 1997. Application of inter simple sequence repeat (ISSR) markers to plant genetics. **Electrophoresis** 18:1524-1528.

Griffin, J.J., T.G. Ranney and D.M. Pharr. 2004. Photosynthesis, chlorophyll fluorescence, and carbohydrate content of *Illicium* taxa grown under varied irradiance. **J. Amer. Soc. Hort. Sci.** 129:46-53.

Gupta, P.K. 1996. Mutation breeding in mungbean. pp.124-136. *In* A.N. Asthana and D.H. Kim, eds. **Recent Advances in Mungbean Research**. Indian Society of Pulses Research, IIPR, Kanpur, India.

Haldane, J.B.S. 1919. The recombination of linkage values, and the calculation of distance between loci of linked factors. **J. Genet.** 8:299-309.

Hanson, W.D. 1991. Seed protein content and delivery of assimilates of soybean seed embryos. **Crop Sci.** 31:1600-1604.

Hartl, L., V. Mohler, F.J. Zeller, S.L.K. Hsan and G. Schweizer. 1999. Identificaion of AFLP markers closely linked to the powdery mildew resistance gene pm1c and pm 4a in common wheat (*Triticum aestivum* L.). **Genome** 42:322-329.

Hedley, C.L. and M.J. Ambrose. 1980. An analysis of seed development in *Pisum sativum* L. **Ann. Bot.** 46:89-105.

Heinz Walz GmbH. 1999. Photosynthesis Yield Analyzer Mini-PAM, Portable Chlorophyll Fluorometer. **Handbook of Operation.** First edition. Eichenring, Germany.

Hermsen, J.G., T. Ramanna and J. Vogel. 1973. The location of a recessive gene for chlorophyll deficiency in diploid *Solanum tuberosum* by means of trisomic analysis. **Can. J. Genet. Cytol.** 15: 807-813.

Hong, T. D. and R.H. Ellis. 1992. Development of desiccation tolerance in Norway maple (*Acer platanoides* L.) seeds during maturation drying. **Seed Sci. Res.** 2:169-172.

Humphry, E., V. Konduri, J. Lambrides, T. Magner, L. McIntyre, B. Aitken and J. Liu. 2002. Development of a mungbean (*Vigna radiata*) RFLP linkage map and its comparison with lablab (*Lablab purpureus*) reveals a high level of colinearity between the two genomes. **Theor. App. Genet.** 105: 160-166.

Hurry, V., J.M. Anderson, W.S. Chow and C.B. Osmond. 1997. Accumulation of zeaxanthin in abscisic acid-deficient mutants of Arabidopsis does not affect chlorophyll fluorescence quenching or sensitivity to photoinhibition *in vivo*. **Plant Physiol.** 113:639-648.

Johnson, S., C.M. Liu, C.L. Hedley and T.L. Wang. 1994. An analysis of seed development in *P. sativum*. XVIII. The isolation of mutants detective in embryo development. **J. Expt. Bot.** 45:1503-1511.

Jones, B.A., R.L. Plaisted and L.C. Peterson. 1963. The behavior of a chlorophyll mutant in *Solanum vernei* (Bitt. Et Wittm). **Am. Potato J.** 40: 109-114.

Keck, R.W., R.A. Dilley and B. Ke. 1970. Photochemical characteristics in a soybean mutant. **Plant Physiol**. 46:699-704.

Kirchhoff, W.R., A.E. Hall and M.L. Roose. 1989. Inheritance of a mutation influencing chlorophyll content and composition in cowpea. **Crop Sci.** 29:105-108.

Kosambi, D.D. 1944. The estimation of map distance from recombination values. **Ann. of Eugenics** 12: 172-175.

Koster, H.L. and A.C. Leopold. 1988. Sugars and desiccation tolerance in seeds. **Plant Physiol.** 88:829-832.

Lakhanpaul, S., S. Chadha and K.V. Bhat. 2000. Random amplified polymorphic DNA (RAPD) analysis in Indian mungbean (*Vigna radiata* (L.) Wilczek) cultivars. **Genetica** 109: 227-234.

Lambrides, C.J., R.J. Lawn, I.D. Godwin, J. Manners and B.C. Imrie. 2000. Two genetic linkage maps of mungbean using RFLP and RAPD markers. **Aust. J. Agric. Res.** 51: 415-425.

