DISSERTATION

DEVELOPMENT OF DNA MARKERS FOR ASSISTING SELECTION OF SOYBEAN RESISTANT TO FIELD WEATHERING IN BACKCROSS PROGENIES

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GRADUATE SCHOOL, KASETSART UNIVERSITY

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THESIS

DEVELOPMENT OF DNA MARKERS FOR ASSISTING SELECTION OF SOYBEAN RESISTANT TO FIELD WEATHERING IN BACKCROSS PROGENIES

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Field weathering is the main limitation for producing high quality soybean seeds in the tropics. The purpose of this study was to identify DNA markers linking to the field weathering resistance genes, and to use the developed markers for assisting selection in breeding programs. The field weathering resistance of soybean *Chiangmai 60 (CM60)* and *GC10981* was tested by seven treatments. The modified incubator weathering (yellow pods were incubated at 30℃ for 7 days) and controlled deterioration (dry seeds were soaked in distilled water for 60 minutes and then incubated at 41℃ for 3 days) showed wide-range differences in germination and viability between *CM60* and *GC10981*. These two methods were further used to evaluate the field weathering resistance of 139 F_2 progenies derived from the cross *CM60/GC10981*. The germination and viability of the seeds subjected to both treatments showed a normal distribution. This result revealed that field weathering resistance might be controlled by polygene. According to the germination and viability of the treated F_2 seeds, six resistant F_2 plants and seven susceptible F_2 plants were pooled for bulk segregant analysis (BSA). The genotypic difference of the DNA pools could not be identified by 198 RAPD primers. However, five field weathering resistance linked polymorphic DNA bands were identified from 2,162 AFLP markers. The 5 DNA fragments were cloned and sequenced. PCR primers were designed from the sequences to amplify the related DNA fragment from the soybean genomic DNA of 139 F_2 progenies. It was found that marker Eaag/Mcac-233 and Eact/Mctt-157 were in the same linkage group with a genetic distance of 25.8 cM. A major QTL controlling the field weathering resistance was identified between these two markers located at 14 cM from marker Eaag/Mcac-233. This QTL explained 29.7% of the variation in weathering resistance. Seven F_2 plants were selected by marker assisted selection (MAS) for backcrossing to *CM60*. The germination and viability of 18 BC_1F_1 progenies were higher than the mean of *CM60* and *GC10981* by controlled deterioration test. The DNA markers developed in this study were used for MAS efficiently.

Ye Changrong — Student's signature

P. Sripichitt — Thesis Advisor's signature

24 / Aug. / 2006

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Ye Changrong

August, 2006

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LIST OF SYMBOLS AND ABBREVIATIONS

AA	=	accelerated aging
AFLP	=	amplified fragment length polymorphism
AOSA	=	Association of Official Seed Analysts
bp	=	base pair
BSA	=	bulked segregant analysis
CD	=	controlled deterioration
CIM	=	composite interval mapping
DNA	=	deoxyribonucleic acid
EC	=	electrical conductivity
IW	=	incubator weathering
MAS	=	marker assisted selection
PAGE	=	polyacrylamide gel electrophoresis
PCR	=	polymerase chain reaction
RAPD	=	random amplified polymorphic DNA
RFLP	=	restriction fragment length polymorphism
RNase	=	ribonuclease
rpm	=	revolutions per minute
QTL	=	quantitative trait locus
RH	=	relative humidity
rpm	=	revolutions per minute
SCAR	=	sequence characterized amplified region
SSR	=	simple sequence repeat
TZ	=	tetrazolium

DEVELOPMENT OF DNA MARKERS FOR ASSISTING SELECTION OF SOYBEAN RESISTANT TO FIELD WEATHERING IN BACKCROSS PROGENIES

INTRODUCTION

Cultivated soybean [*Glycine max* (L.) Merrill] belongs to family *Leguminosae*, subfamily *Papilionoideae*, genus *Glycine*. It has been domesticated from *Glycine soja* Sieb & Zucc. in northeast China where first written record dated back to 2328 B.C. (Hymowitz and Newell, 1980; Shimamoto, 2001; Smith and Huyser, 1987). It has been cultivated at broadly diverse geographical locations and under many different growing conditions, particularly in the America and Asia.

Soybean proteins have high nutritional quality and good functionality for foods. Soybean is also the lowest-cost producer of vegetable oil. Soybean seed contains approximately 37-41% protein, 18-21% oil, 30-40% carbohydrate, and 4-5% ash (Hulse, 1996). It's one of the world's leading sources of vegetable oil and plant protein, both of which are very well adapted to the nourishment of human beings. The increase in soybean production and uses as human food has been very rapid during the last few decades compared with that of many other major crops, and continuous growth is considered very important for stabilizing the world food supply. Its capacity for protein and oil production makes it a significant contributor to human nutrition, and its characteristic symbiosis with root bacteroids makes it a very important crop in research.

Historically, the soybean seed was mainly produced in the temperate zone of the world, where environmental stress was relatively minimal. However, as the world demand for vegetable oil and protein meal continue to increase, soybean production has been spread rapidly into hot and humid production areas, and more recently into the tropical regions (Moore, 1966b; Tekrony *et al*., 1987). Following the expansion,

weather conditions have become the major factor, which affect the soybean seed quality and production in tropical and subtropical regions. Unfavorable weather conditions (mainly temperature and moisture) during the post-maturation and pre-harvest period increase difficulty in consistently producing soybean seed with high quality. The germinablility and vigor of the matured seed decrease rapidly under such conditions. This obstacle in producing good quality seed may be the factor that most limits the distribution of soybean production in the tropics, such as Thailand.

It is well known that soybean seed vigor and viability reach its highest potential quality at physiological maturity (maximum seed dry weight) (Ching *et al*. 1972; Delouche, 1974; Wahab and Burris, 1971). The moisture content of physiological maturated seed is about 50-55%. Due to the high moisture content, the seed cannot be harvested commercially at this growth stage and must remain in storage on the plant through a desiccation period. This period may vary from a few days to over 3 weeks before the seed reaches a harvestable moisture level (about 14%) at which they can be effectively threshed (i.e. harvest maturity). Weather conditions during this post-maturation pre-harvest period have a great influence on the quality of the harvested seed (Delouche, 1980). The soybean seed deteriorates rapidly in the field prior to harvest in hot and humid tropical growing regions.

The detrimental effects of allowing soybean seed to remain in the field after reaching physiological maturity have been known for many decades. As early as 1950, Moorse *et al*. reported that exposure to periods of dampness caused soybean seeds to deteriorate. The deterioration of seed vigor, as well as viability, due to high temperature and high relative humidity during the post-maturation and pre-harvest period is referred to as field weathering (Bhatia *et al*., 1993; Tekrony *et al*., 1980a). Environmental conditions that contribute to field weathering include high temperature and relative humidity, and frequent or prolonged rainfall. These conditions play a critical role affecting soybean seed quality in the tropics (Keigly and Mullen, 1986; Mondragon and Potts, 1974; Nangju, 1979; Tekrony *et al*., 1987).

The severity of weathering and the limitation imposed on seed quality by weathering generally increases from cool to warm areas. The worst situation is in the hot and humid tropics. Tropical weather conditions during the post-maturation and pre-harvest period cause severe seed quality problems in soybean, and the seed deterioration continues at a rapid rate due to the high temperature and humidity. Thus, the quality of soybean seed produced in the tropics is generally low. To overcome the problem, numerous researches have been done on morphological, physiological, biochemical, environmental and genetic aspects of the field weathering of soybeans. However, it is still an urgent task to look for ways to solve the field weathering problems.

In the recent years, as the development of molecular technology, major advances have been recently made in our understanding of soybean genetics and of the application of new technologies to soybean improvement. Many reports of the construction of soybean genetic linkage maps using various markers have been published (Keim *et al*. 1990, 1997; Lark *et al*. 1993; Cregan *et al*. 1999; Song *et al*. 2004). Based on the construction of soybean genetic maps, quantitative trait loci (QTLs) for a number of agronomic traits in soybean have been mapped. For example, there are at least 53 QTL associated with oil content, 61 with protein concentration, and 66 with seed size have been mapped using various population types and different marker-based mapping techniques (Hyten *et al.* 2004). There were also some reports on environmental stress resistance of soybean such as chilling tolerance (Furatsuki *et al*., 2005). Technically, it is possible to identify the QTLs controlling the field weathering resistance. However, genetic analysis of the field weathering resistance using molecular markers has not been reported yet. The purpose of this research was to detect the molecular markers linking to the genes controlling field weathering resistance of soybean, and to make further application of the markers for assisting selection in soybean breeding programs.

In Thailand, Many soybean varieties were found to be resistant to the field weathering (Kaowanant, 2003). Based on the prior researches and a well-designed

study plan, *Chiangmai 60* (*CM60*) and *GC10981* were chosen as susceptible and resistant variety for genetic analysis in this study. Efficient testing methods have been established to evaluate the field weathering resistance of their F_2 progenies. RAPD and AFLP markers combining with BSA were used to identify the markers linking to the field weathering resistance. Furthermore, the obtained markers were used for MAS in a backcross breeding program.

Objectives

Overall Objective

To develop DNA markers associated with field weathering resistance for assisting selection in soybean backcross breeding program.

Specific Objectives

1. To identify DNA markers linking to the soybean field weathering resistance genes by RAPD and AFLP primers;

2. To use the developed DNA markers for assisting selection in backcross breeding program to increase field weathering resistance of soybean.

LITERATURE REVIEW

Origin, Uses and Distribution of Soybean

Soybean is one of the oldest cultivated crops. It is widely believed that soybean originated in northern China where it has been domesticated from 4000 to 5000 years ago (Hymowitz and Newell, 1980; Shimamoto, 2001). The first written record dated back to 2328 B.C. (Morse, 1950; Smith and Huyser, 1987). In 2838 B.C., Chinese emperor Sheng-Nung described the soybean in his book *Pen Tsao Kang Mu* [Materia Medica] – the first written record of soybean cultivation. In that record, he named five sacred plants: soybeans, rice, wheat, barley, and millet. Of them, soybeans were noted as being valued for their medicinal properties. From then on, soybean were domesticated in the eastern half of China and spread into Japan, Korea and the rest of Southeast Asia. Chinese farmers have grown this legume and fed it to their families as well as to their livestock for centuries. The soybean production was more or less localized in China until after the Chinese-Japanese War (1894-1895), when the Japanese began to import soybean oil cake for use as fertilizer (Morse, 1950).

Soybeans were grown for centuries in Asia mainly for their seeds. The soybean seeds were used in preparing a large variety of fresh, fermented and dried food products that were considered indispensable to oriental diets. Soybeans are high in protein and fiber, low in carbohydrates and are nutrient-dense. Scientific studies show that compounds in soybeans may help heart disease, osteoporosis, cancer and diabetes. Soybeans, compared with other legumes, are higher in essential fatty acids, and are a good source of calcium, magnesium, lecithin, riboflavin, thiamin, fiber, folate (folic acid), and iron. It was also used as a specific remedy for the proper functioning of the heart, liver, kidneys, stomach and bowels (Probst and Judd, 1973). In 1904, the famous American chemist, George Washington Carver discovered that soybeans are a valuable source of protein and oil. This significant breakthrough led to the development of what would become soybeans' two main uses — edible oil and meal (Carlson, 1973). Today, soybeans can be found in a wide variety of products,

ranging from soybean sprout, tofu, soymilk and soy sauce to plywood, particle board, printing inks, soap, candy products, cosmetics, and antibiotics. More and more researchers are developing new uses for soybeans. Recent developments include soy diesel, building materials, candles, road dust suppressants, hand cleaners and soy crayons (Probst and Judd, 1973; Smith and Huyser, 1987)

Hymowitz (1970) researched the distribution of *Glycine max* and concluded that historical and geographical evidence points to the eastern half of North China as the area where the soybean was first domesticated around the 11th century B.C. He also indicated that perhaps Manchuria should be designated as a secondary gene center and the eastern half of north China as the primary center. Nagata (1959, 1960) suggested that the cultivated soybean was introduced into Korea from north China about 2000 to 2500 years ago, and then disseminated to Japan sometime between 200 B.C. and the 3rd century A.D. A second route of dissemination may have been from central China to southern Japan. The earliest Japanese reference to the soybean is in the classic Kojiki (Records of Ancient Matters) which was completed in 712 A.C. Soybeans were introduced to Europe in 1712 by Englebert Kaempfer, a German botanist who had studied in Japan. Later Swedish botanist Carl von Linne made the first scientific study of the soybean in the West and named it *Glycine max* because of the unusually large nitrogen-producing nodules on its roots. Soybean was first grown in France in 1740, in England in 1790, in America in 1765, and in Thailand in 1931 (Benjasil *et al*., 1994; Hymowitz and Harlan, 1983; Morse 1950; Probst and Judd, 1973). During the first three decades of the 20th century, soybean production was largely confined to the orient. China, Indonesia, Japan and Korea were the major producers of soybean (Hittle, 1975). Now, soybean has a very broad distribution and is cultivated at broadly diverse geographical locations and under many different growing conditions, particularly in the America and Asia. At present, the world leading producers of soybeans are USA, Brazil, Argentina, China and India (Soystats, 2005).

World Soybean Production and Trade with Emphasis on Thailand

Although soybeans are native to China, and China led the world soybean production until 1954, but after George Washington Carver discovered that soybean is a valuable source of protein and oil in 1904, the U.S. government paid great attention to the soybean production. In the early 1950s, the USA overtook China and eventually the entire orient in soybean production. The USA dominated world soybean production through the 1950's, 60's, and 70's, growing more than 80% of the world soybean production in 1987 (Sun and Xu, 1993). A worldwide shortage of feed protein in the early 1970's led to the initiation of large-scale soybean production in several South American countries, most notably Argentina and Brazil. By 1992, the United States accounted for 51% of the world's soybean production, and soybean became America's second largest crop in cash sales and the number one export crop. In 2001, the world soybean production shared to U.S. 42%, Brazil 24%, Argentina 16%, China 8%, India 3%, Paraguay 2% and other country 5%. By 2003, the U.S. share of the world's soybean production had shrunk to 34%, while Argentina's and Brazil's had increased to 18% and 28%, respectively. However, it was notable that the adoption of biotech-enhanced soybean in these countries was 85%, 99.3% and 34% at the same time (Soystats, 2005).

In 2003, the export value of soybeans in the U.S. was more than 9.7 billion dollars, or about one-sixth of all agricultural exports. About 40 percent of the world's soybean trade originates from the U.S. At the same time, the first time China imported soybeans exceeded domestic production. China has become the largest single country customer for U.S. soybeans with purchases totaling 20.74 million tons (about 3 billion USD). In 2004, the United States exported 29.94 million metric tons of soybeans, which accounted for 48% of the world's soybean trade. The United States, Argentina and Brazil shared more than 93 percent of the world's soybean exports. Some of the leading soybean producers are listed in Table 1.

Table 1 The world's leading soybean producer in 2005.

Country	Area (Ha)	Yield (Kg/Ha)	Production (Mt)
USA	28,842,260	2,872	82,820,048
Brazil	22,895,300	2,192	50,195,000
Argentina	14,037,000	2,729	38,300,000
China	9,500,135	1,779	16,900,300
India	7,000,000	857	6,000,000
Paraguay	1,935,700	1,815	3,513,000
Canada	1,158,300	2,589	2,998,800
World total	91,386,421	1,482 (mean)	209,531,278

Source: FAO statistical database (FAOSTAT, http://faostat.fao.org).

Soybean is also one of the most important grain legume crops in Thailand. It has been cultivated from 1930s in the upper north of the country following the rice harvest. The planted area has expanded to the lower part of the northern area, and was later extended to the northeastern region and central plains. In Thailand, soybean is grown in three main seasons. *i.e*. early rain season (40% of the total planted area), late rain season (35%) and dry season (25%) (Shrivastava, 1997). The average planted area during 1988-1991 was 422,000 ha and this decreased to 251,000 ha during 1996-1999. The average annual soybean production during 1988-1991 was 539,000 tons. Annual production during 1996-2000 decreased due to the decrease in the planted area, it has just been able to supply only 20-30% of the domestic demand. In the earliest years of the 21st century, the Thai soybean production has decreased to nearly half of the 1990s (see Table 2). To meet the country's demand, Thailand imported about 1.0-1.5 million tons of soybean cake at a cost of approximately 9,000-11,000 million Baht during 1997-1998 (Field Crops Research Institute, 2001). The USA share of the Thai soybean market has grew from 0 to 100 percent in the 1990s. The USA soybean exports to Thailand jumped from nothing in 1991 to 53 million USD in 1995 and 126 million USD in 1996. Thailand ranked as the seventh -largest market for U.S. soybeans at the end of 1996 (www.fas.usda.gov). Although soybean was consider as a crop with high potential for expansion in Thailand, but except in its northern region, most area of Thailand is too hot for growing soybeans. With its

limited production capacity and growing needs, Thai soybean demand regularly outstrips supplies. One of the main reasons is the soybean production often meets weathering damage for the tropical and subtropical climate. The tropical and subtropical weather conditions with high temperature and relative humidity prior to harvest result in rapid decrease in germination and vigor of soybean seeds. It also results in decreasing the quality of seed produced, and not conductive to production of high quality seed necessary to establish acceptable stands in Thailand.

Table 2 Thai soybean production and import in the recent years.

Year	Harvest area (ha)	Production (Mt)	Yield (Kg/ha)	Import amount (Mt)	Import value (1000USD)
2001	176,446	260,696	1,478	1,363,224	278,668
2002	174,838	259,863	1,486	1,528,557	324,309
2003	158,412	238,561	1,506	1,689,649	442,007
2004	159,680	244,968	1,534	1,435,803	471,439
2005	160,000	245,000	1,531	-	-

Source: FAO statistical database (FAOSTAT, http://faostat.fao.org).

Weather Requirement for Soybean Cultivation

Soybean development is sensitive to environmental factors. Photoperiod, temperature and water are the main changing soybean productivity in time and space. Mathematical models to express soybean sensitivity to these factors have been developed (Summerfield *et al*., 1989; Wilkerson *et al*., 1989). Hodges and French (1985) studied the climatic effect of different soybean growth stages, their model indicated that the growth stage from planting to emergence is temperature dependent, from emergence to the end of the juvenile phase is temperature dependent, from the end of juvenile phase to floral induction is daylength dependent, and the floral development phase is temperature dependent. The rate of development was also assumed to be sensitive to water stress.

Soybean develops well under a wide range of temperatures, although regions in which the warmest mean monthly temperature below 20°C are considered inappropriate for soybean. Soybeans seed germination occurs at temperature from 5°C to 40°C. However, for rapid germination the temperature should be around 30°C. Generally, soybean begins germination when the soil temperature reaches 10°C. At an average soil temperature of 12°C emergence takes 12 days. At 15.5°C emergence occurs in 7 to 10 days. However, in tropical and subtropical areas (soil temperature above 20°C) the seedling emergence is in 3 to 5 days. The major world soybean producing areas have average mid-season temperature of 23°C to 25°C. Soybean vegetative growth is slow or nil at temperature of 10°C or less and optimum at 30°C, and then decreases as the temperature increases. Soybean growth is the best with summer temperatures of 22°-27°C. Temperatures above 40°C are known to have adverse effect on growth rate, flower initiation and pod-set. Temperature below 22°C decrease pod initiation and no pods are formed when temperatures are lower than 14°C (Hesketh *at al*., 1973; Thomas and Raper, 1981). At the seed developing stage, cool and dry conditions during maturation and harvest are optimum. As a general rule, maturation of soybean seeds should occur when average temperature is 22°C or lower (Franca Neto *at al*., 1994). Soybean seed quality is usually good when produced under favorable environment. However, in many counties, especially in tropical and subtropical countries, the environmental conditions are unfavorable and often detrimental to the production of high quality soybean seed. In many cases, the environment becomes implicated as a major contributing to the rapid deterioration of the seeds (Andrews, 1982).

Soybean Seed Development

Biological and cytological changes in developing soybean seeds have been fine studied. Soybean fertilization occurs within 8-10 hours following the pollination. Fertilization initiates cell division to form the embryo, which occurs about 32 hours after pollination. After 6-7 days, localized division at the opposite side of the embryo results in the initiation of the cotyledons. At the same time, the hypocotyls and the

epicotyls tissues are also differentiated. During early development (15-18 days after flowering), the cells of cotyledon appear to form plastids, mitochondria, and other membranous structures. Only ribonucleo protein particles and nuclei are seen inside the 15-day cells. By 26 days after flowering, many mitochondria, immature chloroplasts, and starch grains are present. A relatively high respiration rate is also observed. Lipid granules and protein bodies begin to appear in the cytoplasm. Subsequently, the lipid and protein structures increase in size and the mitochondria and chloroplasts decrease in number. Dry weight of the developing seed increases slowly until about 20 to 30 days after flowering, while moisture content slowly decrease from about 90% to 80%. Beginning from about 25 to 35 days after flowering, dry matter begins to accumulate rapidly in the seed and reaches a maximum at about 65 to 75 days after flowering. During the period of rapid dry matter accumulation, seed moisture content decreases rather slowly to 40 to 50 % at the time maximum dry weight is attained (Delouche, 1975). When the fresh weight of the developing seed reaches a maximum, the cells of the cotyledon are filled with numerous starch grains, lipid bodies, and protein bodies. The starch grains are converted to other components and disappear during the last few days of seed maturation (Andrews, 1966).