Lander, E.S., P. Green, J. Abrahamson, A. Barlow, M.J. Daly, S.E. Lincoln and L. Newburg. 1987. Mapmaker: An interactive computer package for constructing primary genetic linkage maps of experimental and natural populations. **Genomics** 1: 174-181.

Le Deunff, Y. and Z. Rachidian. 1998. Interruption of water delivery at physiological maturity is essential for seed development, germination and seedling growth in pea. **J. Exp. Bot.** 39:1221-1230.

Le Page-Degivry, T.M. and G. Garello. 1991. Onset of water stress tolerance in developing *Helianthus annuus* embryos. **Seed Sci. Res.** 1:221-227.

Li, C.D., B.G. Rossnagal and G.J. Scoles. 2000. The development of oat microsatellite markers and their use in identifying relationships among *Avena* species and oat cultivars. **Theor. Appl. Genet.** 101: 1259-1268.

Li, C.D., C.A. Fatokun, B. Ubi, B.B. Singh and G.J. Scoles. 2001. Determining genetic similarities and relationships among cowpea breeding lines and cultivars by microstellite markers. **Crop Sci.** 41:189-197.

Mackill, D. J., Z. Zhang, E.D. Redona and P.M. Colowit. 1996. Level of polymorphism and genetic mapping of AFLP markers in rice. **Genome** 39:969-977.

Malushev, S.V. and N.A. Kartel. 1997. Molecular markers in mapping of plant genomes. **Mol. Biol**. 31:163-171.

Maluszynski, M., B.S. Ahloowalia and B. Sigurbjörnsson. 1995. Application of *in vivo* and *in vitro* mutation techniques for crop improvement. **Euphytica** 85: 303-315.

Markwell, J., J.C. Osterman and J.L. Mitchell. 1995. Calibration of the Minolta SPAD-502 leaf chlorophyll meter. **Phosyn. Res.** 46:467-472.

Markwell, J.P., A.N. Webber and B. Lake. 1985. Mutants of sweetclover (*Melilotus alba*) lacking chlorophyll b: studies on pigment-protein complexes and thylakoid protein phosphorylation. **Plant Phyiol.** 77:948-951.

Martin, G.B., J.G.K. William and S.D. Tanksley. 1991. Rapid identification of markers linked to a *Pseudomonas* resistance gene in tomato by using random primers and near-isogenic lines. **Proc. Nat. Acad. Sci.** 88:1336-1340.

Martin, J.P. and M.D. Sanchez-Yelamo. 2000. Genetic relationship among species of the genus *Diplotaxis* (*Brassicaceae*) using inter-simple sequence repeat markers. **Theor. Appl. Genet.** 101:1234-1241.

Mather, K. 1951. **The Measurement of Linkage in Heredity.** John Wiley and Sons, Inc, New York.

Maxwell, K. and G.N. Johnson. 2000. Chlorophyll fluorescence- A practical guide. **J. Expt. Bot.** 51:659-668.

McDonald, R., S. Fieuw and J.W. Patrick. 1996. Sugar uptake by the dermal transfer cells of developing cotyledons of *Vicia faba* L. Mechanism of energy coupling. **Planta** 198:502-509.

Menancio-Hautea, D., C.A. Fatokun, L. Kumar, D. Danesh and N.D. Young. 1993. Comparative genome analysis of mungbean (*Vigna radiata* L. Wilczek) and cowpea (*V. unguiculata* L. Walpers) using RFLP mapping data. **Theor. Appl. Genet.** 86: 797-810.

Michelmore, R.W., I. Para and R.V. Kessli. 1991. Identification of markers linked to disease-resistance genes by bulked segregant analysis: A rapid method to detect markers in specific genomic regions by using segregant populations. **Proc. Nat. Acad. Sci.** 88:9828-9832.

Mohan, M., S. Nair, A. Bhagwat, T.G. Krishna, M. Yano, C.R. Bhatia and T. Sasaki. 1997. Genome mapping, molecular markers and marker-assisted selection in crop plants. **Mol. Br.** 3:87-103.

Moran, R. 1982. Formulae for determination of chlorophyll pigments extracted with *N*,*N*-dimethylormamide. **Plant Physiol**. 69:1376-1381.