The process of soybean maturation can be divided into many stages. Fehr *et al*. (1971) developed a model to describe the maturating stages of soybean (see Table 3). It has been widely used till now. The maximum length of the pod is reached rather early in development, about 20 to 25 days after blooming. The maximum width and thickness of the pod is reached about 30 days after blooming. This corresponds with the time that the seed reaches its maximum size in all dimensions. The maximum fresh weight of the seed and maximum seed size may be obtained 5 to 15 days later (Andrews, 1966). The seed reaches a maximum dry weight at about 65 to 75 days after flowering; this stage (R7) is also called physiological maturity. After that, the seed loses its moisture and the pods change its color into brown till R8 (harvest maturity). Several researchers have reported that soybean seed attains its highest potential quality (including seed vigor and viability) at physiological maturity (maximum dry seed weight) (Andrews, 1966; Ching *et al*. 1972; Delouche, 1974; Trammell 1983; Wahab and Burris, 1971), although harvesting at 3 weeks prior to

physiological maturity did not cause any appreciable loss in seed viability (Dharmasena, 1982).

Table 3 Description of reproductive stages of soybean (single plant).

Stage	Description
R1	One flower at any node on the main stem
R2	Open flower at one of the 2 uppermost nodes on the main stem with a fully developed leaf
R3	Pod 0.5 cm long at one of the 4 uppermost nodes with a completely unrolled leaf
R4	Pods 2 cm long at one of the 4 uppermost nodes with a completely unrolled leaf
R5	Seeds 3 mm long in a pod at one of the 4 uppermost nodes on the main stem with a completely unrolled leaf
R6	Pod containing full-size green beans at one of the 4 uppermost nodes with a completely unrolled leaf
R7	One normal pod on the main stem that has reached its mature pod color
R8	95% of the pods are brown

Source: Gazzoni (1994) adapted from Fehr and Caviness (1977).

Causes of Soybean Field Weathering

Historically, soybean was produced in the northern regions of the temperate climate zones of the world, where environmental stress were relatively minimal. However, as the world demand for vegetable oil and protein continued to increase, soybean production spread rapidly into the hot and humid production areas, and more recently into the tropical regions (Moore, 1966b; Tekrony *et al*., 1987). Since maximum seed quality is acquired by physiological maturity, it is desirable to harvest the seed as soon as possible thereafter. Practically, however, due to high moisture content (about 55%), the seed can not be harvested commercially at this growth stage

and must remain in storage on the plant through a desiccation period till moisture levels are sufficiently low to permit mechanical harvest without causing undue damage to the seed. This period may vary from a few days to over several weeks before the seed reaches a harvestable moisture level (about 14%). Field conditions are seldom favorable for such storage, especially in the tropics (Delouche, 1971). Deterioration of seed in the field prior to harvest (field weathering) begins when the seed reaches physiological maturity, and it continues until the seeds are harvested. Soybean seed quality can be reduced by a wide range of environmental factors during this period (Delouche 1974; Tekrony *et al*., 1980b).

The deterioration of seed vigor, as well as viability, due to high temperature and high relative humidity during the stages of the post-maturation and pre-harvest period is referred to as field weathering (Bhatia *et al*., 1993; Tekrony *et al*., 1980a). Exposure to hot and humid conditions is the major cause of seed quality loss following physiological maturity (Delouche 1980; Nangju 1980; Worrell 1982). Weathering is the major cause of seed quality loss following physiological maturity (Delouche 1980; Nangju *et al*. 1980). Such weathering not only lower seed germination, but also increase susceptibility to mechanical damage (Green *et al*., 1966), and disease infection (Wilcox *et al*., 1974; Tedia, 1976; Paschal and Ellis, 1978). High temperature, humidity, and precipitation play a critical role in field weathering (Mondragon and Potts, 1974; Nangju, 1979; Tekrony *et al*., 1980a; Keigly and Mullen, 1986). The rate of seed quality loss after physiological maturity depends on the degree of unfavorable environmental conditions surrounding the seed. Poor seed quality is an important constraint to soybean production in the humid tropics and subtropics.

Harvest delays beyond optimum maturity extend field exposure and intensify field deterioration (Pashal and Ellis, 1978). Green *et al*. (1965, 1966) reported that when the rain delayed soybean harvest after the seeds had initially declined to 13.5% moisture content, seed quality declined, germination and field emergence subsequently reduced. Mondragon and Potts (1974) reported that seeds subjected to

ambient environmental conditions in the field declined significantly in germination 4 weeks after physiological maturity. Tekrony *et al*. (1980a) reported similar declines in vigor when seeds were harvested 30 days after harvest maturity, especially if hot and humid conditions prevailed, and indicated that soybean seed quality was reduced by environmental factors such as temperature, precipitation, relative humidity and wind during maturation period. Bangwaek (1985) studied the delayed harvest and seed deterioration of 4 soybean varieties and found that the seeds of soybean deteriorated drastically as harvesting date were delayed. Ratanaubol (1988) studied the effect of delayed harvest on soybean seed quality and found that the seed harvested at 30 days after physiological maturity showed the lowest quality among the 3 harvesting times (10,20 and 30 days after physiological maturity). Sanguansat (1996) studied the seed deterioration of 4 soybean varieties and indicated that delayed harvest resulted in a serious reduction in seed quality especially in early rainy growing season in Thailand. The high relative rate of deterioration of soybean seeds makes it difficult to produce high quality seeds in the humid subtropics and tropics, and even in warmer areas of the temperate zone. Indeed, the difficulty in maintaining the viability of soybean seeds from harvest to the next planting season is one of the major impediments to extension of soybean production into the subtropics and tropics (Delouche, 1974).

Environmental Factors and Field Weathering

Soybean seed quality can be reduced by a wide range of environmental factors during pre-harvest period. Environmental moisture, particularly intermittent rainfall, is quite detrimental to seed quality and, in fact, cause rapid deterioration. Soybean seeds are very susceptible to extremes of wetting and drying (rehydration and dehydration) during intermittent rainy days. Frequent or prolonged precipitation during the post-maturation and pre-harvest period results in alternate wetting and drying of the seed in the pod and severe deterioration (Delouche, 1975). Howell *et al*. (1959) concluded that rain in the field or a simulated rain on intact pods increased the moisture content of the seeds, which delayed dehydration and prolonged rapid

respiration that was of a magnitude to reduce the amount of sugars and other stored materials in seeds leading to lower seed quality.

The primary factors that influence the rate of deterioration of seeds are inheritance, temperature, and seed moisture content (Delouche *et al*., 1973; Dickson, 1980; Harrington, 1972). As soybean seeds are hygroscopic, their moisture content is conditioned by the moisture in the surrounding environment. The quality of soybean seed is very much affected by climatic conditions from the time the seeds have dried to below 25 to 30% moisture content during the post-maturity drying phase until the seed is harvested. When exposed to humid conditions, dry seeds will gain moisture and consequently expand in volume. In some extreme situation, such as after a heavy rain, dried seeds can absorb enough moisture to reach 27 percent (Queiroz *et al*., 1978). At this moisture level, seed respiration is accelerated. Cotyledonary reserves will be consumed, not only by the seed itself, but also by fungi associated with the seed. These processes are temperature related, the rate of deterioration increases as temperature and seed moisture content increase. The actions and interactions of all these physiological, physical, entomological and pathological factors contribute to a common result – seed deterioration.

Both high temperature and relative humidity in tropical and subtropical environments make the production of quality soybean seed and the maintenance of seed vigor during storage difficult (Pashal and Ellis, 1978). Mondragon and Potts (1974) reported that adverse weather conditions during the post-maturation and pre-harvest period cause severe seed quality problems in soybean. Fluctuations in temperature and relative humidity determined the degree of soybean seed weathering. High temperature coupled with high moisture exerts severe stresses upon developing soybean seeds. Moorse *et al*. (1950) stated that hot weather during seed maturation often resulted in seed coat wrinkling which reduced germination. Costa (1987) evaluated 18 soybean lines in Brazil, and found that an alternation of rain and hot weather accelerated deterioration, and high temperature during final stages of seed maturation caused green seeds that were low in quality.

In addition, tropical and subtropical climates with high temperature and moisture are also favorable for rapid development of disease and insect pests, and obviously lead to a reduction in seed quality. Some insect pests pierce the pod wall and subsequently the seed coat; they may either transmit diseases directly to the seeds or provide openings, which allow pathogens and moisture to penetrate into the pod cavity, causing deterioration (Kilpatrick and Hartwig, 1955). Several pathogens also affect soybean seed quality. *Phomopsis* spp., *Colletotrichum dematium* var. *truncate*, *Cercospora kikuchii* and *Fusarium* spp. are among the fungi most frequently associated with soybean seed (Henning, 1988). The increase infection obviously leads to a reduction in seed quality. It has been pointed out that delay in harvest results in an increase in seed borne fungi and subsequently a reduction in germination (Hepperly and Sinclair, 1978; Nangju, 1979). Nicholson and Sinclair (1971) reported that the incidence and severity of fungal invasion of seeds was increased by weathering. Hepperly and Sinclair (1978) demonstrated that germination decreases were accompanied by increases in the incidence of *Bacillus subtilis* and *Phomopsis sojae*, and concluded that seeds infected by *Phomopsis sojae* were low in quality, since they were distorted in size and shape, covered by fungal mycelium, and low in seed weight. Wilcox (1974) showed that a loss in seed quality was due to increased incidence of *Phomopsis sojae* and other fungi when soybean harvest was delayed. Paschal and Ellis (1978) determined the effect of fungal infection on seed viability of 24 soybean varieties under tropical conditions, the results showed that the incidence of fungi increased from 9% on seed harvested at maturity to 45% for seed harvested 4 weeks later.

Soybean Seed Deterioration by Field Weathering

Seed quality in soybean encompasses several important attributes including genetic purity, physical purity, germination and vigor. Seed quality is influenced by several factors that occur in the field before harvesting and during harvesting, drying, processing, storage, transit and planting. These factors include extreme temperature during maturation, fluctuations in moisture (including drought), weathering, nutrient

deficiencies, occurrence of insects and diseases, and improper handling, drying and storage. The tropical and subtropical weather conditions with high temperature and relative humidity prior to harvest result in rapid decrease in germination and vigor of soybean seeds. Weathering not only lowers seed germination, but also increases susceptibility to mechanical damage (Delouche, 1972; Franca Neto *et al*., 1984; Green *et al*., 1966), and to disease infection (Wilcox *et al*., 1974; Pashal and Ellis, 1978). It also results in decreasing the quality of seed produced, such as purple stained seeds (contaminated by fungi), wrinkled seeds, fissures in the seed coat, discolored seed, insect damaged seeds, which are typical symptoms of field deterioration (Moore, 1973; Wolf *et al*., 1981; Pereira and Andrews, 1985). Embryonic tissues just beneath the seed coat become damaged during wrinkling, which may result in reduced seed germination and vigor, and a higher percentage of pathogen-infected, abnormal seedlings (Moore, 1972). Moore (1971) proposed that exposure of mature soybean seed to alternate wetting and drying in the field resulted in embryo destruction and lower quality.

Seed deterioration is a natural process that involves cytological, physiological, biochemical and physical changes in each seed. These changes reduce viability and eventually cause death of the seed. The process has been described as progressive, irreversible and inexorable (Delouche, 1973). Deterioration of soybean seed has been widely studied but the exact process of deterioration remains unexplained. Such a process is characterized by a complex of mechanisms and theories, each involving a different facet of the process.

One of the earliest theories about seed deterioration was that food reserves were depleted in the seed. This theory is not valid, however, since non-viable seeds usually have ample food reserves (Roos, 1986). That there are problems associated with the mobilization of these food reserves to the embryonic axis is a theory that is still widely accepted. Other potential causes of seed deterioration include:

- Quantitative and qualitative alterations of the chemical composition of the seed, including storage and functional lipid degradation (oxidation), nucleic acid and protein changes and nucleotide alteration.

- Alterations of membrane systems, such as the tonoplast, plasmalemma and endoplasmic reticulum, which may result in impairment of normal cell function and energy production. Membrane deterioration and loss of permeability occur at an early stage during the seed deterioration (Abu-Shakra and Ching, 1967; Byrd and Delouche, 1971; Delouche and Baskin, 1973).

- Enzyme alterations, such as reduced activity of lipase, ribonuclease, acid phosphatase, protease, catalase, diastase, peroxidase, α and β amylase, DNase, or dehydrogenase enzymes. It is unclear whether loss of enzyme activity is the cause of seed deterioration or if enzyme activity is lost as a result of seed deterioration (Cheah and Osborne, 1978; Petruzzelli and Taranto, 1990; Roos, 1986).

- Genetic damage may lead to seed disfunction. Deteriorating seeds have been reported to produce chromosomal aberrations in root tips during germination (Roos, 1986). Undesirable mutations may lead to metabolic malfunctions, as well as to problems related to the preservation of the genetic identity of germplasm.

- Microorganisms that interact with several of the above-mentioned processes and may cause faster rates of seed deterioration. Microorganisms produce exocellular enzymes and toxins (Halloin, 1986) that may cause damage to membranes, inhibition of chlorophyll action, reduced germination and seedling elongation, degranulation of the endoplasmic reticulum or enhanced solute leakage from the imbibing seeds.

The most widely accepted and useful index to measure seed deterioration is reduction in ability to germination. Based on available evidence and concepts that deterioration of seeds is progressive and that loss of germinability is the final consequence, Delouche (1969) inferred a probable sequence of deteriorative changes in seeds as follows:

1. Degradation of cellular membranes and loss of permeability control;

2. Impairment of energy-yielding and biosynthetic mechanisms;

3. Reduced respiration and biosynthesis;

4. Reduced rate of germination and early seedling growth;

5. Reduced storage potential;

6. Reduced rate of plant growth and development;

7. Decreased uniformity of growth and development within the population;

8. Increased susceptibility to environmental stresses, especially during germination, emergence, and early seedling development;

9. Decreased yield;

10. Decreased emergence percentage;

11. Increased percentage of abnormal seedling; and

12. Loss of the capacity to germinate.

The postulated sequence of deteriorative changes in seeds emphasized the increasing severity of the changes, or rather their consequences, as related to performance capabilities of the seeds. The consequence of deterioration is less disastrous than loss of germinability fall within the providence of vigor (Delouche and Baskin, 1973).

Symptoms of Seed Deterioration

Seed deterioration is an irreversible degenerative change in the quality of a seed after it has reached its maximum quality level (Abdul-Baki and Anderson, 1972). It is an inexorable, irreversible process that progressively impairs the capabilities and performance of the seed and culminates in loss of the germinative capacity (Delouche and Baskin, 1973). Deterioration of seed is usually initiated during the period following the physiological maturity while the seeds continue to dry down to the

harvest maturity stage (Delouche, 1974, 1980; Tekrony *et al*., 1980a). Field weathering of seed in terms of causes and effects is essentially the same as deterioration after harvest because field weathering is actually a kind of storage (Delouche, 1982). Some of the symptoms of the deteriorated seed are as follows:

- *Morphological changes* Seed coat color changes are presumably due to oxidative reactions in the seed coat that are accelerated under conditions of high temperature and relative humidity (Hughes and Sandsted, 1975).

- *Ultrastructural changes* By electron microscopy examination, two general patterns of coalescence of lipid bodies and plasmalemma withdrawal from the cell wall associated with deterioration have been observed. Both of these events influence cell membrane integrity (Villiers, 1980).

- *Cell membrane changes* As seeds deteriorate, their inability to retain cellular constituents decreased which was attributed to cell membrane disruptions associated with the loss of membrane phospholipids (Powell, 1986; Powell and Matthews, 1981a; Priestly and Leopold, 1983). Bewley and Black (1982) listed the consequence of membrane damage, which include (1). breaks in the structure of plasmalemma and its contraction from the cell wall, (2). fragmented endoplasmic reticulum devoid of polyribosomes, (3). monosomes randomly dispersed in the cytoplasm, (4). absence of dicytosomes, (5). disintegration of mitochondria and plastids, (6). condensation of chromatin and lobed nucleus, (7). coalescence of lipid droplets, and (8). lyses of membranes of lysomic structures.

- *Loss of enzyme activity* The most sensitive test for measuring incipient seed deterioration are the tetrazolium test and the glutamic acid decarboxylase activity test (Saxena and Maheshwari, 1980). Other enzymes that have been correlated with seed deterioration are the oxidases such as catalase, peroxidase, amylase and cytochrome oxidase.

- *Reduced respiration* As seeds deteriorate, respiration becomes progressively weaker, and ultimately leads to loss of germination. It appears that reductions in the rate of respiration are closely associated with seed deterioration (Woodstorck and Feeley, 1965; Woodstock *et al*., 1984, 1985).

- *Increase in seed leachates* A frequently observed symptom of deteriorated seeds is their increased leachate content when soaked in water. The leachate concentration has been measured by electrical conductance methods and by determines the soluble sugar content of the leachate (Abdul-Baki and Anderson, 1970). The degree of deterioration is associated with the concentration of seed exudates that may be found in the steep solution. These exudates are a reflection of amount of membrane degradation that has occurred. Ferguson *et al*. (1990) indicated that increases in conductivity of leachate from isolated axes were an early symptom of soybean deterioration.

- *Increase in free fatty acid content* The hydrolysis of phospholipids leads to the release of glycerol and fatty acids, and this reaction accelerates with increasing seed moisture content (Harrington, 1973). The continual accumulation of free fatty acids culminates in a reduction in cellular pH and is detrimental to normal cellular metabolism.

Eventually the deterioration of seeds is observable in their lowered performance during germination such as delayed seedling emergence followed by a slower rate of seedling growth and development. Deteriorated seed is also decreased resistance to environmental stresses during germination and early seedling growth (Isely, 1957; Woodstock and Pollock, 1965). Loss of the capacity to germinate was the final phase of the process of deterioration in soybean seeds and the final indication of vigor loss (Byrd, 1971; Heydecker, 1972). The ultimate performance of seed deterioration is the complete loss of germinability and death of the seed.

Possible Causes of Seed Deterioration

The effects of field weathering or deterioration are physiological, morphological, biochemical, microbiological and mechanical. The field weathering or seed deterioration is a complex process including many aspects. Darkening of seed coat was due to oxidative reactions in the seed coat which are accelerated under conditions of high temperature and relative humidity (Hughes and Sandsted, 1975;

Saio *et al*., 1980). Cell membrane disruption and inability to retain cellular constituents because the loss of membrane phospholipids (Priestley and Leopold,1983), loss of enzyme (amylase, dehydrogenase, oxidases, phospholipase, glutamic acid decarboxylase) and protein activity (Saxena and Maheshwari, 1980), reduced respiration (Woodstock *et al*., 1984, 1985), increases in seed leachates and free fatty acid content (Harrington, 1973).

The mechanism of soybean seed deterioration under high temperature and humidity has not been completely understood. It is now commonly believe that lipid peroxidation plays an important role in initiating the seed aging process. Lipid peroxidation can result in not only destruction of the lipid itself, but also damage to cell membranes and other cellular components (Wilson and McDonald, 1986b). Lambrecht *et al*. (1996) observed that a soybean mutant lacking lipoxygenase isozymes 2 and 3 were more resistant to changes during adverse storage than soybean with all the isozymes. This indirectly supports the theory that lipid oxidation plays a role in increasing cell membrane permeability (Stewart and Bewley, 1980). Smith and Berjak (1995) suggested that the following events are basic to seeds deterioration: (1) chromosome aberrations and damage to the DNA; (2) changes in the synthesis of RNA and protein; (3) changes in the enzymes and food reserves; (4) differences in respiratory activity and ATP production; and (5) membrane alterations. There are still several theories that have been suggested as basic causes of seed deterioration. Such as lipid peroxidation (Bewley, 1986; Harrington, 1973; McDonald, 1999; Stewart and Bewley, 1980; Wilson and McDonald, 1986b), degradation of functional structures (Delouche, 1982; Duke *et al*., 1986; Parrish and Leopold, 1978; Schoettle and Leopold, 1984; Seneratna and McKersie, 1983), inability of ribosomes to dissociate (App *et al*., 1971; Weidner and Zalewski, 1982), enzyme degradation and inactivation (Walter, 1963), formation and activation of hydrolytic enzymes (Elder, *et al*., 1993; Copeland and McDonald, 2001; Vazquez *et al*., 1991), breakdown in mechanisms for triggering germination (Harrington, 1973; Petruzzelli and Taranto, 1985; Takayanagi and Harrington 1971), starvation of meristematic cells (Abdul-Baki and Anderson, 1973; Anderson, 1977, 1986; Ching, 1973; Harrington 1973; Leopold and Musgrave, 1980; Yaklich and Abdul-Baki, 1979), and accumulation of toxic compounds

(Harrington, 1973; Mukhopodhyay *et al*., 1983; Sreeramulu, 1983; Stewart and Bewley, 1980; Wilson and McDonald, 1986b; Woodstock and Taylorson, 1981a, 1981b).

Many physiological and biochemical changes occur in deteriorating seeds, but it is presently difficult to discriminate between primary and secondary events because our researches are too limited to reveal a common pattern of the events. The conclusions from seed deterioration studies are often difficult to evaluate critically because all seed lots are composed of individual seeds, each possessing its own unique capability and the deterioration probably does not occur uniformly, total population studies of seed deterioration do not represent what is occurring in deteriorating soybean seeds (Lawrence and McDonald, 2001). Since the effects of field weathering on seed deterioration are physiological, morphological, biochemical and microbiological, we only have a poor catalog of the adverse metabolic and other events associated with the seed deterioration. In no instance, there are still no any general conclusions can be drawn about the importance of particular process in seed deterioration, for there are too many different events going on at the same time.

Methods for Testing Seed Quality and Field Weathering Resistance

Seed viability test

Seed quality encompasses several important attributes including genetic purity, physical purity, germination and vigor. In a standard germination, the percentage of seeds germinating is a quantitative measure of viability of the seed lot. Seed viability is the property of the seed that enables it to germinate under favorable conditions (ISTA, 1999). Numerous testing methods have been developed for determining seed viability such as:

- *Germination test* Seed viability is used synonymously with germination capacity. Germination is the emergence and development from the seed

embryo of those essential structures that, for the kind of seed in question, are indicative of the ability to produce a normal plant under favorable conditions (AOSA, 2000). A germination test is an analytical procedure to evaluate seed germination under standardized, favorable conditions that are seldom encountered in the field. The AOSA rules for testing seeds described the standard germination testing details such as media, temperature, moisture, light, dormancy breaking and germination counting standard for various crop seeds. These procedures lead to standardized interpretations in routine seed testing.

- *Tetrazolium (TZ) test* The tetrazolium test is widely recognized as an accurate mean of estimating seed viability. This method was developed by Professor Georg Lakon in the early 1940s. Today the test is used throughout the world as a highly regarded method of quick estimating seed viability and is a routine test in many seed testing laboratories (Copeland and McDonald, 2001). The tetrazolium test distinguishes between viable and dead tissues of the embryo on the basis of their relative respiration rate in the hydrated state. Handbooks of instructions for performing TZ tests and interpretation instructions have been published by the AOSA (2000) and International Seed Testing Association (ISTA, 1999).

- *Electrical conductivity test* This method is based on the premise that as seed deterioration progresses, the cell membranes become less rigid and more water permeable, allowing the cell contents to escape into solution with the water and increasing its electrical conductivity. It provides a rapid indication of seed viability for seed lots (McDonald and Wilson, 1979).

- *Vital coloring test* The principle of this method is the differential coloration of live versus dead tissues when exposed to certain dyes such as sulfuric acid, indigo carmine and aniline dyes. These dyes stain the dead tissue blue and the live tissue remains unstained. Gadd (1950) stated that this method is particularly useful for determining viability of tree seeds.

- *Enzyme activity tests* These methods measure enzyme activity (such as lipase, diastase, amylase, catalase, peroxidase and dehydrogenase) of imbibed seeds as an indication of their viability.

- *Other tests* There are still many testing methods such as free fatty acid test, Hydrogen peroxide test, ferric chloride test, indoxyl acetate test, fast green test, sodium hypochlorite test, excised embryo test, and X-ray test. These methods were discussed by Copeland and McDonald (2001).

Seed vigor test

Most people are familiar with the concept of germination testing of seed and have come to recognize a percentage value assigned to a seed lot as one of the indicators of the quality of the seed in the bag. However, under certain conditions in the field, it is often noted by producers that the laboratory germination results often overestimates seedling emergence of the seed lots (Delouche and Caldwell, 1960; Johnson and Wax, 1978; Tekrony, 1983; Tekrony and Egli, 1977; Tao, 1978; Yaklich and Kulik, 1979). As Perry (1973) pointed out, it is largely the lack of consistent relationships between laboratory germination test and field emergence that has led to the development of the concept of seed vigor. It is now increasingly recognized that seed vigor tests can more accurately reflect the ability of a seed to perform in the field. Vigor test is a sensitive index of seed quality; it is also a wide used characteristic to estimate seed deterioration.

Seed vigor refers to both the ability and strength of a seed to germinate successfully and establish a normal seedling. As stated by the Seed Vigor Test Committee of the Association of Official Seed Analysts, seed vigor is those seed properties that determine the potential for rapid, uniform emergence and development of normal seedlings under a wide range of field conditions (McDonald, 1980, 1985; AOSA, 1983). The underlying reason for testing for vigor is to determine the true value of a seed lot. Any of the events that precede loss of germination could serve as a basis for vigor test. The basic premise behind most vigor test is to subject seeds to stress, causing weak seeds to perform poorly. A variety of vigor tests under various kinds of stress have been suggested (Delouche, 1960; Heydecker, 1972; Isely, 1957; Perry, 1972; Wilson and McDonald, 1986a; Woodstock, 1973), however, only a few

have attained acceptance by seed analysts and seed testing organizations (AOSA, 1983; Perry, 1981). Commonly used stress conditions for vigor test are as follows:

- *Seed storage* (*natural aging*) By this method, seeds are subjected to prolonged storage, for example about 6 to 12 months for soybean seeds. If the time is not a limitation, prolonged storage is perhaps the best way to determine seeds with the best storability.

- *Accelerated aging* This method was proposed by Byrd and Delouche (1971) involved keeping seeds at 42 °C and 100% relative humidity (RH) for 48 hours, followed by a laboratory germination test. In this approach, vigor is interpreted as a consequence of normal deterioration, which is being mimicked by the test conditions (Tekrony, 1985, 1993, 2005). Parrish and Leopold (1978) reported physiological changes in seeds subjected to accelerated aging is similar to those prolong stored under normal conditions. Accelerated aging can also be applied to freshly harvested seeds to distinguish which lines have the best inherent seed quality and storability. In regard to soybean seed lots, accelerated aging under the conditions of 40 °C and 100% relative humidity for 48 hours or 72 hours followed by regular germination test have produced results that correlated closely with field emergence. However, difference in initial seed moisture should be considered when interpreting this test (McDonald, 1977). Delouche and Baskin (1973) found that germination after accelerated aging and periods of storage were closely associated. Similarly, Tekrony and Egli (1977) found that accelerated aging could predict how well different seed lots would emerge when planted in the field.

- *Controlled deterioration* This test was proposed by Matthews in 1980. It allows the seeds to absorb moisture to a certain degree before treated by accelerated aging (AA). The controlled deterioration test avoids some of the AA test variables by bringing the seeds to a constant moisture content before being exposed to high artificial aging conditions. Some good correlations have been obtained with field emergence and commercial storage potential on a range of species (Powell and Matthews, 1981b, 1984a, 1984b, 1985). Recent refinements have included the use of saturated salt solutions to control humidity during the test (McDonald, 1998).

- *Cold stress* This stress test involves sowing seeds into soil-filled boxes equilibrated at 10 °C and held at this temperature for 7 days, then transferring the boxes to 25 °C for additional 4 to 7 days for recording the emergence of the seedlings. Although cold stress is very useful for evaluating the seedling emergence ability in the field, the test is undesirable for screening tropical soybeans because the stress is actually not exist in the tropics. Further, it would be difficult to handle a large number of seed samples for the limitation of the cold room facilities.

- *Hot water pregermination* In this method, seeds are soaked in 75 °C hot water for 70 seconds and then rinsed in tap water before evaluating by germination or emergence. Kueneman (1982) found that some soybean lines performed well under ambient storage but poorly under hot water stress. However, lines tolerant to hot water stress also were tolerant to ambient storage. Thus, hot water stress would be an acceptable method for fast screening for seed storability.

- *Osmotic stress* Since standardization of soil conditions is difficult to achieve, a solution system is preferred. Hadas (1977) suggested that germination of seeds in polyethylene glycol solutions might provide an indication of seed vigor. Seeds are germinated in solutions such as sodium chloride, glycerol, sucrose, polyethylene glycol, and mannitol with specific osmotic potentials. Osmotic stress would not be likely to provide the breeder a test for inherent storability, but it could be a more accurate test for predicting seed vigor.

- *Thermo-stress during germination* Cole (1972) developed a seed vigor index based on the rates of germination at different temperatures by planting seeds across a thermo-gradient. The vigor index score gave similar ranking of seed lots of *Zea mays* L. as the accelerated aging test. Thermo-stress during germination might provide valuable information about cultivars that have been aged because it would subject the seeds to a temperature stress that is frequently imposed on soybean sown in the tropics.

- *Methanol stress* This method based on the fact that soaking seeds in 15 to 20 % methanol for 2 hours caused physiological changes in seeds similar to those caused by accelerated aging. Kueneman (1982) compared 51 soybean lines with

unstressed germination greater than 80%, and found a good relationship between emergence following methanol stress and following accelerated aging. It appears that the methanol stress test may provide a rapid screen for inherent storability.

Following the stress treatment, several methods can be used to evaluate the vigor of the seed or seedling:

- *Laboratory germination test* A laboratory germination test is very frequently used to access the effects of stress applied during vigor tests. The germination test has the advantage of providing uniform conditions for all seeds under evaluation. However, for a large number of seed samples, many germination incubators are required. As an expansion of the routine germination test, the normal seedling can be classified into "strong" and "weak" seedling to determine the seed vigor. However, this is a subtle task and can introduce additional variability to the results.

- *Field emergence test* The field emergence test is a good way to evaluate the actual performance of the stressed seeds and large number of seed samples can be performed at the same time. However, the variability of soil characteristics and other environmental conditions in the field can have rather large effects on the seed emergence because the seeds are very weak after stress treatment. It is preferred to perform the test in an artificial seedling bed in a screening house which providing a uniform conditions.

- *Tetrazolium (TZ) test* Lakon (1942) first reported on the use of tetrazolium salts to seed test. This test relies on the action of the TZ molecular to react with hydrogen atoms released as a result of dehydrogenase enzyme activity in living tissue, and results in the formation of a water insoluble red pigment called formazan. It involved soaking stressed seeds in tetrazolium, cutting the soaked seeds and evaluating individual seeds for their staining patterns. The TZ test is most widely used to rapid estimate the germination percentage of seed lots. It is equally applicable for evaluating vigor of seed as has long been advocated by Moore (1962, 1966a, 1985). When conducted by an experienced analyst, it is probably the most informative of all tests for evaluating the physiological quality of seed.

- *Seedling growth rates* Vigorous seeds are able to efficiently synthesize new materials and rapidly transfer these new products to the emerging embryonic axis, resulting in increased dry weight accumulation. This test is generally conducted according to the standards for the routine germination test. After germination evaluations are made, the growing segments of the seedlings are excised from storage organs (cotyledons and endosperm), dried and weighed to determine their increases. Seedling dry weight may reflect general vigor of stressed seeds. However, differences in seedling weight among various genotypes may reflect genotypic difference not related to seed quality because seedling growth can be genetically controlled in specific cultivars (Burris, 1975).

- *Electrical conductivity of seed leachate* It has been well known that cell contents of deteriorated seeds will leach out when soak the seeds, and the amount of solute leached is related to the degree of seed deterioration. Consequently, measuring the electrical conductivity of leachate has been proposed as a vigor test and has been shown to be inversely related to field emergence for soybean (Yaklich and Kulik, 1979, Hampton and Tekrony, 1995). Takayanagi and Murakami (1969) proposed measuring the sugar quantities in leachate using urine sugar analysis paper to detect differences in seed vigor among seed lots. While this method may have merit for within-cultivar comparisons of different seed lots, it would not likely be very efficient for between-cultivar evaluation because of inherent differences in seed composition unrelated to seed vigor.

- *ATP and GADA tests* Abu-shakra and Ching (1967) found evidence of considerable uncoupling of oxygen uptake and ATP synthesis in mitochondria extracted from aged soybean. Oxygen uptake was higher and ATP production was lower than in isolates from high vogue material. Initial ATP production was correlated with seed vigor and was a sensitive index of seed deterioration (Yaklich *et al*., 1979; Lun and Madsen, 1981). Glutamic acid decarboxylase activity (GADA) is important in seed germination because glutamic acid comprises a high percentage of total amino acids in seeds, for example 19% in soybean. GADA test measures the evolution of CO_2 as glutamate is enzymically converted to amino butyric acid. Studies showed a direct relationship between seed

vigor and GADA (Burris *et al*., 1969; Delouche and Baskin, 1973; Ram and weisner, 1988; Van de Venter *et al*., 1989).

The importance of seed vigor as an attribute of seed quality in soybean and other crops has been proposed by seed scientists for many years (Heydecker, 1972; Pollock and Roos, 1972). Several laboratory tests have been proposed to evaluate seed vigor in soybean and the tests have been standardized by seed analysts (AOSA, 1976; McDonald *et al*., 1978). Preliminary results from extensive studies conducted with the objective of establishing a vigor rating system for soybean seed lots showed that three vigor tests are especially promising for soybean: the accelerated aging (AA) test, the tetrazolium (TZ) test and the rate of seedling growth evaluation interpreted for vigor (AOSA, 1976). Deterioration and vigor are essentially antonymous. They represent the negative and positive aspects of the seed physiological conditions. Most of the methods used to evaluate seed vigor are the same as those used in research on seed deterioration (Abdul-Baki and Anderson, 1973; AOSA, 1976; Johnson and Wax, 1978; Pinthus and Kimel, 1979; Tekrony and Agli, 1977; Yaklich and Kulik, 1979; Yaklich *et al*., 1979). Indeed, a substantial portion of the information on seed deterioration derives from studies of seed vigor.

Field weathering resistance test

To improve field weathering resistant cultivars, screening methods that can distinguish resistance are required. Since the field weathering is caused under the hot and humid field condition after the seed physiologically matured, the most common procedure for evaluating resistance to seed weathering is to leave plants in the field beyond the normal harvest period and then access the quality of the seed by visual score, examining seed borne fungi, seed vigor test, or use a combination of these assessment methods. This delayed harvest technique has several limitations, for example, genotypes maturating at different times will be subject to different environmental weathering stress and different period of weathering. A major

difficulty in using the delayed harvest approach is applying the same environmental stress conditions to cultivars of different maturities.

In an attempt to overcome the limitations of delayed harvest, Kueneman (1982) developed spreader row and overhead irrigation techniques to accelerate weathering based on the delayed harvest method and found the cultivar differences were highly significant. Artificial seed weathering methods, such as incubator weathering, can minimize the effects of variable pod maturity. Kueneman (1982) harvested the yellow pods from test plants and placed on a metal incubator tray with 1 cm apart, then incubated at 30°C and 85-90% RH for 7days before final drying and germination test. He found the emergence score decrease significantly after one week incubation. Dassou and Kueneman (1984) compared unweathered seeds with three weathering treatment, i.e. incubator weathering (pods at physiological maturity were detached from the plant and kept at 30 ℃ and 90-95% relative humidity for 10 days), wet-burlap-bag weathering (plants at physiological maturity were placed in wet burlap bags for 10 days) and field weathering (seeds were harvested 3 weeks after physiological maturity). They concluded that the incubator weathering treatment minimized intraplant variability and environmental effects that would confound comparisons among genotypes of different maturity. The incubator weathering method promised a practical screening procedure for identification of resistance to field weathering of soybean seeds and has been widely used since then. However, the incubator conditions are conductive to the rapid growth of pathogens, which is likely to encourage deterioration, thereby neglecting physiological factors (Balducchi and McGee, 1987). Horlings *et al*. (1994) modified the treatment to incubate the pods at 27 ℃ and 90-100% relative humidity for 4 days and indicated that the modified incubator treatment had the most detrimental effect on seed quality with a wide range in germination.

In breeding programs, visual selection, such as rating of defective seed coat, wrinkled seed coat, shriveled cotyledons and green cotyledons may be effective in eliminating the poorest progeny and could be used routinely (Green *et al*., 1971).

Non-visual screening to identify lines with superior quality characteristics usually involves accelerated aging test. It consisted of submitting soybean seeds to 42 °C and 100% relative humidity (RH) for 48 hours prior to evaluation for vigor or viability. For use in breeding programs, more severe stress, such as controlled deterioration, is necessary. Wien and Kueneman (1981) evaluated an aging period of 72 hours, but had problems with fungal growth and subsequent contamination. These problems were overcome by lowering the RH to 75% and extending the aging period to 6 weeks. Kaster *et al*. (1989) treated the seeds at 42°C and 95% RH for 96 hours, the best results were obtained. For seeds recently harvested (up to 3 months), increasing the aging period to 120 hours also produced good results.

Soybean Seed Characters and Field Weathering Resistance

In soybean, some seed characters found to be beneficial under tropical conditions (resistant to field weathering) are hard seed coat (Hartwig and Potts, 1987; Potts *et al*., 1978; Rungsarid, 1982; Suriyon, 2003), small seed size (Dassou and Kueneman, 1984; Edwards and Hartwig, 1971; Horlings *et al*., 1991; Nangju, 1979; Paschal and Ellis, 1978; Tiwari and Joshi, 1998; Wangkam, 1999), and black seed coat (Dassou and Kueneman, 1984; Horlings *et al*., 1994; Kueneman and Costa, 1987; Starzinger and West, 1982). Among these characteristics, hardseedness has been extensively studied because it was also considered to be related to seed dormancy and seed longevity. Gilioli and Franca Neto (1982) studied the effects of weathering on 13 breeding lines with impermeable seed coat. Seeds were severely aged in the greenhouse for 14 days after harvest maturity and then scarified and planted. Seeding emergence of the impermeable lines ranged from 70.5 to 93.5 percent, compared to 14% for the check cultivar *Panana* (permeable). This result showed that the permeability of the seed coat plays an important role on seed weathering.

Hardseedness is the inability of seeds to absorb water due to seed coat impermeability. Impermeability reduces the fluctuation in seed moisture that can occur with alternating wetting and drying conditions in the field, making seeds less

susceptible to weathering (Potts *et al*., 1978; Hartwig and Potts, 1987). The hard seed coat helps to prevent viability loss by limiting the exchange of water and gas between the seed and the environment. The seed coat also has a relevant role in preventing the entry of pathogenic microorganisms. Physiological changes of the seed coat, with or without pathological interactions, generally play a primary role in seed deterioration (Kueneman, 1983). Hard-seeded line also had a lower incidence of seed borne pathogens, higher storage potential, less mechanical damage during harvest and reduced damage by stink bugs (Dassou and Kueneman, 1984; Franca Neto and Potts, 1979; Franca Neto *et al*., 1983; Miranda, 1977; Hill *et al*., 1985; Potts *et al*., 1978). Dassou and Kueneman (1984) found that most lines resistant to field weathering were also resistant to deterioration in post harvest storage.

Seed coat permeability is caused by both genetic and environmental factors, thus, a potential exists for fast selective changes in hardseedness as an adaptation to xeric environments. However, the structural and chemical changes involved in these mutations have not been elucidated. During the late stages of seed maturation, the loss of water and the subsequent shrinkage of cells result in a closer packing of cells, and even in cell collapse and lumen obliteration in the testa. This mechanical compaction of cells drastically reduces water uptake (Kigel, 1995). Hardseedness has been attributed to small-elongated pores and a high density of waxy material embedded in the testa epidermis (Calero *et al*., 1981; Tully *et al*., 1981; Yaklich *et al*., 1986; Mugnisjah *et al*., 1987). However, the role of the cuticle and waxy surface layers is also doubted since their removal by organic solvents or superficial scratches did not improve the water uptake (Rolston, 1978). Climatic conditions during seed development influence seed coat structure and permeability of the seeds. The incidence of hardseedness is greatest when seed maturation occurs under high temperature and dry conditions (Dassou and Kueneman, 1984; Hill *et al*., 1986; Potts *et al*., 1978). Hill *et al*. (1986) concluded that high soil moisture availability during soybean seed fill reduced seed coat impermeability. Reduces supply of cytokinins and minerals to the developing soybean seeds, which may occur under water stress, causes the development of thicker and less permeable seed coat (Nooden *et al*., 1985).

Temperature fluctuations also result in the fracture of the strophiole and in subsequent water uptake by the seed (Hagon, 1971), which may lead to faster loss of impermeability (Kigel, 1995).

Although soybean cultivars with high percentages of impermeable seed coats are less prone to weathering, however, there are some undesirable attributes that may restrict its use. Expression of this character is influenced by several environmental factors. Water stress, seed size and field environment all have marked effects on seed coat permeability (Hartwig and Potts, 1987; Hill *et al*., 1986a, 1986b; Minor and Paschal, 1982). Hard seed coat is also not desired in commercial soybean varieties because it causes un-uniform germination and emergence. Disadvantages include an increased number of volunteer plants in later crops, a reduced rate of stand establishment and the need to scarify seed lots having high levels of hardseedness (Potts *et al*., 1978). Small seed size and black seed coat are also not desired in the market even they are not influence yield (Hartwig and Edwards, 1970). There are also some restrictions on the physical characteristics of the seed. For example, yellow seed coat and cotyledon color are considered essential. Colorless hilum is also desirable since there is a tendency for hilum pigments to diffuse into the seed coat resulting in mottled seed coat color. Special carefulness is needed to use these characteristics in breeding programs.

Heredity of Soybean Field Weathering Resistance

Genotypic differences in resistance to field weathering have been observed (Green and Pinnel, 1968a; Pashal and Ellis, 1978, Potts *et al*., 1978; Ndimande *et al*., 1981; Korte *et al*., 1983; Kadhem *et al*., 1985). Some cultivars of soybean do appear to be inherently more susceptible to field deterioration than others. Nangju (1977) and Paschal and Ellis (1978) reported that soybean variety *Improved Pelican* was resistant to purple seed and field weathering. Korte *et al*. (1983) and Kadhem *et al*. (1985) found that soybean *Elf*, *Will* and *Hobbitt* exhibited better visual seed quality than *Nebsoy* and *Amcor*. Ndimande *et al*. (1981) found two blackseeded accessions of Indonesian origin, *TGM685* and *TGM686*, are also resistant to field weathering.

Lassim *et al*. (1982) compared the rate of field deterioration of 3 soybean varieties (*Mack*, *Dare* and *Forrest*), the germination of *Mack* seeds dropped from over 90% to 80% at three weeks after maturity and further dropped to 68% at six weeks after maturity. However, *Dare* and *Forest* seeds remained above 80% germination until over 6 weeks after maturity under the same field conditions. The results clearly indicated that certain cultivars possess differential rates of deterioration even they maturated at the same time and exposed to the same weathering stresses, the seed of *Mack* decreased much more rapidly in germination than other varieties, and the *Mack* seeds were more adversely affected by delays in harvest and weathering than *Dare* and *Forrest* seeds. It appears that the seed of *Mack* cultivar is inherently more susceptible to weathering than the seed of *Dare* and *Forrest*. Paschal and Ellis (1978) and Costa (1987) provided additional evidence that substantial genetic variation exists in different cultivars for seed quality characteristics measured under tropical conditions. Cultivars with small seed size appear to be better adapted to some tropical climates and to be resisted weathering and invasion by pathogens. Kaowanant (2003) also found that smallseeded *GC10981* and *GC10848* are more resistant to field weathering than *Chiangmai 60*. This is the material basis of this study as well.