Morgante, M., M. Hanafey and W. Powell. 2000. Microsatellites are preferentially associated with nonrepetitive DNA in plant genomes. **Nat. Genet.** 30: 194-200.

Muehlbauer, G.J., J.E. Specht, M.A. Thomas-Compton, P.E. Staswick and R.L. Bernard. 1988. Near-isogenic lines- a potential resource in the integration of conventional and molecular marker linkage maps. **Crop Sci.** 28:729-735.

Munier-Jolain, N.G., B. Ney and C. Duthion. 1993. Sequential development of flowers and seeds on the mainstem of an indeterminate soybean. **Crop Sci.** 33:768-771.

Nair, S.K. and S.M.S. Tomar. 2001. Genetical and anatomical analysis of a leaf flecking mutant in *Triticum aestivum* L. **Euphytica** 121: 53-58.

Negi, M.S., M. Devic, M. Delseny and M. Lakshmikumaran. 2000. Identification of AFLP fragments linked to seed coat colour in *B. Juncea* and conversion to SCAR marker for rapid selection. **Theor. Appl. Genet.** 101:146-152.

Nelson, O.E. 1967. Biochemical genetics of higher plants. **Ann. Rev. Genet.** 1:245-268.

Ney, B., C. Duthion and E. Fontaine. 1993. Timing of reproductive abortions in relation to cell division, water content, and growth of pea seeds. **Crop Sci.** 33:267-270.

Ney, B. and O. Turc. 1993. Heat-unit-based description of the reproductive development of pea. **Crop Sci**. 33:510-514.

Nobel, P.S. 1999. **Plant Physiology.** Academic Press, San Diego, California.

Offler, C.E., E. Liet and E.G. Sutton. 1997. Transfer cell induction in cotyledons of *Vicia faba*. **Protoplasma** 200:51-64.

Oliker, M., A. Poljakoff-Mayber and A.M. Mayer. 1978. Changes in weight, nitrogen accumulation, respiration and photosynthesis during growth and development of seeds and pods of *Phaseolus vulgaris*. **Amer. J. Bot.** 65:366-371.

Ott, J. 1985. **Analysis of Human Genetics Linkage.** The John Hopkins Press Ltd, London.

Oxborough, K. 2004. Imaging of chlorophyll a fluorescence: theoretical and practical aspects of an emerging technique for the monitoring of photosynthetic performance. **J. Expt. Bot.** 55:1195-1205.

Pate, J.S. and B.E.S. Gunning. 1972. Transfer cells. **Ann. Rev. Pl. Physio.** 23:173-196.

Poehlman, J.M. 1991. **The Mungbean.** Oxford and IBH Publishing Co., New Delhi.

Powell, W., G.C. Machray and J. Provan. 1996. Polymorphism revealed by simple sequence repeats. **Trends Plant Sci.** 1: 215-222.

Provan, J., W. Powell and R. Waugh. 1996. Microsatellite analysis of relationships within cultivated potato (*Solanum tuberosum*). **Theor. Appl. Genet.** 92: 1078-1084.

Qiu, N., Q. Lu and C. Lu. 2003. Photosynthesis, photosystem II efficiency and xanthophylls cycle in the salt-adapted halophyte *Atriplex centralasiatica*. **New Phytologist** 159:79-486.

Rallo, P., G. Dorado and Martin A. 2000. Development of simple sequence repeats (SSRs) in olive tree (*Olea europaea* L.). **Theor. Appl. Genet.** 101:948-989.

Rascher, U., M. Liebig and U. Lüttge. 2000. Evaluation of instant light-response curves of chlorophyll fluorescence parameters obtained with a portable chlorophyll fluorometer on site in the field. **Plant Cell Environ.** 23:1397-1405.

Ratnaparke, M.B., D.K. Santra, A. Tullu and F.J. Muehlbeur. 1998. Inheritance of inter-simple sequence repeat polymorphism and linkage with a fusarium wilt resistance gene in chickpea. **Theor. Appl. Genet.** 96:48-353.

Rolfe, S.A. and J.D. Scholes. 1995. Quantitative mapping of chlorophyll fluorescence. **New Phytologist** 131:69-79.