Genetic variability for resistance to deterioration exists. Genetic studies on components of resistance to field weathering have already been mentioned. Kilen and Hartwig (1978) studied the heritability of impermeable seed coat and assumed that the trait was controlled by maternal tissue. They also concluded that at least three major genes control the permeability characteristics. Green and Pinnel (1968a and 1968b) evaluated the inheritance of resistance to field weathering by crossing the resistant cultivars with the susceptible cultivars; they also assumed maternal control of the resistance. Broad sense and narrow sense heritability estimates based on field emergence and laboratory germination were very low. Their results also showed low narrow sense heritability for wrinkled seed coats, shriveled cotyledons, green cotyledons, average visual rating and overall visual rating. However, genetic differences appeared to be small in comparison with the effect of environmental stress (Tekrony, 1980b). A significant genotype and environment interaction was detected following selection of 20 cultivars for improved seed germination ability (Unander *et*

al., 1983). Genetic variability for seed quality exists but degree of potential improvement is small in comparison with the main effect of environment. Like some other environmental stress resistance in plants, the field weathering resistance of soybean may also be controlled by polygene (QTLs).

Reducing Field Weathering Damage by Breeding and Field Management

Historically, breeders have used selection and crossing techniques to enhance the agronomic performance of soybean. The basic methods used in breeding for improved varieties of soybeans are the same as used in other crops including pedigree, bulk-population, and various modifications of pure line methods of plant breeding. However, breeding objectives have changed from the initial primary emphasis on yield to include other traits believed necessary for wider adaptability to expanding production. Progress has come mainly from breeding for resistance to disease and shattering and to suitability to mechanical harvesting. Improvement in physical and chemical attributes of the seed has also been attained. As in other crop species, continued improvement in yield appears to be more and more difficult to attain. Some argue that further progress must come from increase emphasis on disease resistance, insect resistance, and environmental stress resistance (Brim, 1973). To improve seed quality in subtropical and tropical soybean production areas, breeding programs should incorporated resistance to unfavorable conditions while continuing to stress the necessity for improved management and production practices (Andrews, 1982).

Soybean breeders in the tropics have made significant efforts towards developing adapted cultivars with genetically improved seed quality. This task has received greatest emphasis at the International Instituted of Tropical Agriculture (IITA) in Nigeria and at the National Soybean Research Center of the Brazilian Agricultural Research Enterprise. At the IITA, several soybean lines from Southeast Asia with small, black seeds were found to have good seed quality. Eight months after ambient storage reduced the emergence of the cultivar *Bossier* to nearly zero, whereas

the selected lines maintained at least 50% emergence (Wien and Kueneman, 1981). Researches in IITA have confirmed the possibility of selecting for improved seed quality among soybean lines, and several sources of high seed quality have been identified (Gilioli *et al*, 1982; Paludzyszyn *et al*., 1987; Kaster *et al*., 1989a, 1989b). *Doko*, released in 1980 in Brazilian, is an example of a tropically adapted cultivar with good seed quality. Fungicide-treated seeds of this cultivar could overcome severe accelerated aging condition (41°C for 96 hours) without any decrease in germination (Franca Neto *et al*., 1985).

As mentioned above, several attributes of soybean that may contribute to improved seed quality are seed-coat properties, pod-wall permeability, resistance to fungi, seed size and cell-wall permeability. To increase the soybean seed production and quality in the tropics, cultivars with inherently good seed quality should be used. In low-latitude tropical areas, it is practical to import good quality seeds from higher latitude location, where environmental conditions are favorable for seed production. Regions best suited for seed production should be selected. The best areas are characterized by cool and dry conditions during maturation and harvest, These conditions are not easily found in the tropics, but they may be achieved in areas of higher altitude (above 800 m) or sometimes, by adjusting planting dates. As a general rule, maturation of soybean seeds should occur when average temperatures are 22°C or lower. Clearly, as suggested by TeKrony *et al*. (1980b), predicting the effects of alternating wetting and drying in the field on seed quality must take into consideration with the temperature at which it takes place.

Altering planting dates to allow the critical stages of seed maturation to coincide with favorable segments of the field environment may prove feasible (Green *et al*., 1965). In the tropics, there can be distinct planting dates for the production of grain and seed. For grain production, planting dates should be adjusted so that maximum yields of good quality grain are obtained. For seed production, however, quality comes before quantity and yields must often be sacrificed to produce seeds of high quality. By carefully matching cultivar maturity with rainfall patterns, planting

dates that will minimize field deterioration can often be selected (Franca *et al*., 1994). In the low-latitude tropics, where soybean can be planted at any time of the year, there are also specific periods for production of high quality seeds. Seasons in many of these areas are often characterized by the extent of the rainy period. In West Africa, where a bimodal rainfall pattern exists, soybean should be planted in the major rainy season for grain production and the minor rainy season for seed production. Although yields are higher in the major season, seeds produced are frequently purple-stained, cracked and discolored (Nangju *et al*., 1980).

Seeds should be harvested promptly when their moisture content first reaches 12 to 15%. A delay in harvest will result in reduced germination and vigor of the seeds, especially when weather conditions are adverse (Costa *et al*., 1983). Additionally, seed infection by pathogenic and saprophytic microorganisms will increase with harvest delay. Several practices can be suggested to lessen the deleterious consequences of field deterioration. Foliar fungicides may improve the quality of soybean seed. The application of defoliants may permit earlier harvest, and, theoretically, if soybean seeds are removed earlier from adverse field conditions, seed quality should be better. Andreoli and Ebeltoft (1979) reported that by applying defoliants that could speed up the plant maturation and the dry-down time of soybean seeds, which resulted in better seed quality. Nevertheless, Costa *et al*. (1983) found no substantial increase in soybean seed germination and vigor and so did not recommend the use of defoliants. In fact, they reported greater losses during harvest, since some plants were run over by the tractor tyres while the defoliant was being applies. Durigan (1979) also found no improvement in seed quality and detected paraquat residues in the seeds, making them unsuitable for use by humans or animals. Others (Bovey, 1969; Metcalfe *et al*., 1956) have warned about the risk of residue and about the possibility of a decrease in seed germination, depending on the desiccant and dosage used. The use of systemic fungicide also provides some degree of protection against weathering pathogens.

Genetic Markers and Marker Assisted Selection in Crop Breeding

Genetic markers

Genetic markers are those biological characteristics indicate the genetic diversity of organisms. A genetic marker must be polymorphic, that is, it must exist in different forms so that chromosome carrying the mutant gene can be distinguished from the chromosome with normal gene by the form of marker it also carries (Chawla, 2004). Genetic markers include morphological marker, cytological marker, biochemical marker and DNA marker.

- Morphological markers are mainly the visible characteristics of organisms such as plant height, flower color, and seed color. Morphological markers are easy to be detected, but they are also easy to be effected by the environment and just limited number of characteristics can be found and used.

- Cytological markers are the characteristics of chromosomal variation. Cytological markers include the nucleic pattern (the structure and number of chromosomes) and the banding pattern (C-band, N-band and G-band) of chromosomes. They are not affected by the environment, but it's difficult to develop marked materials (such as triploid material).

- Biochemical markers are the pattern of proteins. They include enzyme markers (isozymes and allozymes) and non-enzyme markers (storage proteins). There are more than 50 enzyme systems (about 100 gene loci) have been identified and widely used (Vallegos and Chase, 1991). Morphological attributes and protein loci (isozymes) were typically used in linkage studies in many crop species. However, the number of biochemical marker is still not enough for further researching and breeding. The usefulness has been limited because of the limited number of loci and limited level of polymorphism associated with them.

- The era of DNA markers began two decades ago when Bostein *et al*. (1980) highlighted the potential of DNA markers and unleashed the

possibility of innumerable restriction length fragment polymorphism (RFLP) markers due to variation of restriction sites in natural populations. Soon after the discovery of RFLP, numerous marker types were recognized and widely reviewed for their competence in crop improvement (Tanksley *et al*., 1989; Paterson *et al*., 1991; Burrow and Blake, 1998; Joshi *et al*., 1999).

- The most recent additions for DNA markers are single nucleotide polymorphism (SNP) (Brookes, 1999; Marshall, 1997; Rafalski, 2002) and the markers based on miniature inverted repeat transposable element (MITE) (Casa *et al*., 2000).

DNA markers

DNA markers are the DNA fragments that can detect the genetic diversity between organisms at DNA level. DNA markers include DNA-DNA hybridization based markers, polymerase chain reaction (PCR) based markers, and target sequence based markers.

- DNA-DNA hybridization based markers are DNA fragments that can be used denatured and hybridized with genomic DNA or other sources of DNA fragments, including restriction fragment length polymorphism (RFLP) (Botstein *et al*., 1980), variable number of tandem repeats (VNTR) and *in situ* hybridization probes.

- PCR based DNA markers are primers using for PCR or PCR amplified DNA fragments, including random primers and sequence specific primers. Random amplified polymorphic DNA (RAPD) (Williams *et al*., 1990), Arbitrarily primed polymerase chain reaction (AP-PCR) (Welsh and McClelland, 1990), DNA amplification fingerprinting (DAF) (Caetano-Anolles *et al*., 1991, 1993, 1994), arbitrarily primed polymerase chain reaction (AP-PCR) (welsh and McClelland, 1990) and inter-simple sequence repeat (ISSR) primers are random primers. Simple sequence repeat (SSR) (Litt and Luty, 1989; Zietiewicz *et al*., 1994), amplified fragment length polymorphism (AFLP) (Vos *et al*., 1995),

random amplified microsatellite polymorphism (RAMP) (Wu *et al*., 1994), selective amplification of microsatellite polymorphism loci (SAMPL) (Vogel and Morrante, 1995), random amplified hybridization microsatellite (RAHM) (Cifarelli *et al*., 1995), single strand conformation polymorphism (SSCP) (Orita *et al*., 1989) and resistance gene analog (RGA) primers are sequence specific markers.

- Target sequence based markers are DNA markers developed from specific DNA sequence, including sequence targeted site (STS) (Olson *et al*., 1989), sequence tagged microsatellite (STM) (Litt and Luty, 1989), sequence characterized amplified region (SCAR) (Martin *et al*., 1991) and cleaved amplified polymorphic sequence (CAPS) (Konieczny and Asubel, 1993).

AFLP technology and bulk segregant analysis

Among the DNA markers, AFLP has proven its advantages in many aspects of genome analysis as a high throughput marker. AFLP is a method that combines PCR and RFLP technology developed by Zabeau (1993) and Vos *et al*. (1997). By combining different restriction enzymes and the number of selective nucleotides in the primers for PCR, the AFLP method offers a larger number of genetic markers from a single PCR than previously available methods. Thus, it is a more efficient and less costly method. The level of polymorphism detected by AFLP is lower than those with other mapping techniques, such as RFLP and SSR. However, because of its robustness, reliability, and efficiency, it is gaining popularity among researchers for many types of investigation, including mapping, fingerprinting, positional cloning and phylogenetics (Ridout and Donini, 1999). The AFLP technology, generally used for the fingerprinting of genomic DNA, can also be used with cDNA preparations for transcript profiling. This cDNA AFLP technology permits the display and quantification of transcripts based on AFLP fingerprinting of double-stranded cDNA. The transcript profile obtained by using this technique is a reliable and efficient tool for the identification of differentially expressed mRNAs. The AFLP marker system appears to be a useful approach for generating high-density genetic maps in soybean

(Keim *et al*., 1997). It was concluded that, among the RFLP, RAPD, and AFLP techniques, AFLP is the most useful (Lin *et al*., 1996).

Bulked segregant analysis (BSA) has been widely used as a tool to find markers linked to target genes or linked to other markers since Michelmore *et al*. (1991) described the technique. BSA is effective for high-resolution mapping to find genes controlling simple inherited traits, such as disease resistance genes. Indirectly, BSA can be used to saturate linkage maps and to screen for markers linked to QTLs. In BSA, the samples from different individual progeny in a family are pooled into bulked samples by genotypic class or by phenotypic class. A specific target allele will occur in one bulked sample, but not the other. It is a strategy to select individuals from a segregating population that are homozygous for a trait of interest. In the pooled DNA sample, most of the genomic region will be homozygous, only the genomic region encompassing the trait or marker of interest will be polymorphic, which can be used as a target for screening DNA markers rapidly (Michelmore *et al*., 1991; Giovanonni *et al*., 1992). A polymorphic marker which shows a clear difference between the two bulks is likely to be linked to the target genes or nearby markers. This means that any trait that can be scored in an F2, backcross, or RI population can be rapidly targeted with DNA markers by BSA (Zhang *et al*., 1994). However, the success of bulking by phenotypes is dependent on the correspondence of genotype and phenotype. PCR based markers have been commonly used in BSA. The BSA used in conjunction with AFLP markers, make it possible to identify large number of DNA markers in a region of interest in a short time.

Molecular marker assisted selection in plant breeding

Breeders have traditionally improved plant varieties by selecting on the basis of phenotype. Although it is possible to undertake breeding programs using only phenotypic selection, however, the phenotype of a plant is determined not only by its genotype but also by the environment in which it is grown. To circumvent such problems, plant breeding techniques based on detailed statistical inference have been

developed. Molecular marker technology-empowered genome analysis offers a new level of resolution to crop improvement efforts. It has been well accepted as a valuable adjunct to classical breeding tools (Burr *et al*., 1983; Beckmann and Soller, 1986; Tanksley *et al*., 1989; Paterson *et al*., 1991; Young, 1992, 1999a, 199b; Paterson, 1996, 1998). Molecular markers can facilitate selection for both single gene traits as well as polygenic inherited traits for agronomic crop improvement (Bent and Yu, 1999; Kumar, 1999). Molecular markers have been rapidly adopted by crop improvement researchers globally as an effective and appropriate tool for basic and applied studies addressing biological components in agricultural production systems (Jones *et al*., 1997; Mohan *et al*., 1997; Prioul *et al*., 1997) as they offer specific advantages in assessment of genetic diversity and in trait-specific crop improvement (Edwards, 1992; Paterson *et al*., 1991). It was stated that the molecular markers can mask the effect of minor linked genes making it possible to identify desirable linkages for selection (Tanksley *et el*., 1989).

Integration of novel techniques and methodologies into conventional breeding programs is needed to facilitate the identification, the characterization, and the manipulation of genetic variation for continued and accelerated progress (Wilson, 1993; Sorrels and Wilson, 1997). Biotechnology offers two new ways for improving crops: one through the development and application of molecular markers, and the other through genetic engineering. Molecular markers technology offers a novel approaches to improve efficiency of selection, and have become a critical tool in studies of genetic variation in crops. The development of comprehensive genetic maps by molecular markers has enormously improved the power of genetic analysis (Snape *et al*., 2001). In addition, the use of marker assisted selection (MAS) will enhance selection efficiency not only for Mendelian traits for which individual phenotypes provide large information about the underlying genotypes, but also for most of the complex traits of agronomic interest for which phenotypes are less informative about the underlying genotypes (McCouch, 2001).

Use of markers in applied breeding programs can range from facilitating appropriate choice of parents for crosses, to mapping and tagging gene blocks associated with economically important traits (Doerge *et al*., 1997). Tanksley *et al*. (1989) have developed application in backcross breeding programs where effective selection for the recurrent parental background genotype can be accelerated. Gene tagging and QTL mapping in turn permit marker assisted selection (MAS) in backcross (Frisch *et al*., 1999a, b; Ribaut *et al*., 1997, 2002) and pedigree programs (Mohan *et al*., 1997). An understanding of the number and location of quantitative trait loci controlling performance for a target trait can markedly enhance the efficiency of breeding. Marker assisted selection appears to be especially useful for crop traits that are otherwise difficult or impossible to deal with conventional means (Moreau *et al*., 1998). Near-Isogenetic products of a marker assisted backcrossing program in turn provide genetic tools for crop physiologists and crop protection scientists to use in improving understanding of the mechanisms of tolerance to various abiotic stresses such as extreme of temperature and water availability (Jones *et al*., 1997; Prioul *et al*., 1997). The primary challenge faced by plant breeders today is how to develop superior cultivars by transferring a repertoire of useful genes in a cost effective, timely, and precise manner. Although the greatest benefit of using molecular markers in breeding is the considerable savings in time required to attain a certain genetic gain for the agronomic traits that are difficult to evaluate (Burr *et al*., 1983; Tanksley *et al*., 1989). Marker assisted selection is definitely useful to manipulate chromosomal regions and rapidly design new genotypes combining favorable QTLs or genes.

The most interesting use of molecular markers is in the more efficient selection that they make possible for polygenic characters controlled by quantitative trait loci or QTLs. The use of molecular markers such as isozymes and RFLP has made it possible to differentiate the quantitative effects of different loci on the same character (Stuber, 1992, 1995), and recent advances in plant molecular genetics have eventually allowed the accurate mapping, cloning and transferring of QTL (El-Assal *et al*., 2001; Frary *et al*., 2000; Johanson *et al*., 2000; Lin *et al*., 2000; Takahashi *et a*l., 2001; Van Berloo *et al*., 2001; Yano *et al*., 2000). The detection and location of

quantitative trait loci enables the use of MAS for attributing difficult to manage by conventional breeding approaches, leading to a potentially more reliable, quick, and efficient selection. However, unless the mapping population sizes are very large, the confidence intervals associated with QTL location can also be large. The lower the heritability of the trait and the smaller affect of the QTL, the larger the confidence interval. For typical population sizes of 100 to 200 RILs, the confidence intervals for detectable QTL are seldom less than 5 cM and often 20 cM or more (Kearsey and Farquhar, 1998). Currently, the development of chromosome substitution lines, near isogenic lines and stepped aligned inbred recombinant strains enable QTL to be mapped to a relative small region of a chromosome, and such a accuracy may be adequate for marker assisted selection (Kearsey, 2002; Koumproglou *et al*., 2002).

The identification and fixation of some economic important QTLs have been successfully studied on tomatoes (Tanksley *et al*., 1982), maize ((Stuber *et al*., 1987), rice (McCouch and Doerge, 1995) and potatoes (Bradshaw *et al*., 1998). The identification of molecular markers linked to traits of economic importance has allowed the development of strategies for manipulating the genotypes or introducing genes that enable the plant to tolerate stress (Snape *et al*., 2001). Functional genomics has the potential to reveal the genetic basis phenotypic response to the environment and, hence, open up the possibility for genetic improvement by transformation. However, adaptation to stress at the whole plant level involves the interaction of many genes that are expressed at multiple levels. The challenge now is to shift through the massive database to identify genes associated with QTLs for stress tolerances (Forster *et al*., 2000). A deeper crop ecophysiological understanding is required to take full advantage of biotechnology on crop breeding (Araus *et al*., 2003; Slafer, 2003). The rapid pace of changes and innovation in the field of molecular biology will undoubtedly result in faster, more efficient and more informative methods applicable to improving the speed and precision of plant breeding.

Although the application of the marker assisted selection in crop Improvement has not been striking for complex quantitative traits. It has been largely limited to

simply inherited traits (Hittalmani *et al*., 1995; Huang *et al*., 1997; Mudge *et al*., 1997; Davierwala *et al*., 2001; Wang *et al*., 2001). Since QTLs are affected by the degree of linkage disequilibrium between the QTL and the markers, and the environment x genotype interaction (Gillespie and Turelli, 1989). Therefore, the direct application of molecular markers in constructing desirable QTL allelic combinations, across a wide range of genotypes and environment, is probably quite limited. The difficulty of manipulating quantitative traits is related to their genetic complexity, principally the number of genes involved in their expression and interactions between genes. As several genes are involved in the expression of polygenic traits, they generally have smaller individual effects on the plant phenotype and are cross- dependent. This implies that several regions must be manipulated simultaneously to have a significant impact, and that the effect of individual regions is not easily identified. Hospital *et al*. (1997, 2000) studied selection on marker pairs flanking 50 QTL identified in a F2 population and concluded that the efficiency of marker based selection is bounded by the recombination taking place between the markers and the QTL. Current progress in mapping consistent QTL for yield and stress tolerance characteristics can potentially lead to successful MAS for complex traits in the future (Subudhi and Nguyen, 2004).

Molecular Biological Researches on Soybean

Soybean genetic mapping

The soybean genome contains an estimated 1.1 x 10^9 bp for 1n DNA content (Arumuganthan and Earle, 1991). In soybean, several genetic linkage maps including various molecular markers have been constructed since the early 1990s (Akkaya *et al*., 1995; Cregan *et al*. 1999; Diers *et al*., 1992; Keim *et al*. 1989, 1990a, 1997, 2000; Kim *et al*. 2000; Lark *et al*. 1993; Mansur *et al*. 1996; Morgante *et al*., 1994; Palmer and Shoemaker 1998; Rafalski *et al*., 1993; Shoemaker *et al*. 1992, 1996a, 1996b; Shoemaker and Olson 1993; Shoemaker and Specht 1995; Song *et al*. 2004). The first soybean genetic linkage map of molecular markers was reported by Keim *et al*. (1990a). This map consisted of 26 genetic linkage groups containing 150 RFLP loci.

Later, more markers were added into this map, and it includes 252 RFLP markers with a map length of 2147cM (Diers *et al*., 1992; Shoemaker and Olson 1993). Lark *et al*. (1993) constructed a genetic map containing 132 RFLP, isozyme, protein and morphological markers with a map length of 1550 cM. Rafalski *et al*. (1993) and Morgante *et al*. (1994) developed a genetic map containing 600 RFLP and SSR markers with a map length of 2679 cM. Akkaya *et al*. (1995), Shoemaker and Specht (1995) and Palmer and Shoemaker (1998) developed a map containing 530 RAPD, RFLP, SSR, and morphological markers with a map length of 1486 cM. Mansur *et al*. (1996) developed a map containing 220 RFLP, SSR, and morphological markers with a map length of 2000 cM. Keim *et al*. (1997, 2000) developed a map consisting 840 markers (165 RFLP, 25 RAPD, and 650 AFLP markers), which spread over 28 linkage groups representing 3,441 cM distance. Cregan *et al*. (1999) constructed a map of twenty consensuses molecular linkage groups based on three mapping populations contain 689 RFLP, 606 SSR, 79 RAPD, 11 AFLP, 10 isozyme and 26 classical loci. The resulting map was about 2400 cM in length. Recently, Song *et al*. (2004) combined 5 maps into an integrated genetic map spanning 2523.6 cM of Kosambi map distance across 20 linkage groups that contained 1849 marker, including 1015 SSR, 709 RFLP, 73RAPD, 6 AFLP, 10 isozyme, 24 classical traits and 12 other markers.

Soybean QTL identification

Saturated genetic maps allow us to evaluate genetic regulation and interactions of genes or chromosome regions that affect complex agronomic traits. A broad range of studies have been conducted to identify quantitative trait loci (QTLs) affecting agronomically important traits in soybean. Based on the construction of soybean genetic maps, quantitative trait locus (QTL) for a number of agronomic traits in soybean have been mapped (Arahana *et al*. 2001; Brummer *et al*. 1994; Concibido *et al*., 1994; Csanadi *et al.* 2001; Diers *et al*. 1992a,b,c; Funatsuki *et al.* 2005; Guo *et al*. 2005; Hyten *et al.* 2004; Iqbal *et al*. 2001; Keim *et al*. 1990b; Kim and Diers 2000; Landau-Ellis *et al*. 1991; Lewers *et al*. 1999; Mansur *et al*., 1993; Nickell *et al*. 1994;

Njiti *et al*. 1998; Panthee *et al.* 2005; Polzin *et al*. 1994; Schuster *et al*. 2001; Tamulonis *et al*. 1997a,b; Zhang *et al.* 2004). For example, there are at least 53 QTL associated with oil content, 61 with protein concentration, and 66 with seed size have been mapped using various population types and different marker-based mapping techniques currently (Hyten *et al.* 2004). QTLs have been identified for resistance to a number of soybean diseases including brown stem rot (Lewers *et al*., 1999), Sclerotinia stem rot (Kim and Diers, 2000; Arahana *et al*., 2001); soybean cyst nematode (Schuster *et al*., 2001); sudden death syndrome (Njiti *et al*., 1998; Iqbal *et al*., 2001), and root knot nematodes (Tamulonis *et al*., 1997a,b). During the last decade, several research groups, using various population types and different marker-based mapping techniques, have also identified many QTLs governing soybean seed hardness, seed protein, oil, and yield (Diers *et al*., 1992; Keim *et al*., 1990). Some of the identified QTLs are listed in Table 4.

Rapidly growing soybean EST database (Shoemaker *et al*., 2002) and mapping of these ESTs onto the soybean genetic map (Matthews *et al*., 2001) promised to be a valuable public resource for efficient gene discovery, study of evolution, and comparative analysis between genera to identify candidate genes for important biological functions.

Application of identified QTLs in soybean breeding program

DNA marker technology has been developed and integrated into soybean breeding programs (Boerma and Mian, 1999; Cregan *et al*., 1999). Increasing emphasis in breeding programs to produce high quality soybean seed with specific protein or oil content for specialty markets demands more efficient manipulation of the traits were achieved. A number of QTL affect these traits and the markers could potentially be used in breeding programs (Brummer, 1997). Diers *et al*. (1992) identified two QTLs for protein and oil concentration in *G. soja*. These QTLs were subsequently transferred to the background of *G. max* and their effect on protein and oil concentration and on other agronomic traits was reexamined (Sebolt *et al*., 2000).

The effect of increased protein concentration of the QTL on chromosome 1 was confirmed in three out of four genetic backgrounds. However, the QTL was also associated with negative effects on oil concentration and yield. Recently, a QTL for increased yield originated from *G. soja* was identified and mapped in linkage group B2 (Concibido *et al*., 2002). Heterozygous BC2F1 lines containing the wild allele at the QTL had 9.3% yield increase (an average of 10 locations) compared to homozygous BC_2F_1 lines containing the cultivated parent allele. The QTL was subsequently transferred to 6 additional commercial soybean cultivars. In two backgrounds, the wild QTL allele showed 5% and 9% yield increase over haplotype containing the cultivated parental allele. Despite the limited adaptability of the QTL across genetic backgrounds, this study demonstrated the potential of using exotic germplasm for yield improvement in soybean. The use of markers in breeding for resistance to the soybean cyst nematode is also widely spread in soybean breeding programs (Young, 1999a, 199b).

Table 4 Some identified QTLs in soybean genome.

Trait	Linkage group	Reference
Chlorimuron ethyl sensitive	E	Mian *et al*., 1997
Canopy height	D1a/Q, F	Keim *et al*., 1990
Drought tolerance	G, H, J	Mian *et al*., 1996
Fe efficiency	A1, A2, D1a/Q, G, H, I, L, N, p/B2	Lin *et al*., 1997; Diers *et al*., 1992
First flower (R1)	C2, L, M, p/B2	Keim et al., 1992; Mansur et al., 1993, 1996
Hardseededness	A2, L	Keim *et al*., 1990
Leaf ash	D2, G, J	Mian *et al*., 1996
Lodging	A2, C1, C2, G, J, L,	Lee *et al*., 1996; Mansur *et al*., 1993, 1996
Leaf area (mm^2)	A2, C2, E, F, H, M,	Mian *et al*., 1998; Mansur *et al*., 1993, 1996
Leaf weight (dw/lf area)	B1, C2, H, L	Mian *et al*., 1998
Leaf length (mm)	D1a/Q, G, L, N	Keim *et al*., 1990; Mansur *et al*., 1996

Table 4 (cont'd).

Trait	Linkage group	Reference
Leaf width (mm)	A2, E, F, H, M, W/D1b	Keim *et al*., 1990; Mansur *et al*., 1996
Linoleate (% of oil)	A1, B1, E	Diers and Shoemaker, 1992
Linolenate (% of oil)	E, K, L	Diers and Shoemaker, 1992
Oil (% of seed dw)	A1, A2, B1, E, G, H, I, K, L, p/B2	Mansur *et al*., 1993, 1996; Brummer *et al*., 1997; Diers *et al*., 1992
Oleate (% of oil)	A1, E, p/B2	Diers and Shoemaker, 1992
Palmitic (% of oil)	J, p/B2	Diers and Shoemaker, 1992
Plant height (cm)	B1, C1, C2, D1a/Q, H, J, L, M, w/ D1b	Lee *et al*., 1996; Mansur *et al*., 1993, 1996; Lark *et al*., 1995
Pod dehiscence	E, J	Bailey *et al*., 1997
Pod maturity (R8)	A1, B1, C1, C2, D1a/Q, K, L, M	Lark *et al*., 1994; Lee *et al*., 1996; Keim *et al*., 1990; Mansur *et al*., 1993, 1996
Protein(%of seed dw)	A1, A2, B1, C1, D1a/Q, E, G, I, L	Mansur *et al*., 1996; Brummer *et al*., 1997; Diers *et al*., 1992
Beginning seed (R5)	C2, L	Mansur *et al*., 1993
Reproductive period	L, M	Mansur *et al*., 1996
Seed filling (R1-R8)	C2, J, M	Keim *et al*., 1990; Mansur *et al*., 1993
Seed number	C2, M	Mansur *et al*., 1996
Seed weight	A1, A2, B1, C1, C2, D2, E, G, J, L	Mansur *et al*., 1996; Manghan *et al*., 1996; Mian *et al*., 1996
Seed yield	C2, M, N	Mansur *et al*., 1993, 1996; Chang *et al*., 1996
Stearate (% of oil)	J	Diers and Shoemaker, 1992
Stem diameter	L	Keim *et al*., 1990

Source: sorted out from Shoemaker *et al*., 2001.

Summary

As reviewed above, the importance of soybean production has pushed its cultivation from the temperate zone into the subtropical and tropical areas during the last century. Weather conditions, especially high temperature and humidity that cause serious seed weathering, have become the major factors affecting the soybean production and seed quality in the tropics. Although great amount of researches have been done on the mechanism of soybean seed deterioration by weathering, our present knowledge would only indicate that no one aspect of seed metabolism is likely to provide a clear universal index of seed deterioration. Many seed testing methods have been developed to evaluate the seed quality and weathering, but for a specific breeding program, the seed test should be modified to adapt the local conditions and the germplasm used. It is well known that we can reduce the damage of field weathering by choosing resistant varieties, adjusting the planting date and enhancing the field management. Among these ways, breeding new resistant varieties is the most important and essential way to solve the field weathering problem. At present, the field weathering breeding is limited by the lack of genetic resources because just a few resistant varieties have been identified. It is important to identify and to create more gene sources for breeding programs. As the development of molecular biology, genetic marker systems have improved dramatically. Significant progress has been made in soybean genomics to target important genes, which provides a deeper insight into its genome structure and organization. DNA marker technology has also been developed and integrated into soybean breeding programs. Theoretically, it is possible to analyze the genes related to the field weathering resistance and to make more efficient manipulation of this trait. However, up to now, very little work has been done on the actual mechanism of the seed damage in the field, and there is no any molecular analysis of soybean field weathering has been previously reported. To solve the problem of field weathering, more researches are expected to seek the mechanism of the seed deterioration caused by weathering, to identify DNA markers associated with field weathering resistance genes, and to make further application of the markers for soybean breeding programs.

MATERIALS AND METHODS

Plant Materials, Equipments and Chemicals

Plant materials

Soybean *Chiangmai 60* (*CM60*), *GC10981* and their progenies were used as plant materials in this study. *CM60* (*Williams* x *SJ.4*) was released by the Department of Agriculture, Ministry of Agriculture and Co-operatives in 1987. It is widely adaptable in Thailand with high yield. *GC10981* was an early maturing line developed by the breeders of Asian Vegetable Research and Development Center (AVRDC). Kaowanant (2003) had evaluated the field weathering resistance of these two varieties and indicated that *CM60* was susceptible and *GC10981* was resistant to field weathering.

Equipments

1. Extra low temperature freezer (-80 ℃) and common refrigerator
2. Centrifuge
3. Water bath (37 ℃ and 65 ℃)
4. Autoclave
5. Pestles and mortars
6. Microwave oven
7. PH meter
8. Magnetic mixer
9. Electrophoresis apparatus
10. Incubator

11. Shaker
12. Thermocycler (PCR apparatus)
13. Ultraviolet lamp and gel photography apparatus
14. Scanner, computer,

Chemicals

1. CTAB (Cetyltrimethyl ammonium bromide)
2. EDTA (Disodium ethylenediaminetetra-acetate.2H2O)
3. Tris Base (Tris [hydroxymethyl] aminomethane)
4. Chloroform
5. Isoamyl alcohol
6. Ethanol
7. NaAcO (Sodium acetate)
8. KAcO (Potassium acetate)
9. SDS (Sodium dodecyl sulfate)
10. Isopropanol
11. Agarose
12. Polyacrylamide
13. APS(ammonium persulfate),
14. TEMED
15. BPB (Bromophenol blue)
16. EtBr (Ethidium bromide)
17. Urea
18. PCR kit

19. AFLP primers

20. pGEM®-T Easy Vector Systems (Promega)

Methods

Hybridization and F_1 hybridity identification

Soybean *CM60* and *GC10981* were grown in the greenhouse at the Department of Agronomy, Kasetsart Univewrsity, Bangkok as parents for pollination. The pollination was done by removing the anthers from *CM60* and receiving pollens from *GC10981*. The obtained F_1 seeds along with their parents were grown in a field at National Corn and Sorghum Research Center, Nakorn Rachasima Province. Three weeks after planting, leaf samples were collected from each F_1 plant for checking the hybridity using SSR primers. The plants that showed DNA bands from both *CM60* and *GC10981* were regarded as real hybrid. The pods from each hybrid plants were harvested separately at harvest maturity for developing F_2 population. The pods of *CM60* and *GC10981* were harvested at physiological maturity for confirming their difference in field weathering by a serial of treatments as follows.

Field weathering test on parents and testing method establishment

The yellow pods harvested at physiological maturity from *CM60* and *GC10981* were submitted to the following 7 treatments (3 for incubator weathering, 1 for accelerated aging and 3 for controlled deterioration) to evaluate the difference in field weathering resistance between these two varieties.

1. *Incubator weathering*: Fresh yellow pods (36 pods for each treatment) were placed upright in the cells of a grid to avoid pod contact, and then sealed in a plastic box with 1 cm water under the grid to ensure a high relative humidity (90-

100%) during the incubation. The boxes with pods inside were incubated by three treatments as follows:

- 30℃ for 10 days;
- 35℃ for 7 days;
- 30℃ for 7 days.

After the incubation, the pods were dried, threshed and germinated between wet papers at 25˚C for 5 days.

2. *Accelerated aging test and Controlled deterioration*: Fresh yellow pods were dried and threshed. The seeds obtained (50 seeds for each treatment) were subjected to the following four treatments:

- Seeds were put in a wire-mesh tray. The trays were then sealed in a plastic box with 1 cm water under the trays to ensure a high relative humidity (90-100%) during the incubating. The boxes with seeds inside were incubated at 41˚C for 3 days (standard AA test);
- Seeds were soaked in distilled water for 15 minutes, and then put in wire-mesh tray and sealed in plastic box with 1 cm water under the trays. The boxes with seeds inside were incubated at 41˚C for 7 days;
- Seeds were soaked in distilled water for 30 minutes, and then put in wire-mesh tray and sealed in plastic box with 1 cm water under the trays. The boxes with seeds inside were incubated at 41˚C for 4 days;
- Seeds were soaked in distilled water for 60 minutes, and then put in wire-mesh tray and sealed in plastic box with 1 cm water under the trays. The boxes with seeds inside were incubated at 41˚C for 3 days.

After the treatment, the seeds from each treatment and 50 non-treatment seeds (control) were germinated between wet papers at 25˚ C for 5 days. The normal seedlings, abnormal seedlings, fresh ungerminated seeds, hard seeds and dead seeds were counted (AOSA, 2000). The field weathering resistance of the variety was evaluated by germination (percentage of normal seedlings and hard seeds) and viability (percentage of normal seedlings, abnormal seedlings, fresh ungerminated and

hard seeds) of the treated seeds. According to the germination and viability of the field weathering tests, the treatments that showed wide-range difference between *CM60* and *GC10981* was selected for evaluating the field weathering resistance of their F_2 progenies.

F_2 population development and sampling

The F_2 seeds from hybrid F_1 plant were grown in a field at National Corn and Sorghum Research Center, Nakorn Rachasima Province. Water, fertilizer, pesticide and fungicide were applied when it is necessary. After 3 weeks from planting, every F_2 plant was numbered and about 1g leaves of each F_2 plant were collected for DNA extraction. At the physiological maturity, yellow pods of each F_2 plant were harvested separately for field weathering test. The plant height was measured at the same time.

Field weathering test on F_2 population

Based on the tests on *CM60* and *GC10981*, the field weathering resistance of the F_2 progeny was evaluated by incubator weathering and controlled deterioration method as follows.

1. *Incubator weathering test*: Eighteen fresh yellow pods harvested from each F_2 plant were placed upright in the cells of a grid to avoid pod contact, and then sealed in a plastic box with 1 cm water under the grid to ensure a high relative humidity (90-100%) during the incubation. The boxes with pods inside were incubated at 30℃ for 7 days. After incubation, the pods were dried and threshed, and then the seeds were germinated between wet papers at 25°C for 5 days.

2. *Controlled deterioration test*: The yellow pods were harvested, dried and threshed. Twenty-five seeds from each F_2 plant were soaked in distilled water for 60 minutes, and then the seeds were put in a wire-mesh tray. The trays with seeds inside were sealed in a plastic box with 1 cm water under the trays to ensure a high relative humidity (90-100%) during the incubation. The boxes were then incubated at 41°C for 3 days. After incubation, the seeds were germinated between wet papers at 25°C for 5 days.

After germination, the normal seedlings, abnormal seedlings, hard seeds, fresh ungerminated seeds and dead seeds were counted. The field weathering resistance was evaluated by the germination and the viability of the treated seeds. According to the germination and viability of the seeds from incubator weathering and controlled deterioration test, equal amount of leaf tissue from extreme resistant F_2 plants (0.2g/plant) were pooled as bulked resistant sample, while the same equal amount of leaf tissue from extreme susceptible F_2 plants were pooled as bulked susceptible sample for DNA extraction.

DNA extraction

Total genomic DNA of *CM60*, *GC10981*, bulked samples and each F2 plants were extracted using a SDS extraction protocol modified from that described by Keim *et al*. (1988) and Doyle *et al*. (1990).

1. 0.5g leaf tissue was freezed rapidly by liquid nitrogen in a mortar, grinded with a pestle till the tissue became powder. A little more liquid nitrogen was added, if necessary, to keep the powder from thawing while grinding.

2. The frozen powder transferred into a micro centrifuge tube containing 1 ml DNA extraction buffer (100mM Tris, 50mM EDTA, 500mM Nacl, 1.25% SDS and 10mM β-mercaptoetanol).

3. The mixture was incubated at 65 ℃ for 30 minutes with inverting tubes a few times every 10 minutes.

4. 300µl 5M KAcO was added and the mixture was incubated on ice for 1 hour.

5. The mixture was centrifuged at 12,000 rpm for 15 minutes. The supernatant was transferred into a new micro centrifuge tube.

6. 300µl chloroform / isoamyl alcohol (CIA, V/V=24:1) was added to the supernatant and mixed. The mixture was centrifuged at 12,000 rpm for 15 minutes, and then the supernatant was transferred into a new 15 ml tube.

7. Same volume of cold isopropanol was added to the supernatant and mixed. The mixture was placed at room temperature for 1 hour or at 4 ℃ overnight.

8. The DNA was hooked into a new micro centrifuge tube and washed 3 times by 75% alcohol to remove salts.

9. The DNA was dried at room temperature for 30 minute and then dissolved in 200µl TE buffer (10mM Tris-Cl, pH8.0 and 1mM EDTA, pH 8.0).

10. 3µl DNase free RNase (10mg/ml) was added to the DNA, mixed and incubated at 37 ℃ for 30 minutes.

11. 200µl phenol was added, mixed and centrifuged at 12,000 rpm for 15 minutes, and then the supernatant was transferred into a new micro centrifuge tube.

12. 200µl chloroform / phenol (24:1, V/V) was added to the supernatant, mixed and centrifuged at 12,000 rpm for 15 minutes, and then the supernatant was transferred into a new micro centrifuge tube.

13. 200µl chloroform was added to the supernatant, mixed and centrifuged at 12,000 rpm for 15 minutes, and then the supernatant was transferred into a new micro centrifuge tube.

14. 20µl 3M NaAcO and 500µl ethanol was added to the supernatant, mixed and placed at -20 ℃ for 30 minutes.

15. The DNA was hooked into a new micro centrifuge tube and washed with 65% alcohol for 1 time and 85% alcohol for 2 times.

16. The DNA was dried at room temperature and 100µl TE buffer was added to dissolve the DNA.

17. The presence of DNA was monitored by subjecting 1µl of each DNA sample to 1% agarose gel and electrophoresed in 1xTBE buffer. The gel was stained in ethidium bromide (0.1g/ml) and the visual bands under UV lamp were compared with standard DNA marker by photography. The DNA concentration was adjusted to 100ng/µl, and then stored at -20 ℃ for further using.

SSR procedure

SSR analysis was conducted to check the hybridity of the F_1 plants. The procedure was the same as described by Yu *et al*. (1994) and Cregan *et al*. (1999). PCR reaction was performed in a volume of 12.5µl containing 10xPCR buffer1.25µl, 2.5mM dNTPs 0.5µl, 25mM $MgCl_2$ 1.0µl, 1µM forward primer 1.25µl, 1µM reverse primer 1.25µl, 5U/µl Taq Polymerase 0.1µl, 50ng/µl template DNA 1.0µl and ddH_2O 6.15µl. The mixture were put in a thermocycler and the following temperature profile was run: 3 minutes for denaturation at 94 ℃; 35 cycles of 30 seconds for denaturation at 94 ℃, 30 seconds for annealing at 55 ℃ (vary with primer), 1 minutes for extension at 72 ℃; and 10 minutes for final extension at 72 ℃, then hold at 4 ˚C.

PCR products were separated by denatured 6% polyacrylamide gel and visualized by the method described by Saghai Maroof *et al*., 1994. A 6% (w/v) polyacrylamide gel (19:1 crosslinking ratio) with 7M urea was pre-run in 1xTBE buffer (89mM Tris, 89mM boric acid, 2,0mM EDTA, pH8.3) at 1500V for 30 minutes. Then a mixture including 10µl denatured PCR production and 2µl loading buffer was loaded into the well of the polyacrylamide gel. The gel was electrophoresed at 1500V for 3 hours. After the electrophoresis, the gel was soaked in the following solutions and shaken gently: distilled water for 5 minutes, 0.1% CTAB for 30 minutes, 0.3% ammonia for 15 minutes, 0.2% silver staining solution for 20 minutes, distilled water for 30 seconds, developing solution for 10~20 minutes (till the bands are clear), stop

solution for 5minutes, distilled water for 30 minutes, and then dried at room temperature.