Ronnenkamp, R.R., H.J. Gorz and F.A. Haskins. 1975. Genetics studies of induced mutants in *Melilotus alba*. IV. Inheritance and complementation of six additional chlorophyll-deficient mutants. **Crop Sci.** 15:187-188.

Ruban, A.V. and P. Horton. 1995. Regulation of non-photochemical quenching of chlorophyll fluorescence in plants. **Aust. J. Plant. Physiol.** 22:221-230.

Russell, J.R., J.D. Fuller, M. Macaulay, B.G. Hatz, A. Jahoor, W. Powell and R. Waugh. 1997. Direct comparison of levels of genetic variation among barley accessions detected by RFLPs, AFLPs, SSRs and RAPDs. **Theor. Appl. Genet.** 95: 714-722.

Sangsiri, C., W. Sorajjapinun and P. Srinives. 2005. Gamma radiation induced mutations in mungbean. **ScienceAsia** 31:251-255.

Sanhewe, A.J. and R.H. Ellis. 1996. Seed development and maturation in *Phaseolus vulgaris*. 1. Ability to germinate and to tolerate desiccation. **J. Expt. Bot.** 47:949-958.

SAS Institute. 1989. **SAS/STAT guide for personal computers**, **version 6 ed.** SAS Inst, Cary, NC.

Saunder, A.R. 1960. Inheritance in the cowpea (*Vigna sinensis* End.). III. Mutation and linkages. **S. Afr. J. Agric. Sci.** 3:327-348.

Senaratna, T. and B.D. McKersie. 1983. Dehydration injury in germinating soybean (*Glycien max* L. Merr) seeds. **Plant Physiol**. 72:620-624.

Shanmugasundaram, S. and D.H. Kim. 1996. Mungbean. pp.137-173. *In* **P.N. Bahl and P.M. Salimath, eds. Genetics, Cytogenetics and Breeding of Crop Plants (Volume1) Pulses and Oilseeds**. Oxford and IBH Publishing Co, New Delhi.

Smith, D.L. 1973. Nucleic acid, protein, and starch synthesis in developing cotyledons of *Pisum arvense*. **Ann. Bot.** 37:795-804.

Smith, J.S.C. and O.S. Smith. 1991. Restriction fragment length polymorphism can differentiate among U.S. maize hybrids. **Crop Sci.** 31:893-899.

Southern, E. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. **J. Mol. Bio.** 98:503-517.

Specht, J.E., F.A. Haskins and H.J. Gorz. 1975. Contents of chlorophyll a and b in chlorophyll-deficient mutants of sweetclover. **Crop Sci.** 15:851-853.

Srinives, P., N. Hual-alai, S. Saengchot and S. Ngampongsai. 2000. The use of wild relatives and gamma radiation in mungbean and blackgram breeding. pp.205-218. *In* **Proc. of the 7th MAFF International Workshop on Genetic Resources**. **Part1. Wild Legumes**. AFFRC and NIAR, Japan.

Subramanian, D. 1980. Effect of gamma radiation on *Vigna*. **Indian J. Genet. Pl. Br.** 40: 187-194.

Sun, W.Q., T.C. Irving and A.C. Leopold. 1994. The role of sugar, vitrification and membrane phase transition in seed desiccation tolerance. **Physiol. Plant.** 90:621-628.

Tegeder, M., X.D. Wang, W.B. Frommer., E.O. Offler and J.W. Patrick. 1999. Sucrose transport into developing seeds of *Pisum sativum*. **Plant J.** 18:151-161.

Tekrony, D.M., D.B. Egli, J. Balles, T. Pfeiffer and R.J. Fellows. 1979. Physiological maturity in soybean. **Agron. J.** 71:771-775.

Vogelmann, T.C. and J.R. Evans. 2002. Profiles of light absorption and chlorophyll within spinach leaves from chlorophyll fluorescence. **Plant Cell Environ.** 25:1313-1323.

Vos, P., R. Hogers, M. Bleeker, M. Reijans, T. van de Lee, M. Hornes, A. Frijters, J. Pot, J. Peleman, M. Kuiper and M. Zabeau. 1995. AFLP: A new technique for DNA fingerprinting. **Nucleic Acids Res.** 23: 4407-4414.