RAPD procedure

RAPD technique was used to identify the polymorphism among *CM60*, *GC10981*, resistant DNA pool and susceptible DNA pool. The PCR was performed in a volume of 12.5µl containing 10 X PCR buffer 1.25µl, 2.5mM dNTPs 0.625µl, 25mM $MgCl_2$ 0.625µl, 1µM RAPD primer 1µl, 5U/µl Taq Polymerase 0.1µl, 50 ng/µl template DNA 1.0µl and ddH_2O 7.9µl. The mixture were put in a thermocycler and the following temperature profile was run: 3 minutes for denaturation at 94 ℃; 40 cycles of 30 seconds for denaturation at 94 ℃, 30 seconds for annealing at 36 ℃, 1 minutes for extension at 72 ℃; and 5 minutes for final extension at 72 ℃, then hold at 4 ˚C. The PCR products were separated by 1.5% agarose gel electrophoresis and visualized under UV lamp by ethidium bromide stain.

AFLP procedure

AFLP analysis was conducted to identify the polymorphism among *CM60*, *GC10981*, resistant DNA pool and susceptible DNA pool. The used procedure was the same as described by Vos *et al*. (1995) and Maughan *et al*. (1996). For restriction enzyme digesting and adapter linking, the following solution were added into a micro centrifuge tube: 5µl OnePhorAll (10xOPA), 2µl *Mse*I (4U/µl), 4µl *EcoR*I (10U/µl), 10µl *Mse*I buffer (10ug/µl), 1µl *EcoR*I adapter, 1µl *Mse*I adapter, 1µl T4 DNA ligase (3U/µl), 2µl T4 DNA ligase buffer (10x), 10µl bulked genomic DNA (50ng/µl) and 19µl ddH_2O. The mixture was incubated at 37 ˚C for 3 hours and then stored at 4°C or –20°C for pre-amplification.

Pre-selective PCR was set up with 1µl E-A oligo (50ng/µl), 1µl M-C oligo (50ng/µl), 2.5µl dNTPs (2mM), 2.5µl 10xPCR buffer, 0.1µl Taq polymerase (5U/µl), 0.75µl $MgCl_2$ (50mM), 2µl template DNA from restriction-ligation and 15.15µl ddH_2O. For amplification the following cycle profile was used: 2minutes for denaturation at 94°C; 25 cycles of 30 seconds for denaturation at 94°C, 1minutes for annealing at 60°C and 1min for extension at 72°C; then final extension at 72°C for 5 minutes and hold at 4°C. To verify successful amplification, 10 µl of the PCR product was separated on a 1% agarose gel. The remaining 15 µl PCR product was diluted 20-fold by adding 285µl of ddH_2O and stored at 4°C or –20°C for selective amplifying.

Selective PCR was carried out using 1µl E-ANN oligo (50ng/µl), 1µl M-CNN oligo (50ng/µl), 2µl dNTPs (2mM), 2µl 10xPCR buffer, 0.1µl Taq polymerase (5U/µl), 0.6µl $MgCl_2$ (50mM), 5µl diluted template DNA from pre-selective PCR and 8.3µl ddH_2O. High selectivity was obtained with the following cycle profile: 2 minutes for denaturation at 94 ℃; one cycle of 30 seconds for denaturation at 94°C, 30 seconds for annealing at 65°C, 1 minutes for extension at 72°C, followed by 12 cycles of a 0.7°C decreasing annealing temperature per cycle; and then 35 cycles of 30 seconds for denaturation at 94°C, 30 seconds for annealing at 56°C, 1min for extension at 72°C; and 10 minutes for final extension at 72 ℃, then hold at 4 ˚C. The PCR products were stored at 4 ˚C for polyacrylamide gel electrophoresis.

PCR products were separated by denatured 6% polyacrylamide gel as described above for SSR procedure. After the electrophoresis, the gel was soaked in the following solutions and shaken gently: 10% acetic acid for 20 minutes, distilled water for 30 minutes, 0.2% silver staining solution for 30 minutes, distilled water for 30 seconds, developing solution for 10~20 minutes (till the bands were clear), stop solution for 5 minutes, distilled water for 30 minutes, and then the gel was dried at room temperature.

DNA cloning, sequencing and marker designing

Based on the bulk segregant analysis, DNA band of interest which appeared to be linked to the field weathering resistance was excised from the gel and eluted in 20µl of ddH_2O or TE buffer by incubating in a water bath at 55 ℃ for at least 3 hours (Upender *et al*., 1995; Hayes and Saghai Maroof, 2000). The eluate was re-amplified by PCR using the primers that generated the polymorphic product under the same conditions as the selective amplification. After confirming the correct size of the re-amplified DNA, the PCR product was then cloned into the pGEM®-T Easy Vector Systems (Promega) and transformed into *E.coli* XL-1 competent cell following the manufacturer's protocols. The white clones were selected by culture on LA medium with ampicillin, X-Gal and IPTG. The selected clones were proliferated by LB medium culture and the plasmid DNA was isolated by standard alkaline-lyses method (Birnboim and Doly, 1979). Subsequent PCR was conducted on plasmid DNA using the same AFLP primers to confirm that the proper band was cloned. The recombinant plasmid DNA was then sequenced using ABI3100 DNA sequencer (Bioservice Unit, Thailand). Sequence characterized amplified region (SCAR) primers were designed from the sequence of the clone to check the relationship between the marker and the field weathering resistance by screening all the F_2 progenies. The SCAR primers were synthesized by KU-VECTOR Custom Synthesis Service Unit (Kasetsart University).

Marker assisted selection, backcross and efficiency evaluation

The SCAR primers were used for selecting field weathering resistant plant from F_2 progenies. The PCR was performed in a volume of 12.5µl containing 10xPCR buffer1.25µl, 2.5mM dNTPs 0.75µl, 25mM $MgCl_2$ 0.75µl, 1µM forward primer 0.8µl, 1µM reverse primer 0.8µl, 5U/µl Taq polymerase 0.1µl, 50ng/µl template DNA 0.5µl and ddH2O 7.55µl. The mixture was put in a thermocycler and the following temperature profile was run: 3 minutes for denaturation at 94 ℃; 40 cycles of 30 seconds for denaturation at 94 ℃, 30 seconds for annealing at 50 ℃

(depend on the primer), 30 seconds for extension at 72 ℃; and 5 minutes for final extension at 72 ℃, then hold at 4 ° C. The PCR products were separated by 6% polyacrylamide gel and visualized by silver nitrate stain as described above for AFLP procedure.

Based on the band pattern of the DNA amplified by SCAR primers, the F_2 plants that showed same DNA pattern as *GC10981* were considered to be resistant to field weathering. By comparing the field weathering resistance (germination and viability) of and the DNA pattern of these plants, the seeds from those F_2 plants with high germination and viability were grown in a greenhouse as male parent to cross with *CM60*.

The obtained BC_1F_1 seeds along with *CM60*, *GC10981* and relative F_3 lines (*CM60*/*GC10981*) were grown in a field to compare the field weathering resistance of BC_1F_1 plants and their parents. The physiological mature pods were harvested from each plant for field weathering test. The pods from each BC_1F_1 plant were treated separately; the pods from each F_3 line were mixed as one sample. The pods were dried and threshed, and then 50 seeds of each line (or BC_1F_1 plant) were treated by controlled deterioration (dry seeds were soaked in water for 60 minutes and then incubated at 41℃ for 3 days) as described above. The efficiency of the marker assisted selection was evaluated by comparing the backcross progenies to *CM60*, *GC10981* and corresponding F_3 lines.

Data analysis and QTL identification

The frequency distribution, Chi-square test, t-test, one way ANOVA and correlation analysis of data for germination and markers were carried out by using Microsoft Excel and SPSS 11.5 for Windows (SPSS Inc.). Genetic map distances were calculated using Mapmaker/EXP 3.0 computer program (Lander *et al*., 1987; Lincoln *et al*., 1993). Information from Mapamker was used in windows QTL

Cartographer V2.5 (Wang *et al*., 2005) to verify the candidate QTL by composite interval mapping (CIM). A standard model (model 6) in the CIM procedure with a 10 cM window size and a 2 cM walking speed were used to identify the related QTL. Threshold value of LOD for the trait was set at a minimum of 3.0. The location with a LOD score greater than this level was considered to identify QTL significantly associated with the trait.

RESULTS AND DISCUSSION

Agronomic Characters of Parents and F1 Plants

Soybean *CM60*, *GC10981* and their hybrid seeds were grown in a field at National Corn and Sorghum Research Center on March 2, 2004. The growing habits of the parents and F_1 plants were investigated. *CM60* was 29 cm higher than *GC10981*. GC10981 was 12 days later to flowering and 15 days later to harvest than *GC10981*. Some of their agronomic characteristics are shown in Table 5. The agronomic characteristics of F_1 plants were intermediate between their parents except the color of seed coat. The seed color of F_1 seeds were same as their maternal parents (green). Basically, the hybridity of the F_1 plants can be determined by these agronomic characteristics. However, it is also possible to check the hybridity at early growing stage by some codominant DNA markers, such as SSR markers. In this study, the hybridity of F_1 plants were confirmed by SSR primer soypro-1 (Figure 1). All the F_1 plants showed the DNA bands from both parents. It was clear that all plants were hybrid.

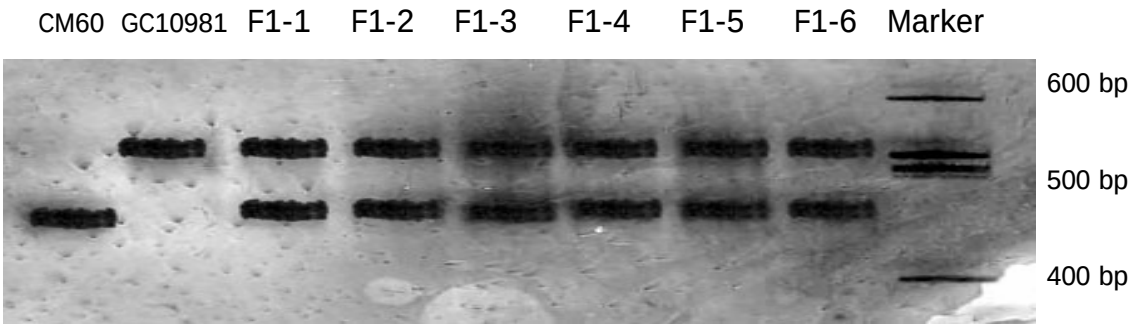

Figure 1 Hybridity of F_1 plants identified by SSR primer. The PCR amplification was carried out by SSR primer soypro-1. PCR products were separated by 6% denatured polyacrylamide gel and visualized by silver stain.

Table 5 Some agronomic characters of the parents and the F1 plants

Name	Plant height (cm)	Days to flowering	Days to harvest	Flower color	Pods per plant	100 seeds weight (g)	Seed color
CM60	57	41	105	White	97	21.2	Yellow
GC10981	28	29	90	White	31	12.7	Green
F_1-1	43	35	96	White	85	16.5	Green
F_1-2	46	35	96	White	91	15.5	Green
F_1-3	41	34	98	White	76	17.3	Green
F_1-4	40	33	95	White	43	15.1	Green
F_1-4	42	33	97	White	61	17.1	Green
F_1-6	45	34	98	White	78	18.4	Green

The Efficiency of Different Weathering Treatment

To find out optimum test methods for evaluating the field weathering resistance of soybean varieties, the seeds of *CM60* and *GC10981* were subjected to seven treatments. The germination and viability of the seeds of *CM60* and *GC10981* after being subjected to seven treatments are shown in Table 6. For incubator weathering, after the pods were incubated, the germination of *CM60* and *GC10981* seeds was 0~9.2% and 0~42.9%, and the viability of *CM60* and *GC10981* seeds was 19.5~41.5% and 27.3~72.5%. The most serious seed deterioration was caused by incubating the pods at 30 ℃ for 10 days due to a serious pathogen infection and germination during incubation. The seeds of both varieties/lines had lost their germinability (0% germination). By incubating the seeds at 35 ℃ for 7 days, the pathogen infection and germination during incubation were controlled, but the difference between *CM60* and *GC10981* was still small (8.6 for germination and 11.2 for viability). Decrease in both treating temperature and time could increase the germination and viability of the treated seeds. After incubating the pods at 30 ℃ for 7 days, the difference between *CM60* and *GC10981* in germination (33.7%) and viability (31.0%) were greater than the other two treatments. Therefore, this treatment was considered to be more efficient to distinguish the seed weathering of *CM60* and *GC10981* than the other two treatments.

For the standard accelerated aging (AA) test, the germination of *CM60* and *GC10981* were decreased (from 92% to 60% and from 94% to 84%, respectively), but the viability was only decreased slightly (from 100% to 92%) for *CM60* comparing with the control. For controlled deterioration treatments, soaking the seeds in distilled water prior to incubation further decreased the germination and viability of the treated seeds. The lowest germination and viability were caused by soaking the seeds in distilled water for 15 minutes and incubating at 41 ℃ for 7 days. Soaking the seeds in distilled water for 60 minutes and then incubating at 41 ℃ for 3 days showed the widest difference in seed germination (38.0%) and viability (17.0%) between *CM60*

and *GC10981*. Thus, this treatment was considered to be an optimum one to distinguish the seed weathering of *CM60* and *GC10981*.

Since the incubator weathering at 30 ℃ for 10 days could not identify the difference between *CM60* and *GC10981* (both 0% germination). If ignore this treatment and compare the means of the other 6 treatments (paired t-test), there was significant difference between *CM60* and *GC10981* (t_5=5.825, p=0.002 for germination and t_5=4.083, p=0.01 for viability). It was clear that the field weathering resistance of *CM60* and *GC10981* is significantly different. They could be used for mapping the field weathering resistance genes.

Table 6 The germination and viability of *CM60* and *GC10981* by different treatment.

Treatment*	Germination (%)**			Viability (%)		
	CM	GC	Dif.	CM	GC	Dif.
IW: 30 ℃, 10 days	0.0	0.0	**0.0**	19.5	27.3	**7.8**
IW: 35 ℃, 7 days	6.6	15.2	**8.6**	35.6	46.8	**11.2**
IW: 30 ℃, 7 days	9.2	42.9	**33.7**	41.5	72.5	**31.0**
CD: Water 15 min. + 41 ℃, 7 days	8.0	32.0	**24.0**	52.0	60.0	**8.0**
CD: Water 30 min. + 41 ℃, 4 days	28.0	48.0	**20.0**	64.0	76.0	**12.0**
CD: Water 60 min. + 41 ℃, 3 days	32.0	70.0	**38.0**	68.0	85.0	**17.0**
AA: 41 ℃, 3 days	60.0	84.0	**24.0**	92.0	100.0	**8.0**
Control (no treatment)	92.0	94.0	**2.0**	100.0	100.0	**0.0**

* IW= incubator weathering, CD= controlled deterioration, AA= accelerated aging;

** CM= *CM60*, GC= *GC10981*, Dif.=difference= GC-CM.

Since the field weathering occurs under the hot and humid conditions in the field after the seeds are physiologically mature, the most common procedure for evaluating seed resistance to field weathering is to leave the plants in the field beyond the normal harvest period and then assess the quality of the seed by visual score, examining seed-borne fungi, seed vigor, or use a combination of these assessment

methods. However, this delayed harvest technique has several limitations, for example, genotypes matured at different times are subjected to different environmental weathering stresses and different periods of weathering. It is difficult to apply the same environmental stress conditions to cultivars of different maturity by delayed harvest. In an attempt to overcome the limitations of delayed harvest, Kueneman (1982) developed spreader row and overhead irrigation techniques to accelerate weathering based on the delayed harvest method and found the cultivar differences were highly significant. Artificial seed weathering methods, such as incubator weathering, can minimize the effects of variable pod maturity. In this study, three incubator weathering treatments were carried out to identify the difference in seed quality between susceptible variety *CM60* and resistant variety *GC10981*. After the fresh yellow pods were incubated at 30 ℃ and 90-100% relative humidity for 10 days, a serious pathogen infection occurred, and some seeds even germinated during the incubation. The remaining seeds had lost their germinating ability (0% germination) and showed a very low viability (19.5% and 27.3% abnormal seedlings). Increasing the temperature and shortening the incubating time (35 ℃, 7 days) reduced the pathogen infection and seed germination during incubation, but the treatment still caused serious damage to the seeds. This treatment had been successfully used to identify the field weathering difference between *CM60* and *GC10981* by Kaowanant (2003). However, the constant temperature at 35 ℃ practically does not occur in the soybean field. By shortening the incubating time (30 ℃, 7 days), the pathogen growth and seed germination during incubation were controlled. The germination and viability of the treated seeds showed an obvious difference between *CM60* and *GC10981* which could be used to identify the field weathering resistance of these varieties.

On the other hand, since the ability of seed coat to absorb moisture from the environment is a decisive factor of field weathering, the faster the seed absorbs moisture from the environment, the more serious the weathering may be. Based on this hypothesis, the controlled deterioration test method developed by Matthews (1980) was modified to compare the seed weathering. The modified treatments emphasized the relationship between seed moisture absorbing speed and seed

weathering. The original controlled deterioration method was modified into three combinations of water soaking time and incubating time to compare with the standard accelerated aging test. Soaking the seeds in distilled water for 60 minutes and incubating at 41 ℃ under 90-100% relative humidity for 3 days showed a wide-ranging difference in germination and viability between *CM60* and *GC10981*. Since the different field weathering resistances of these two soybean varieties had been stated by Kaowanant (2003), the treatments that showed more difference between these varieties should be more efficient for distinguishing the field weathering resistance of soybean varieties. Thus, this treatment was considered to efficient for field weathering resistance test.

The modified incubator weathering (pods at physiological maturity were incubated at 30℃ for 7 days) and the controlled deterioration (dry seeds were soaked in water for 1 hour and then incubated at 41℃ for 3 days) showed a wide-range difference in seed germination and viability between *CM60* and *GC10981*. By comparing the field weathering resistance of *CM60* and *GC10981* from controlled deterioration and that from incubator weathering with the results from Kaowanant (2003), the difference on field weathering resistance of these two soybean varieties was further confirmed. In practical application, both methods may be used to determine the field weathering resistance of soybeans. However, it is difficult to treat a great number of pods at the same time due to the laboratorial limitation, especially in the large-scale breeding programs. The modified controlled deterioration method makes it possible to harvest the pods at physiological maturity, dry to a similar moisture content level, and then store for testing. This will be very benefit to large-scale screening in breeding programs. This method emphasized the relationship between seed moisture absorbing speed and seed weathering. It is a new applicable way to evaluate the field weathering resistance of soybean varieties.

Field Weathering Resistance of F_2 Progenies

The field weathering resistance of 139 F_2 plants was evaluated by modified incubator weathering and controlled deterioration methods established upon their parents. The germination and viability of the treated seeds were used as indicator for field weathering resistance (Appendix Table A). For incubator weathering test, the germination of the F_2 plants ranged from 21.3 to 81.6%, whereas those of *CM60* and *GC10981* were 34.7% and 75%, respectively; the viability of the F_2 plants ranged from 47.8 to 95.6%, whereas those of *CM60* and *GC10981* were 59.7% and 93.4%, respectively. For controlled deterioration test, the germination of the F_2 plants ranged from 20 to 82%, whereas those of *CM60* and *GC10981* were 32% and 72%, respectively; the viability of the F_2 plants ranged from 44 to 90%, whereas those of *CM60* and *GC10981* were 54% and 94%, respectively. The distribution of the germination and viability of the F_2 progenies are shown in Table 7, Figure 2 and Figure 3. All the indicators showed normal distribution. The field weathering resistance of soybean appeared to be a quantitative trait controlled by polygene.

Table 7 Statistical description of the germination and viability of 139 F_2 progenies.

Indicator	Minimum	Maximum	Mean	Variance	Skewness	Kurtosis
IW	21.3	81.6	50.2	168.7	0.079	-0.523
IW viability	47.8	95.6	70.5	78.2	0.199	0.656
CD	20.0	82.0	52.4	204.4	-0.319	-0.742
CD viability	44.0	90.0	70.3	78.2	-0.395	0.721
Mean*	36.5	83.6	60.9	78.7	-0.006	0.526

* Mean is the average of the germination and viability of both treatments.

By comparing the means of the germination and viability of the two treatments (paired t-test), there is no significant difference between two treatment ($t_{138}=1.685$, $p=0.094$ for germination and $t_{138}=0.174$, $p=0.862$ for viability). However, a highly significant correlation was also observed between the incubator weathering and the controlled deterioration (germination $r=0.331^{**}$, viability $r=0.425^{**}$, $n=139$, $P<0.001$). The efficiency of using the modified controlled deterioration method for

evaluating the field weathering resistance of soybeans was further confirmed. Both testing methods worked well for evaluating the field weathering resistance of F_2 progenies.