Wang, X.W., A. Kaga, N. and Tomooka, D.A. Vaughan. 2004. The development of SSR markers by a new method in plants and their application to gene flow studies in azuki bean (*Vigna angularis* (Wild.) Ohwi & Ohashi). **Theor. Appl. Genet.** 109: 352-360.

Waugh, R. and W. Powell. 1992. Using RAPD markers for crop improvement. **Trends in Biot.** 10:186-191.

Weber, H., L. Borisjuk and L. Wobus. 1997a. Sugar import and metabolism during seed development. **Trends Plant Sci.** 22:169-74.

Weber, H., L. Borisjuk, Heim, N. Sauer and U. Wobus. 1997b. A role for sugar transporters during seed development: molecular characterization of a hexose and a sucrose carrier in Faba bean seeds. **Plant Cell** 9:895-908.

Weber, H., U. Heim, S. Golombek, L. Borisjuk and U. Wobus. 1998. Assimilate uptake and the regulation of seed development. **Seed Sci. Res.** 8:331-345.

Weis, E. and J.A. Berry. 1987. Quantum efficiency of photosystem 2 in relation to energy dependent quenching of chlorophyll-fluorescence. **Biochem Biophys Acta** 894:189-208.

Westman, A.L. and S. Kresovich. 1998. The potential for cross-taxa simple sequence repeat (SSR) amplification between *Arabidopsis thaliana* L. and crop brassicas. **Theor. Appl. Genet.** 96: 272-281.

Wettstein, D.V. 1961. Nuclear and cytoplasmic factors in development of protoplast structure and function. **Can. J. Bot.** 39: 1537-1545.

Willaims, J.G.K., A.R. Kubelik, K.J. Livak, J.A. Rafalski and S.V. Tingey. 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. **Nucleic Acids Res.** 18:6531-6535.

Williams, N.D., L.R. Joppa, M.E. Duysen and T.P. Freeman. 1985. Inheritance of three chlorophyll-deficient mutants of common wheat. **Crop Sci.** 5:1023-1025.

Zietkiewicz, E., J.A. Rafalski and D. Labuda. 1994. Genome fingerprinting by simple sequence repeat (SSR) anchored polymerase chain reaction amplification. **Genome** 20:176-183.

APPENDIX

Appendix Table 1 Chlorophyll a, b, total (a+b) contents (g/m^2) and a/b ratio of opaque leaf mungbean, normal mungbean, and their progenies. The data are expressed as mean ±sd.

Genotypes	Chl a	Chl b	Total (a+b)	a/b ratio
Berken	0.135±0.01	0.041±0.00	0.176±0.02	3.336±0.14
Opaque	0.089±0.01	0.027±0.00	0.118±0.01	3.272±0.20
N531	0.133±0.01	0.040±0.01	0.174±0.02	3.351±0.13
OP531	0.112±0.01	0.036±0.00	0.148±0.01	3.172±0.09
N541	0.139±0.01	0.043±0.01	0.183±0.02	3.229±0.17
OP541	0.111±0.01	0.035±0.00	0.146±0.01	3.159±0.13

Appendix Table 12 SSR primers used for screening of opaque and normal leaf parents.

Primer	Primer sequence	Repeat	DNA band
VM1	5′CAC CCG TGA TTG CTT GTT G 5′GTC CCC TCC CTC CCA CTG	(TC)20	-
VM2	5′GTA AGG TTT GGA AGA GCA AAG AG 5′GGC TAT ATC CAT CCC TCA CT	(AG)32	-
VM3	5′GAG CCG GGT TCA ATA GGT A 5′GAG CCA GGG CAC AGG TAG T	(AG)27	-
VM4	5′AGT AA TCA CCC GCA CGA TCG 5′AGG GGA AAT GGA GAG GAG GAT	(CT)20	-
VM5	5′AGC GAC GGC AAC AAC GAT 5′TTC CCT GCA ACA AAA ATA CA	(AG)32	-
VM6	5′GAG GAG CCA TAT GAA GTG AAA AT 5′TCG GCC AGC AAC AGA TGC	(AG)26	-
VM7	5′CGC TGG GGG TGG CTT AT 5′AAT TCG ACT TTC TGT TTA CTT G	(AG)13	-
VM8	5′TGG GAT GCT GCA AAG ACA C 5′GAA AAC CGA TGC CAA ATA G	(AG)16	-
VM9	5′ACC GCA CCC GAT TTA TTT CAT 5′ATC AGC AGA CAG HCA AGA CCA	(CT)21	-
VM10	5′TCC CAC TCA CTA AAA TAA CCA ACC 5′GGA TGC TGG GGG CGG AAG G	(AC)3(CT)10(AC)3	-
VM11	5′CGG GAA TTA ACG GAG TCA CC 5′CCC AGA GGC CGC TAT TAC AC	(TA)4..(AC)12	-
VM12	5′TTG TCA GCG AAA TAA GCA GAG A 5′CAA CAG ACG CAG CCC AAC T	(AG)27	M
VM13	5′CAC CCG TGA TTG CTT GTT G 5′GTC CCC TCC CTC CCA CTG	(CT)21	M
VM14	5′AAT TCG TGG CAT AGT CAC AAG AGA 5′ATA AAG GAG GGC ATA GGG AGG TAT	(AG)24	-
VM15	5′CGG CTG CAG CAA ACA AGA G 5′AAA CCC GTG CAA GAA ACC AA	(AG)4…(GT)10	-
VM16	5′TCC TCG TCC ATC TTC ACC TCA 5′CAA GCA CCG CAT TAA AGT CAA G	(CT)7..(CT)7	-

Appendix Table 12 (Continued)

Primer	Primer sequence	Repeat	DNA band
VM17	5′GGC CTA TAA ATT AAC CCA GTC T 5′TGT GTC TTT GAG TTT TTG TTC TAC	(CT)12	-
VM18	5′AGC CGT GCA AGC AAT GAT 5′TGG CCT CTA CAA CAA CAC TCT	(GA)13	-
VM19	5′TAT TCA TGC GCC GTG ACA CTA 5′TCG TGG CAC CCC CTA TC	(AC)7..(AC)5	-
VM20	5′GGG GAC CAA TCG TTT CGT TC 5′ATC CAA GAT TCG GAC ACT ATT CAA	(GT)17	-
VM21	5′TAG CAA CTG TCT AAG CCT CA 5′CCA ACT TAA CCA TCA CTC AC	(AT)17	-
VM22	5′GCG GGT AGT GTA TAC AAT TTG 5′GTA CTG TTC CAT GGT AGA TCT	(AG)12	S
VM23	5′AGA CAT GTG GGC GCA TCT G 5′AGA CGC GTG GTA CCC ATG TT	(CT)16	M
VM24	5′TCA ACA CCT AGG AGC CAA 5′ATC GTG ACC TAG TGC CCA CC	(AG)15	-
VM25	5′CCA CAA TCA CCG ATG TCC AA 5′CAA TTC CAC TGC GGG ACA TAA	(TC)18	-
VM26	5′GGC ATC AGA CAC ATA TCA CTG 5′TGT GGC ATT GAG GGT AGC	(TC)14	-
VM27	5′GTC CAA AGC AAA TGA GTC AA 5′TGA ATG ACA ATG AGG GTG C	(AAT)5…(TC)14.(AC)3	S*
VM28	5′GAA TGA GAG AAG TTA CGG TG 5′GAG CAC GAT AAT ATT TGG AG	(TC)20	-
VM29	5′CGT GAC ACT AAT AGT AGT CC 5′CGA GTC TCG GAC TCG CTT	(TC)11	-
VM30	5′CTC TTT CGC GTT CCA CAC TT 5′GCA ATG GGT TGT GGT CTG TG	(TC)10	-
VM31	5′CGC TCT TCG TTG ATG GTT ATG 5′GTG TTC TAG AGG GTG TGA TGG TA	(CT)16	S
VM32	5′GAA AAA GGG AGG AAC AAG CAC AAC 5′AGC GAA AAC ACG GAA CTG AAA TC	(AG)10	-
VM33	5′GCA CGA GAT CTG GTG CTC CTT 5′CAG CGA GCG CGA ACC	(AG)18.(AC)8	-

Appendix Table 12 (Continued)