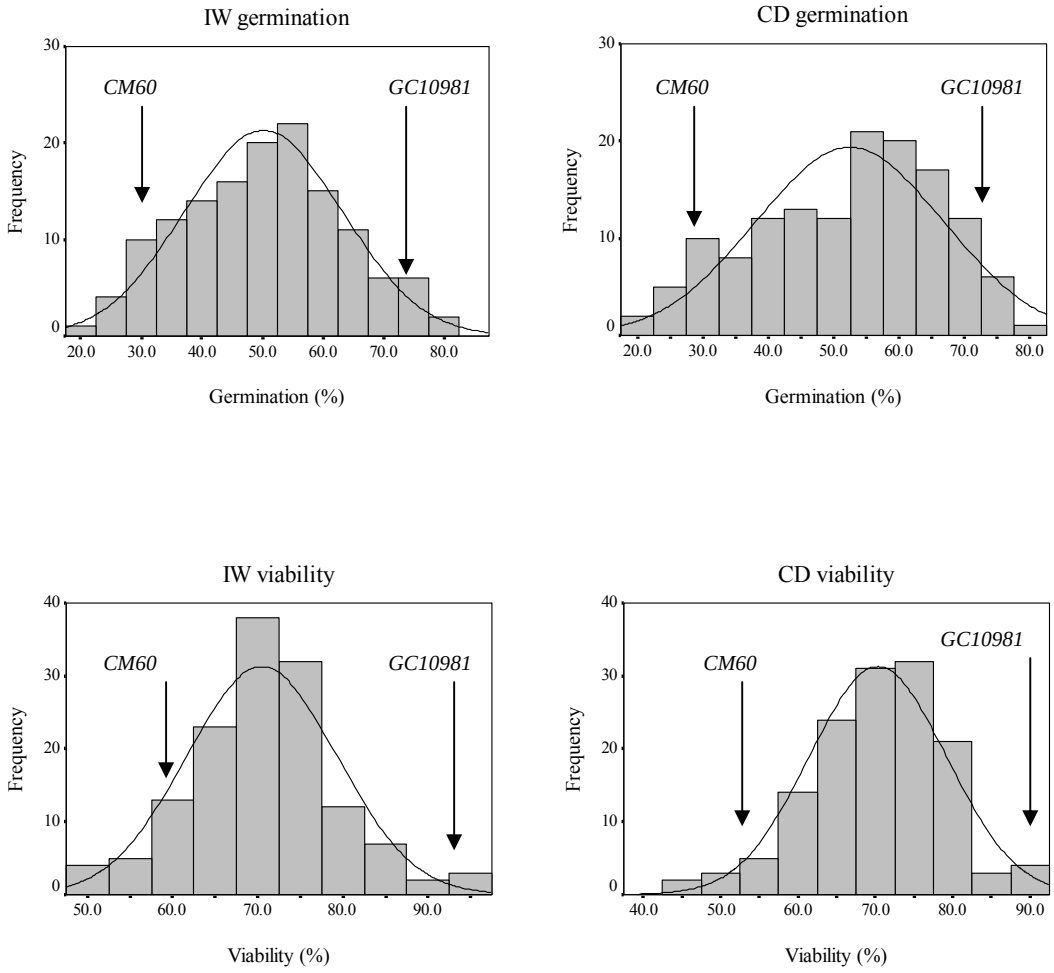

Figure 2 The distribution of the germination and viability of the seeds of F_2 progenies after being subjected to incubator weathering (IW) and controlled deterioration (CD).

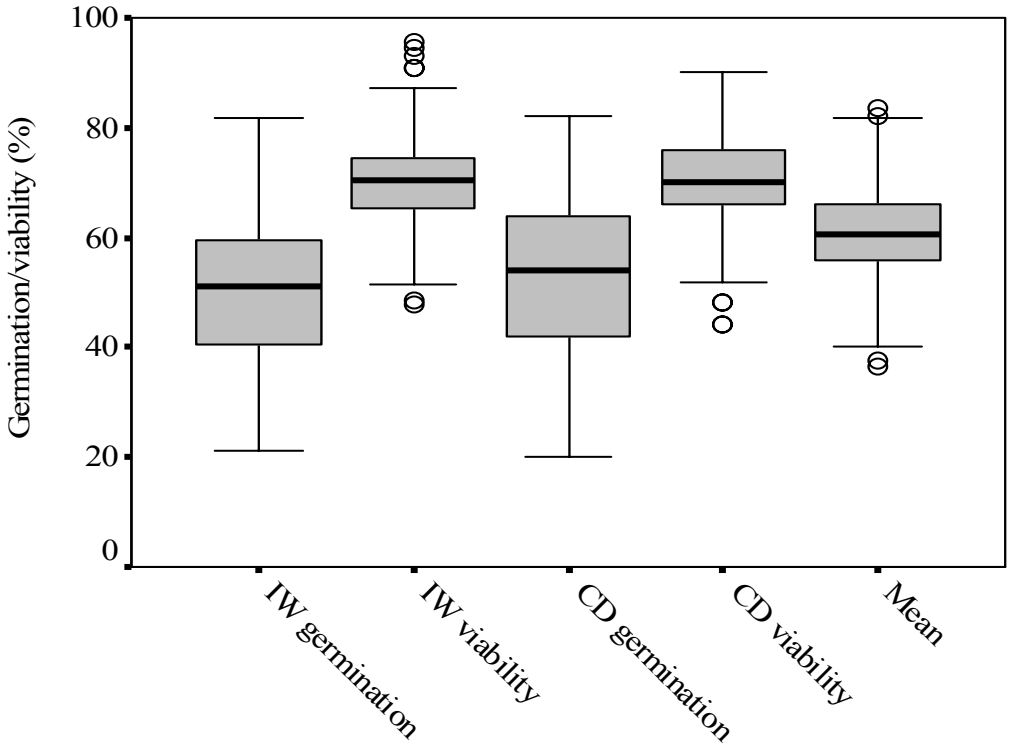

Figure 3 Boxplots of the germination and viability of 139 F2 progenies by incubator weathering and controlled deterioration treatment. IW=incubator weathering, CD=controlled deterioration. Mean is the average of the germination and viability of both treatments.

According to the germination and viability of the F_2 progenies following the field weathering tests, six F_2 plants with extremely high germination and viability were bulked as resistant pool, while seven susceptible F_2 plants with extremely low germination and viability were bulked as susceptible pool for bulk segregant analysis. The germination and the viability of the pooled F_2 plants are shown in Table 8. There were significant differences on germination and viability between the two bulked pools ($p<0.001$).

Table 8 The germination and viability of the pooled F_2 plants.

Bulking no.	F_2 plant no.	IW* test (%)		CD test (%)	
		Germination	Viability	Germination	Viability
R1**	A10	67.7	80.6	82.0	90.0
R2	A11	72.7	90.9	74.0	86.0
R3	A35	75.0	93.2	76.0	90.0
R4	A63	77.1	85.4	72.0	90.0
R5	C2	70.4	90.7	76.0	90.0
R6	G20	81.6	94.7	70.0	82.0
S1	A8	25.7	54.3	22.0	44.0
S2	A14	28.2	64.1	32.0	44.0
S3	A16	26.7	60.0	26.0	48.0
S4	A38	32.3	58.1	32.0	68.0
S5	C26	27.3	48.5	20.0	54.0
S6	C37	21.3	61.7	28.0	66.0
S7	G4	29.8	74.5	28.0	64.0
Independent samples t-test	t_{11}	20.030	7.475	19.671	7.248
	P value	<0.001	<0.001	<0.001	<0.001

* IW= incubator weathering, CD= controlled deterioration;

** R = resistant; S = susceptible.

Polymorphism Detection by RAPD Primers

To seek for the DNA markers linked to the field weathering resistance of soybean, total 198 RAPD primers were used to detect the polymorphism between parental varieties, thirty-nine of them amplified polymorphic bands between *CM60* and *GC10981* (Table 9). The polymorphic markers were further used to detect the polymorphism between bulked resistant and susceptible DNA pool of F_2 progenies,

no difference could be found between the bulked DNA pools. This might be due to the low polymorphism between the bulked DNA pools.

Table 9 Polymorphic RAPD bands between *CM60* and *GC10981*.

Primer no.	Primer	Primer sequence	Polymorphic band size (bp)
1	OPA05	5'-AGGGGTCTTG-3'	950
2	OPA16	5'-AGCCAGCGAA-3'	650, 1120
3	OPB06	5'-TGCTCTGCCC-3'	450
4	OPC07	5'-GTCCCGACGA-3'	650
5	OPD01	5'-ACCGCGAAGG-3'	800, 1180
6	OPD04	5'-TCTGGTGAGG-3'	850
7	OPD11	5'-AGCGCCATTG-3'	250, 1100
8	OPD12	5'-CACCGTATCC-3'	350
9	OPD15	5'-CATCCGTGCT-3'	400, 900
10	OPD16	5'-AGGGCGTAAG-3'	350, 650
11	OPD19	5'-CTGGGGACTT-3'	980
12	OPE10	5'-CACCAGGTGA-3'	480, 1120
13	OPE11	5'-GAGTCTCAGG-3'	300, 500
14	OPE16	5'-GGTGACTGTG-3'	400, 600, 850
15	OPE20	5'-AACGGTGACC-3'	350, 700
16	OPF03	5'-CCTGATCACC-3'	1000
17	OPF05	5'-CCGAATTCCC-3'	480
18	OPF07	5'-CCGATATCCC-3'	450, 800
19	OPF09	5'-CCAAGCTTCC-3'	420, 1100
20	OPF10	5'-GGAAGCTTGG-3'	900
21	OPF16	5'-GGAGTACTGG-3'	250
22	OPH02	5'-TCGGACGTGA-3'	850
23	OPI04	5'-CCGCCTAGTC-3'	900
24	OPI14	5'-TGACGGCGGT-3'	600
25	OPN3	5'-GGTACTCCCC-3'	890
26	OPX05	5'-CCTTTCCCTC-3'	750, 1250
27	OPAA16	5'-GGAACCCACA-3'	1300
28	OPAA19	5'-TGAGGCGTGT-3'	750, 1200
29	OPAB01	5'-CCGTCGGTAG-3'	800
30	OPAC02	5'-GTCGTCGTCT-3'	600, 700, 1100, 1150
31	OPAC03	5'-CACTGGCCCA-3'	500, 650
32	OPAC05	5'-GTTAGTGCGG-3'	300, 1100
33	OPAC15	5'-TGCCGTGAGA-3'	650
34	OPAC19	5'-AGTCCGCCTG-3'	900
35	OPAD05	5'-ACCGCATGGG-3'	1100
36	OPAF07	5'-GGAAAGCGTC-3'	750, 1200
37	OPAI08	5'-AAGCCCCCCA-3'	850
38	OPAI12	5'-GACGCGAACC-3'	1100
39	OPAI13	5'-ACGCTGCGAC-3'	1250

Polymorphism Detection by AFLP

The AFLP technology was used to detect DNA markers linked to the field weathering resistance. Parental and bulked DNA samples were digested by restriction enzyme E*coR* I and M*se* I. Sixty-four E(ANN)-M(CNN) marker combinations were used to amplify DNA fragments from the digested DNA samples. Totally 2,162 DNA fragments were amplified, 120 of them (5.6%) were polymorphic fragments between *CM60* and *GC10981* (Table. 10). Based on the bulk segregant analysis (BSA), five DNA fragments appeared to be related to the field weathering resistance. One of the linked polymorphism is shown in Figure 4.

Table 10 The number of AFLP amplified DNA bands and polymorphic bands (in brackets).

Marker	M-caa	M-cac	M-cag	M-cat	M-cta	M-ctc	M-ctg	M-ctt
E-aac	31(0)	20(2)	27(1)	41(2)	29(2)	26(0)	22(2)	27(0)
E-aag	17(0)	47(3)	37(2)	37(2)	38(0)	30(0)	24(2)	47(4)
E-aca	38(0)	52(0)	31(2)	36(1)	44(0)	24(0)	36(1)	42(2)
E-acc	37(1)	39(0)	29(1)	9(0)	27(3)	25(2)	29(3)	28(4)
E-acg	21(5)	30(0)	18(0)	36(9)	18(1)	25(0)	18(0)	47(2)
E-act	52(5)	51(5)	40(4)	45(4)	48(1)	37(2)	24(2)	34(1)
E-agc	47(2)	42(2)	38(4)	35(0)	33(2)	32(1)	15(0)	43(2)
E-agg	37(1)	44(5)	38(3)	52(7)	44(6)	45(0)	26(0)	21(2)

Although BSA is effective for high resolution mapping to find genes controlling simple inherited traits, such as disease resistance genes (Michelmore *et al*., 1991; Giovanonni *et al*., 1992). Any trait that can be scored in a segregating population can be rapidly targeted with DNA markers by BSA (Zhang *et al*., 1994). The BSA used in conjunction with AFLP markers, make it possible to identify large number of DNA markers in a region of interest in a short time. However, the success of bulking by phenotypes is dependent on the correspondence of genotype and

phenotype. There is a contradiction between the number of the bulked progenies and the polymorphism of the bulked pools. When too many progenies are bulked, the polymorphism between the pools is low, only some of the major genes or QTLs can be identified. However, if too few progenies are bulked, a higher polymorphism between the pools can be observed, but some of the polymorphisms may not be related to the target genes. In this study, the AFLP analysis showed a low polymorphism (5.6%) between the parents and a very low linked polymorphism (0.2%) between the pools. The low linked polymorphism may also be due to the low polymorphism between the parents. The low polymorphism between the bulked DNA pools also explained the reason that the difference could not be identified by RAPD.

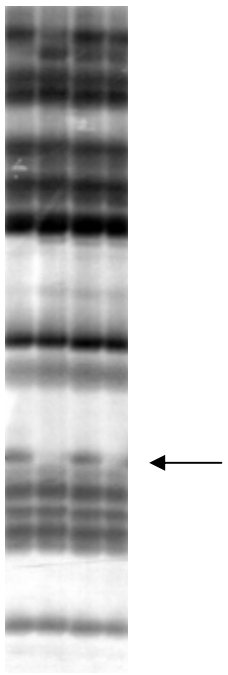

Figure 4 An example of detecting linked polymorphism by bulk segregant analysis using AFLP primer combination Eagg/Mcac. Lanes from the left to right are *GC10981*, *CM60*, resistant bulk and susceptible bulk. The arrow indicates the linked polymorphism that *GC10981* and resistant bulk showed DNA band, but *CM60* and susceptible bulk showed no band.

DNA Cloning and Sequencing

The 5 DNA fragments showed linked polymorphism with the field weathering resistance were cloned and sequenced. The sequence of the 5 cloned DNA fragments are shown in Table 11. The sequence data were compared with the soybean sequence data from DNA Database of Japan (DDBJ) by BLAST (Altschul *et al*., 1997) and FASTA (Pearson and Lipman, 1988) analysis. By BLAST searching, only accession BU765372 and BU549864 were found to have 102 bp same as marker Eact/Mctt-157 within 118 bp overlap (86.4%). By FASTA searching, accession AF180335 and AF186186 were found to have discontinuous similarity (80bp and 129bp) with marker Eaag/Mcac-233, accession AF243378 has partly discontinuous similarity (46bp) with marker Eact/Mctt-157 (Table 12). An example of the sequence similarity is shown in Figure 5. No sequence was found similar to marker Eaag/Mcag-180, Eagc/Mcag-104 and Eagg/Mcac-115 by both BLAST and FASTA. All the found accessions are cDNA clones from soybeans. It was clear that these sequences were not same as the existed sequences in the database. The sequences of 5 cloned fragments were then registered to DDBJ. The database accession numbers and the sequence of the clones are shown in Table 13.

Marker Development and Application

According to the sequence of the cloned DNA fragments, Sequence characterized amplified region (SCAR) primers were designed from the sequence data (Table 14) to amplify the related DNA fragment from the soybean genomic DNA. The primers from Eaag/Mcag-180 and Eagg/Mcac-115 showed a very small difference with a co-amplified band, which made it difficult to check the segregation among F_2 progenies. The primers from Eagc/Mcag-104 could not amplify the correct fragment. The primers from Eaag/Mcac-233 and Eact/Mctt-157 showed a very clear polymorphic band with a co-amplified band, and then these two markers were used to check the segregation of all the F_2 progenies.

The genomic DNA from 139 F_2 progenies were amplified by SCAR primer from Eaag/Mcac-233 and Eact/Mctt-157. For the primers from Eaag/Mcac-233, 108 F_2 progenies showed the same genotype as *CM60* (presented band), the other 31 F_2 progenies showed the same genotype as *GC10981* (no band) (3.48**:**1). For the primers from Eact/Mctt-157, 102 F_2 progenies showed the same genotype as *CM60* (presented band), the other 37 F2 progenies showed the same genotype as *GC10981* (no band) (2.76**:**1). These two SCAR markers developed from AFLP markers were inherited in a Mendelian manner with dominant segregation patterns (3:1; χ^2=0.540, p=0.463 for Eaag/Mcac-233 and χ^2=0.194, p=0.659 for Eact/Mctt-157). A t-test was carried out to compare the means of the germination and viability between the two genotypes, the P values for all tests were less than 0.001. There was a significant difference between the two genotypes. The germination and viability of the *GC10981* genotype were higher than the *CM60* genotype.

Table 11 The sequence of the cloned DNA fragment.

Clone name	Sequence
Eaag/Mcac-233	5'_gaattcaaggacccttactttagccttcccacacctatttatagcaaaacaaggcatttgg gatgaaggtagctcgcctgggcgagctggttacttcatcatgaagttatctaggtggttcagg ggctgaaaaatgccccaagagtgacccttttcccccattttgggtattttgcgtatttacttccaa aacgtcaaaaaaccttacggaatgcacgacaattggtgttaa_3'
Eaag/Mcag-180	5'_ttaacagcagctgcaacaacaataaactccccttctgccgtagattgagcaacaatgtctt gttttttgagctccatgagaaacacctgatccaaagctaaaacaataccctaaagtactcttcat atcatcaattgaaccaccccaatcactgccagagaaacttgttagcttgaattc_3'
Eact/Mctt-157	5'_ttaacttgtccagcatgataaacactcatgaaataatgttagtagtttcactttatagacaac aaggcaattatagaaagggttcttgtatagctatcatgcggctgttgacaggacacaaattgtt ggtgatatcaatgtaacagctgagtgaattc_3'
Eagc/Mcag-104	5'_gaattcagcccttcttcgtgactctagatgatttcataaatattctcagcataaagcagttca taggaatgcgtcgatgtttgaaatgtacctttcctgttaa_3'
Eagg/Mcac-115	5'_ttaacaccataagaggttataatatcatagattcatcattcatatgttcatcacagatcatga atccacagtatagttcaataggtatagcaagacatttcaacaccctgaattc_3'

Table 12 Sequence similarity of the cloned DNA fragments and the accessions searched from the database by BLAST and FASTA.

Clone name	aligned accession	Overlap location (bp)	Similarity (%)
Eaag/Mcac-233	AF180335	107-224	67.2
	AF186186	3-225	54.9
Eact/Mctt-157	AF243378	1-59	78.0
	BU549864	1-118	86.4
	BU765372	1-118	86.4

Table 13 Registering information of cloned and sequenced DNA fragments.

Clone name	Origin	Length (bp)	Database accession no.
Eaag/Mcac-233	*CM60*	233	AB213662
Eaag/Mcag-180	*CM60*	180	AB213663
Eact/Mctt-157	*CM60*	157	AB213664
Eagc/Mcag-104	*GC10981*	104	AB213665
Eagg/Mcac-115	*GC10981*	115	AB213666

```
>BU765372|BU765372.1 sas17d06.y1 Gm-c1080 Glycine max cDNA clone
          SOYBEAN CLONE ID:TRANSFERASE, GST 10B ;, mRNA sequence.
         Length = 617

 Score = 83.8 bits (42), Expect = 2e-14
 Identities = 102/118 (86%), Gaps = 3/118 (2%)
 Strand = Plus / Plus


Query: 1   gaattcactcagctgttacattgatatcaccaaaatttgtgtcctgtcaacagccgcatg 60
            |||||||||||||| |||||||||  |||||||||||||| |||||   | |||| ||||
Sbjct: 260 gaattcactcagc-gttacattggcatcaccaaaatttgcgtcctag-agcagctgcatc
317


Query: 61  atagctatacaagaacccttttctataattgccttgttgtctataaagtgaaactacta 118
            |||||| || |||||||||||| | ||||||| |||||||||| ||||||||||||||
Sbjct: 318 atagctctaaaagaacccttt-tgtaattgcattgttgtctagaaagtgaaactacta 374
```

Figure 5 An example of the sequence similarity compared by BLAST. The sequence (1-118) of Eact/Mctt-157 is overlapped with the sequence (260-374) of BU765372. One hundred and two bases (86%) are the same.

Table 14 SCAR markers designed from the sequence of cloned DNA fragments.

Marker	Primer	Sequence
Eaag/Mcac-233	Forward	5'- ttaacaccaattgtcgtgcat -3'
	Reverse	5'- gaattcaaggacccttttact -3'
Eaag/Mcag-180	Forward	5'- gaattcaagctaacaagtttctct -3'
	Reverse	5'- ttaacagcagctgcaacaacaat -3'
Eact/Mctt-157	Forward	5'- gaattcactcagctgttacat -3'
	Reverse	5'- ttaacttgtccagcatgat -3'
Eagc/Mcag-104	Forward	5'- ttaacaggaaaaggtacat -3'
	Reverse	5'- gaattcagcccttct -3'
Eagg/Mcac-115	Forward	5'- gaattcagggtgttgaaat -3'
	Reverse	5'- ttaacaccataagaggttat -3'

According to the DNA band pattern of the two SCAR markers, among the 139 tested F2 progenies, 91 progenies presented bands by both markers (AABB, AABb, AaBB or AaBb), 28 progenies presented bands by only one marker (AAbb, Aabb, aaBB or aaBb), and 20 progenies showed no band by either marker (aabb). By one-way ANOVA test (Table 15), all the P values were less than 0.05, most of the P values were less than 0.001. There was a significant difference in germination and viability among these three genotypes. In both treatments, the aabb genotype had a higher germination and viability than other heterozygotes and homozygotes. A boxplot of the different genotype is shown in Figure 6.

The main limitation of AFLP markers was the difficulty of using this technology on large segregating population. By transferring the AFLP markers to SCAR marker could overcome this limitation, but the procedure was more complex and time consuming. Meksem *et al*. (2001b) had successfully converted AFLP band into STS, which provides an efficient tool for genomic mapping, and marker assisted breeding. Stackelberg *et al*. (2003) reported a simplified procedure to convert AFLP bands into STS markers and named the loci as sequence specified AFLPs (ssAFLPs). These methods offer a simple and reliable way to focus on the interesting polymorphism, which allowed us to analyze these markers as single bands in normal agarose gel, instead of the time-consuming and costly AFLP polyacrylamide gels.

Table 15 One way ANOVA test on marker segregation and field weathering resistance of 139 F2 progenies.