Primer	Primer sequence	Repeat	DNA band
VM34	5´AGC TCC CCT AAC CTG AAT 5´TAA CCC AAT AAT AAG ACA CAT A	(CT)14	-
VM35	5´GGT CAA TAG AAT AAT GGA AAG TGT 5´ATG GCT GAA ATA GGT GTC TGA	(AG)11.(T)9	-
VM36	5´ACT TTC TGT TTT ACT CGA CAA CTC 5´GTC GCT GGG GGT GGC TTA TT	(CT)13	-
VM37	5´TGT CCG CGT TCT ATA AAT CAG C 5´CGA GGA TGA AGT AAC AGA TGA TC	(AG)5.(CCT)3.(CT)13	S
VM38	5´AAT GGG AAA AGA AAG GGA AGC 5´TCG TGG CAT GCA GTG TCA G	(AG)10..(AC)5	-
VM39	5´GAT GGT TGT AAT GGG AGA GTC 5´AAA AGG ATG AAA TTA GGA GAG CA	(AC)13.(AT)5.(TACA)4	-
VM40	5´TAT TAC GAG AGG CTA TTT ATT GCA 5´CTC TAA CAC CTC AAG TTA GTG ATC	(AC)18	-
VM68	5´CAA GGC ATG GAA AGA AGT AAG AT 5´TCG AAG CAA CAA ATG GTC ACA C	(GA)15	-
VM69	5´CAA AGC ATT GGG CCC TTG T 5´GGC TTT GGG ACC TCC TTT CC	(AG)19	-
VM70	5´AAA ATC GGG GAA GGA AAC C 5´GAA GGC AAA ATA CAT GGA GTC AC	(AG)20	-
VM71	5´TCG TGG CAG AGA ATC AAA GAC AC 5´TGG GTG GAG GCA AAA ACA AAA C	(AG)12.(AAAG)3	S
VM72	5´TGC TGA AGT GAA CAA TCG C 5´CCT TCT CCA ACA ACT CTA C	(AG)20	-
VM73	5´CGG CGT GAT TTG GGG AAG AAG 5´CTA GTA ACG GCC GCC AGT GTC CTG	(AG)15	-

- = no DNA band, M= multiple band, S= single band, * = polymorphism

Appendix Table 13 ISSR primers used for parents screening of opaque and normal leaf parents.

Primer	Primer sequence	DNA band
$(AC)_8G$	ACA CAC ACA CAC ACA CG	M
$(AG)_8C$	AGA GAG AGA GAG AGA GC	M
$(CA)_8T$	CAC ACA CAC ACA CAC AT	-
$(CT)_8A$	CTC TCT CTC TCT CTC TA	-
$(GA)_8A$	GAG AGA GAG AGA GAG AA	-
$(GA)_9AC$	GAG AGA GAG AGA GAG AGA AC	M
$(GA)_9AT$	GAG AGA GAG AGA GAG AGA AT	M*
$(GA)_9C$	GAG AGA GAG AGA GAG AGA C	M*
$(GA)_9R$	GAG AGA GAG AGA GAG AGA R	M*
$(GA)_9T$	GAG AGA GAG AGA GAG AGA T	M*
$(GA)_9Y$	GAG AGA GAG AGA GAG AGA Y	M
$(GC)_9A$	GCG CGC GCG CGC GCG CGC A	-
$(TC)_9G$	TCT CTC TCT CTC TCT CTC G	M*
$(TC)_9A$	TCT CTC TCT CTC TCT CTC A	M*
$(TG)_8A$	TGT GTG TGT GTG TGT GA	M*
$(TG)_8G$	TGT GTG TGT GTG TGT GG	S
$(AAC)_6T$	AAC AAC AAC AAC AAC AAC T	M*
$(AAC)_6Y$	AAC AAC AAC AAC AAC AAC Y	M*
$(ACC)_6C$	ACC ACC ACC ACC ACC ACC C	M*
$(ACC)_6T$	ACC ACC ACC ACC ACC ACC T	M*
$(ATG)_6G$	ATG ATG ATG ATG ATG ATG G	M*
$(ACT)_6G$	ACT ACT ACT ACT ACT ACT G	-
$(CCG)_6T$	CCG CCG CCG CCG CCG CCG T	M
$(CCT)_6T$	CCT CCT CCT CCT CCT CCT T	M
$(CGG)_6T$	CGG CGG CGG CGG CGG CGG T	M
$(CGT)_6A$	CGT CGT CGT CGT CGT CGT A	-
$(CGT)_6G$	CGT CGT CGT CGT CGT CGT G	M
$(CTG)_6A$	CTG CTG CTG CTG CTG CTG A	-

- = no DNA band, M= multiple band, S= single band, * polymorphism, R= purine, Y= pyrimidine

Appendix Table 14 Nucleotide sequences of adapter and primers used for AFLP analysis.

Adapter/ Primer	Sequences
*Mse*I adapters	5´GAC GAT GAG TCC TGA G
	3´TAC TCA GGA CTC AT
*Eco*RI adapters	5´CTC GTA GAC TGC GTA CC
	5´CTG ACG CAT GGT TAA
*Mse*I primers	5´ GAT GAG TCC TGA GTA A
*Eco*RI primers	5´ACT GCG TAC CAA TTC

Appendix Table 15 AFLP combination primers used for PCRI and PCRII reactions.

Selective primer for PCRI		Selective primer for PCRII	
*Eco*RI	*Mse*I	*Eco*RI	*Mse*I
A	A	AA	AAA
		AC	AAC
		AAA	AAG
		AAC	AAT
		AAG	ACA
		ACA	ACC
		ACC	ACG
		ACT	ACT
		ACG	ATC
		AGG	ATG
			ATA
			ATT
			AGG
			AGT
C	C	CT	CAA
		CAG	CAC
		CGT	CAG
			CAT
			CTA
			CTC
			CTG
G	G	GA	GCC
		GCC	GTA
		GCA	GTC
T	T	TAC	TAC
		TCG	TGA

Appendix Table 16 Ninety-six AFLP combination primers (*Eco*RI/ *Mse*I) obtained from parental screening.

Primer	Polymorphic bands	Primer	Polymorphic bands	Primer	Polymorphic bands
AA/AAT	6	AC/TAC	8	TAC/AAC	6
AA/ACT	9	AC/TGA	10	TAC/AAG	3
AA/ACG	8	AAA/ TAC	31	TAC/AAT	4
AA/AGG	6	AAA/TGA	22	TAC/ACA	8
AC/ACA	8	AAC/ TAC	13	TAC/ACT	5
AC/ACT	6	AAG/TAC	19	TAC/ACG	3
AC/ACG	12	ACA/TAC	12	TAC/ATG	9
AC/AGG	11	ACA/TGA	12	TAC/ATC	4
AAA/AAG	8	ACC/TGA	4	TAC/ATA	8
AAA/AAT	7	ACT/TAC	14	TAC/ATT	10
AAA/ACA	9	ACT/TGA	15	TCG/AAA	7
AAA/ACC	9	AA/CAA	10	TCG/AAG	9
AAA/ACT	16	AAA/CAA	5	TCG/AAT	3
AAA/ACG	15	ACA/CAA	7	TCG/ACT	3
AAA/AGG	7	ACC/CAA	10	TCG/ATG	6
AAC/AGG	8	ACT/CAA	19	TCG/ATC	6
AAG/AGG	7	GA/AAC	6	TCG/ATA	5
ACA/AGG	17	GA/AAG	9	TAC/GCC	4
AGG/ATA	5	GA/ACA	3	TAC/GTC	5
AA/GCC	7	GA/ACC	8	TAC/TAC	7
AA/GTC	7	GA/ ACT	6	TAC/TGA	4
AC/GTC	9	GA/ ACG	4	TCG/TAC	3
AAA/GTA	9	GA/ ATG	4	TAC/CAA	7
AAA/GTC	10	GA/ATT	5	TAC/CAC	7
AAC/GTA	7	GCC/ATC	5	TAC/CAG	10
AAC/GTC	21	GCC/AGT	3	TAC/CAT	11
AAG/GTA	5	GCA/ACA	8	TAC/CTA	5
ACA/GTA	12	GCA/ACT	5	TAC/CTG	3
ACA/GTC	8	GCA/ACG	9	CT/GCC	4
ACC/GTA	11	GCA/AGT	10	CT/TAC	11
ACT/GTA	20	GA/TGA	10	CAG/TAC	10
AA/TAC	9	TAC/AAA	3	CGT/TGA	15