Weathering indicator*	Marker pattern**	Minimum	Maximum	Mean	One way ANOVA	
					$F_{2,136}$	P value
IW germination	1	21.3	66.7	47.7	7.762	0.001
	2	26.3	72.5	51.9		
	3	29.2	81.6	59.6		
IW viability	1	47.8	81.0	67.9	22.549	<0.001
	2	51.4	85.0	71.7		
	3	58.3	95.6	80.6		
CD germination	1	20.0	66.0	48.7	12.272	<0.001
	2	26.0	72.0	56.2		
	3	38.0	82.0	64.0		
CD viability	1	44.0	80.0	67.0	26.803	<0.001
	2	54.0	82.0	74.9		
	3	54.0	90.0	78.9		
Mean	1	36.5	70.8	57.8	25.969	<0.001
	2	47.6	76.9	63.6		
	3	48.7	83.6	70.8		

* IW=incubator weathering; CD=controlled deterioration; Mean is the average of the germination and viability of both treatments.

** 1=both markers presented bands (AABB, AABb, AaBB or AaBb); 2= one of the markers presented bands (AAbb, Aabb, aaBB or aaBb); 3=neither markers presented bands (aabb).

In this study, though we successfully identified five polymorphic AFLP markers linked to the field weathering resistance of soybean in this study, the AFLP analysis showed a low polymorphism (5.6%) between the parent and a very low

linked polymorphism (0.2%) between the pools. The loss of the original polymorphism during generation of the STS and loss of the locus specificity of the STS is also an experimental challenge in generating sequence-specific STSs from AFLP bands (Meksem *et al*., 2001b). Only two of the five sequenced fragments were successfully transfer into SCAR markers. The segregation and linkage analysis indicated that the SCAR markers developed from AFLP markers were inherited in a Mendelian manner with dominant segregation patterns. The segregate ratio was 3.48**:**1 for marker Eaag/Mcac-233 and 2.76**:**1 for marker Eact/Mctt-157. Dominant heterozygotes and homozygotes could not be distinguished by these markers. However, since the recessive homozygotes were considered to be resistant to field weathering, it is possible to select the resistant homozygotes by these markers.

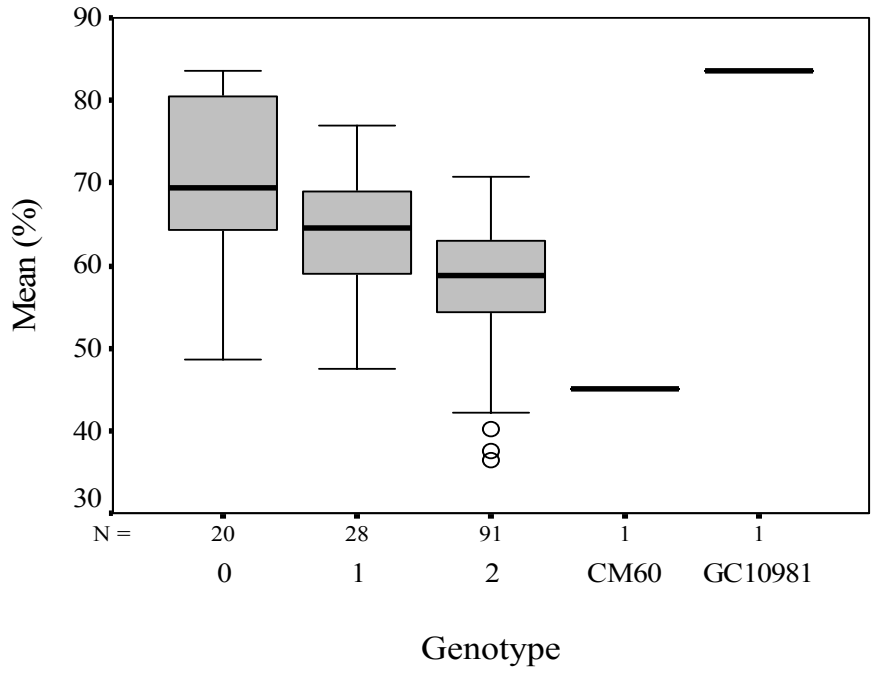

Figure 6 Boxplot of the field weathering resistance of different genotype of marker Eact/Mctt-157 and Eaag/Mcac-233. Mean is the average of the germination and viability of incubator weathering and controlled deterioration treatment. Genotype 1 indicate both markers presented bands (AABB, AABb, AaBB or AaBb); 2 indicate one of the markers presented bands (AAbb, Aabb, aaBB or aaBb); 3 indicate neither markers presented bands (aabb). N is the number of the F_2 progenies in same genotype.

By checking the segregation of the bulked F_2 progenies, it was found that both markers were co-segregated. For marker Eact/Mctt-157, all the 6 resistant F_2 plants showed no DNA band which were the same as *GC10981*, and all the 7 susceptible F_2 plants showed a DNA band which were the same as *CM60*. For marker Eaag/Mcac-233, all the 6 resistant F_2 plants showed no DNA band which were the same as *GC10981*, but only 6 susceptible F_2 plants showed a DNA band which were the same as *CM60*, one susceptible F_2 plants (S4) showed no DNA band (Figure 7). However, since only one plant (S4) was bulked with the F2 plants that presented DNA bands, the possibility to identify the polymorphism between the resistant and susceptible DNA pool was not affected.

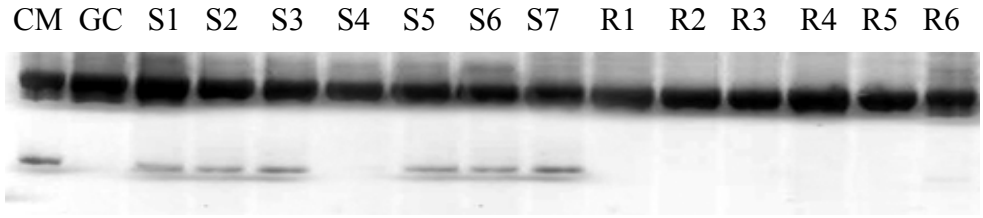

Figure 7 The co-segregation of the bulked F_2 progenies by marker Eaag/Mcac-233. CM = *CM60*, GC = *GC10981*, S = Susceptible plant, R = Resistant plant.

Genetic Mapping and QTL Identification

By analysis the genotypic segregation of the two SCAR markers in F_2 population, the segregation and linkage analysis indicated that the developed SCAR markers were inherited in a Mendelian manner with dominant segregation patterns, the segregating ratio was 3.48**:**1 for marker Eaag/Mcac-233 and 2.76**:**1 for marker Eact/Mctt-157. The genetic map distances were calculated using Mapmaker/EXP 3.0. The marker Eaag/Mcac-233 and Eact/Mctt-157 were in the same linkage group. The genetic distance between the two markers was 25.8 cM.

To identify the location of the markers on the chromosome and to develop more markers for mapping QTL, Michelmore *et al*. (1991) suggested that BSA can be made for "genomic walking" to develop markers for genetic mapping. In order to look

for more markers linked to the two markers developed from this study for QTL analysis of the field weathering resistance of soybean, the sequence data from this study were compared with the soybean sequence data from DDJB by BLAST (Altschul *et al*., 1997) and FASTA (Pearson and Lipman, 1988) analysis. No similar continuous sequence was found. Database accessions BU765372, BU549864 and AF243378 were partly similar to marker Eact/Mctt-157; AF180335 and AF186186 were partly similar to marker Eaag/Mcac-233. All these accessions are cDNA clones from soybeans, but there is no chromosomal information about these clones. And further information was not available due to the low map density and limited sequence database. Thus, the chromosomal positions of the markers developed from this study could not be identified by current searching from the database.

Mapping information from Mapmaker was used to verify the QTLs controlling the field weathering resistance (germination and viability) by composite interval mapping (CIM). The location with a LOD score greater than 3.0 was considered to identify QTL significantly associated with the trait. A QTL was identified by all the four field weathering resistance indicators, i.e. IW germination, CD germination, IW viability and CD viability. The distribution of LOD scores are shown in Figure 8 and the position and genetic effect of the QTLs are shown in Table 16. The QTL explained 29.2% and 31.8% of the variation in the viability of the two weathering treatments; it was higher than those in the germination of these treatments (11.9% and 18.7%). This QTL might contribute more for seed viability than for seed germination. If the average score of IW germination, CD germination, IW viability and CD viability was considered to be a composite index for field weathering resistance, a QTL also could be identified to be located at 14 cM from marker Eaag/Mcac-233 with a LOD score of 9.4 (Table 16). This QTL explained 29.7% of the variation in weathering resistance.

Table 16 QTL position and the genetic contribution for the field weathering resistance. The map distance was 25.8 cM beginning from marker Eaag/Mcac-233 (0.0 cM) to marker Eact/Mctt-157 (25.8 cM), and the QTL position is the peak LOD score from marker Eaag/Mcac-233.

Weathering indicator*	QTL Position (cM)	LOD score	Additive effect	R^2 (%)
IW germination	18.0	3.4	-5.518	11.9
CD germination	10.0	5.1	-7.850	18.7
IW viability	16.0	9.0	-6.002	29.2
CD viability	12.0	9.0	-6.270	31.8
Overall resistance	14.0	9.4	-6.107	29.7

* IW=incubator weathering; CD=controlled deterioration; Overall resistance is the mean of the germination and viability of both treatments.

Though BSA was considered to be limited in its application to QTL mapping (Mansur *et al*., 1996). Michelmore *et al*. (1991) stated that BSA could be extended to the analysis of genetically complex traits, such as QTL. If a quantitative trait is controlled by a few major genes (QTL), comparison of the bulks of extreme individuals could rapidly identify markers linked to QTL. AFLP combined with bulk segregant analysis has been effective to identify unknown genes in materials with low genetic diversity. By using this technique, BSA combined with AFLP has been successfully used to target some genes for simple traits. Hayes and Saghai Maroof (2000) bulked 12 homozygous resistant and susceptible progenies of a F_2 population to target the soybean mosaic virus resistance gene (*Rsv*1) using modified AFLP. They found four markers tightly linked to the *Rsv*1 gene within 6 cM, the closest one is just 0.4 cM. Meksem *et al*. (2001a) identified molecular markers closely linked with the two major QTL associated with soybean cyst nematode resistance. In this study, by QTL analysis using two SCAR markers developed from AFLP markers, Eaag/Mcac-233 and Eact/Mctt-157, a QTL was identified for all the four indicators of field weathering resistance. The resistant allele of QTL derived from parent *GC10981* because all the detected QTLs had a negative additive effect. The R^2 for all indicators were greater than 10, it might be a major QTL between the marker Eaag/Mcac-233

and Eact/Mctt-157 which controlled the field weathering resistance with a quite high effect. Even though the QTL for each indicator was located in a different position, if the mean of germination and viability of both treatments was considered to be an overall resistance for field weathering, a QTL was also found located at 14.0 cM from marker Eaag/Mcac-233. The QTL explained 29.7% of the variation. By combining the germination, viability and band pattern of the SCAR markers, it is possible to use these markers to assist selection in breeding programs.

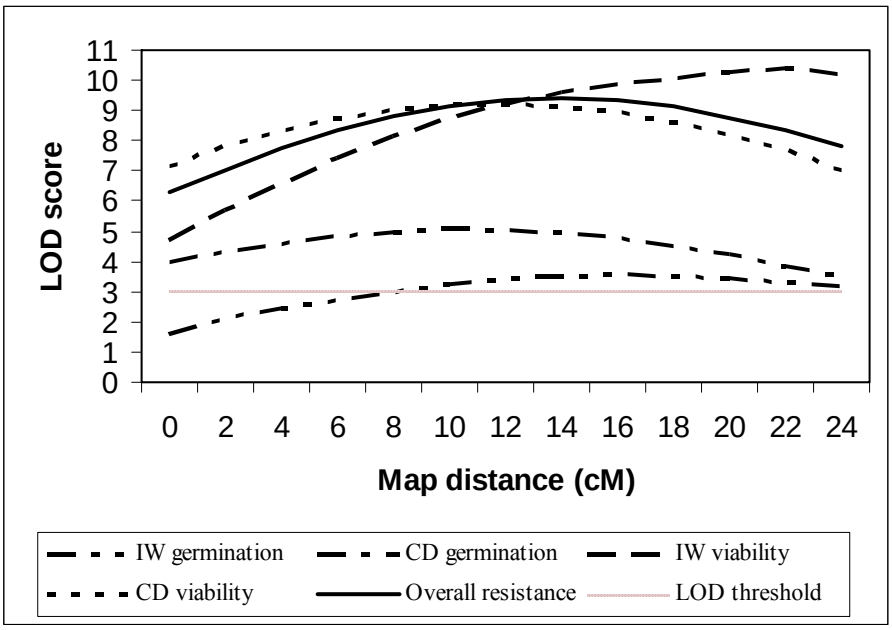

Figure 8 The LOD score distribution of the field weathering resistance along the genetic map. Map distance is from Eaag/Mcac-233 (0.0 cM) to marker Eact/Mctt-157 (25.8 cM). LOD threshold = 3.0.

Marker Assisted Selection and Backcrossing

By using marker Eaag/Mcac-233 and Eact/Mctt-157 to screen the F_2 progenies, it was found that dominant homozygotes and heterozygotes could not be distinguished by these markers. However, since the recessive homozygotes were considered to be resistant to field weathering, it was possible to select the resistant homozygotes from the F_2 progenies. There were 20 F_2 progenies absented DNA band by both markers

(Table 17). These progenies showed a higher resistance to field weathering by considering the seed germination and viability of incubator weathering and controlled deterioration treatment. By combining the germination, viability and DNA band pattern of the SCAR markers, seven F2 plants were selected for backcrossing. Some agronomic characters of these 7 selected F2 progenies are shown in Table 18.

Dudley (1993) outlined the steps in marker assisted selection as identifying associations between marker alleles and QTL and using the associations to develop improved lines or populations. However, most of the reports in the literature have moved to the second step in the process without developing a saturated genetic map, and without establishing strong relationship between marker alleles and QTL by adequately measuring the phenotype, like the procedures in this study. Furthermore, a selection index that includes both phenotypic measurement and a molecular marker score can increase the selection response relative to phenotypic selection alone, particularly if much of the additive genetic variance in a character can be explained using the molecular markers (Lander, 1992). Although it is possible to use either markers or agronomic character for assisting selection in breeding programs, especially at an earlier stage, but sometimes quite large number of progenies could be selected by only one indicator. If the marker assisted selection is used in backcrossing program, when too much progenies were selected, it is difficult to make a successful pollination, especially for those plants with a low hybrid seed production, such as soybean. In this case, if combine the genotype selection (marker) and phenotype selection (agronomic characters) for a complex screening, the candidate progenies will be reduced. As same as in this study, when use only the markers for selection, 20 F_2 progenies were selected. But if combine the marker selection with the field weathering resistance (germination and viability) selection, the F_2 progenies with low or medium resistance were removed, only seven progenies that highly resistant to the field weathering were selected for backcrossing.

Table 17 Some agronomic characters and the field weathering resistance of the F_2 progenies selected by marker Eaag/Mcac-233 and Eact/Mctt-157.

F_2 plant no.	Plant height (cm)	Plant yield (g)*	Seed size (g/100 seeds)	IW germination (%)	IW viability (%)	CD germination (%)	CD viability (%)
A3	29	27.49	14.76	53.7	75.6	54	72
A10	37	22.39	15.16	67.7	80.6	82	90
A11	42	27.33	15.32	72.7	90.9	74	86
A35	33	34.51	14.84	75.0	93.2	76	90
A39	26	26.48	13.12	44.4	58.3	38	54
A49	34	25.01	12.44	29.2	62.5	76	86
A50	35	27.56	13.84	39.0	75.6	74	82
A63	25	42.13	14.52	77.1	85.4	72	90
C2	43	38.5	16.00	70.4	90.7	76	90
C3	20	15.26	12.64	58.3	72.9	46	78
C9	32	17.13	12.52	50.0	69.0	74	80
C15	48	29.04	14.16	60.5	76.7	62	76
C19	23	17.88	11.12	57.4	87.2	66	74
C29	36	11.9	10.40	75.8	84.8	46	76
C33	37	28.42	12.48	52.5	77.5	62	68
C36	44	37.24	14.56	44.7	83.0	70	80
C45	36	36.03	13.72	31.8	75.0	62	74
G20	52	20.18	12.72	81.6	94.7	70	82
G21	46	20.12	14.88	70.0	82.0	62	86
G23	42	29.55	15.8	80.0	95.6	38	64

* Plant yield = total seed weight of the F_2 plant.

Table 18 Some agronomic characters and the field weathering resistance of the F_2 progenies selected for backcross.

F_2 no.	Plant height (cm)	Plant yield (g)*	Seed size (g/100 seeds)	IWG (%)	IWV (%)	CDG (%)	CDV (%)
A10	37	22.4	15.2	67.7	80.6	82.0	90.0
A11	42	27.3	15.3	72.7	90.9	74.0	86.0
A35	33	34.5	14.8	75.0	93.2	76.0	90.0
A63	25	42.1	14.5	77.1	85.4	72.0	90.0
C02	43	38.5	16.0	70.4	90.7	76.0	90.0
G20	52	20.2	12.7	81.6	94.7	70.0	82.0
G21	46	20.1	14.9	70.0	82.0	62.0	86.0

* Plant yield = total seed weight of the F_2 plant.

Efficiency Evaluation of the Marker Assisted Selection

The F_3 seeds from seven selected F_2 plants were grown in the greenhouse as male parent to backcross with the recurrent parent *CM60*. The obtained BC_1F_1 seeds and the seeds from their parents were grown in a field in the same greenhouse. Totally 43 BC_1F_1 plants were developed. The yellow pods from the 43 BC_1F_1 plants were harvested separately for field weathering evaluation by controlled deterioration test. The germination and viability of the BC_1F_1 plants are listed in Appendix Table B. The germination of BC_1F_1 plants ranged from 28 to 78%, while those of *CM60* and *GC10981* were 30% and 74%, respectively. The viability of the BC_1F_1 plants ranged from 58 to 94%, while those of *CM60* and *GC10981* were 56% and 90%, respectively. A boxplot of the germination and viability of different BC_1F_1 family is showed in Figure 9. The average germination and viability of the BC_1F_1 plants derived from the same F_2 plant (F_3/*CM60*) were calculated and compared with the germination and viability of the relative F_2 progenies and F_3 lines (Table 19). By paired t-test (Table 20), the correlation between the different generations was not significant ($p > 0.05$). The difference between F_2 progenies and F_3 lines was not significant ($t_6=2.228$, $p=0.067$ for germination and $t_6=0.834$, $p=0.436$ for viability), but the difference

between F_2 progenies and BC_1F_1 families was significant (t_6=7.862, p<0.001 for germination and t_6=4.948, p=0.003 for viability). There was also a significant difference between F_3 lines and BC_1F_1 families (t_6=6.992, p<0.001 for germination and t_6=3.744, p=0.010 for viability). The germination and viability of the treated BC_1F_1 seeds were lower than those of F_2 progenies and F_3 lines. By comparing the germination and viability of different generations, the field weathering resistance of F_3 lines was weaker than the relative F_2 progenies due to the segregation (Table 19). After the F_3 plants derived from the selected F_2 progenies were backcrossed to *CM60*, the field weathering resistance of BC_1F_1 families (mean) further decreased due to the permeation of the *CM60* background. However, 41 of the 43 BC_1F_1 progenies still showed higher germination and viability than *CM60*. The germination and viability of 18 BC_1F_1 progenies were higher than the mean of *CM60* and *GC10981*. Consequently, it has a high potential to develop field weathering resistant lines by carrying out further selections.

A marker-facilitated method for detecting specific genotypes in segregating populations is very useful during backcrossing, not only to expedite the introgression of a donor parent gene into the recurrent parent, but also to accelerate the recovery of the recurrent parent's genome (Specht and Graef, 1996). In this study, after backcrossing, the field weathering resistance of the BC_1F_1 progenies was still maintained at a high level. It is obvious that using the markers developed in this study for MAS is efficient. For further usage, it is desirable to use these markers to screen the progenies from the resistant BC_1F_1 plants, and then select the resistant progenies to backcross with *CM60* to improve the agronomic characters and the field weathering resistance of the progenies, and finally, to develop field weathering resistant pure lines with good agronomic characters similar to the recurrent parent *CM60*.

Table 19 A comparison of the germination and viability of the F_3 line and the BC_1F_1 family derived from the same F_2 plant. All the seeds were treated by controlled deterioration method.

F2 plant no.	F_2		F_3		BC_1F_1 (average)	
	Germination (%)	Viability (%)	Germination (%)	Viability (%)	Germination (%)	Viability (%)
A10	82	90	56	78	49.3	77.7
A11	74	86	66	88	56.8	85.2
A35	76	90	68	82	50.0	78.5
A63	72	90	70	88	53.1	77.8
C2	76	90	58	84	45.3	73.3
G20	70	82	72	92	50.3	77.1
G21	62	86	62	86	50.0	74.9
CM60	32	54	26	58	30.0	56.0
GC10981	72	94	76	96	74.0	90.0

Table 20 Paired t-test of the germination and viability of the F_3 lines and the BC_1F_1 families derived from the same F_2 plants.

Paired samples*	N	Correlation	P value	t	df	P value
F_2G & F_3G	7	-0.374	0.409	2.228	6	0.067
F_2G & BC_1F_1G	7	-0.139	0.767	7.862	6	<0.001
F_3G & BC_1F_1G	7	0.508	0.244	6.992	6	<0.001
F_2V & F_3V	7	-0.754	0.050	0.834	6	0.436
F_2V & BC_1F_1V	7	-0.178	0.702	4.948	6	0.003
F_3V & BC_1F_1V	7	0.170	0.715	3.744	6	0.010

* G= germination, V= viability.

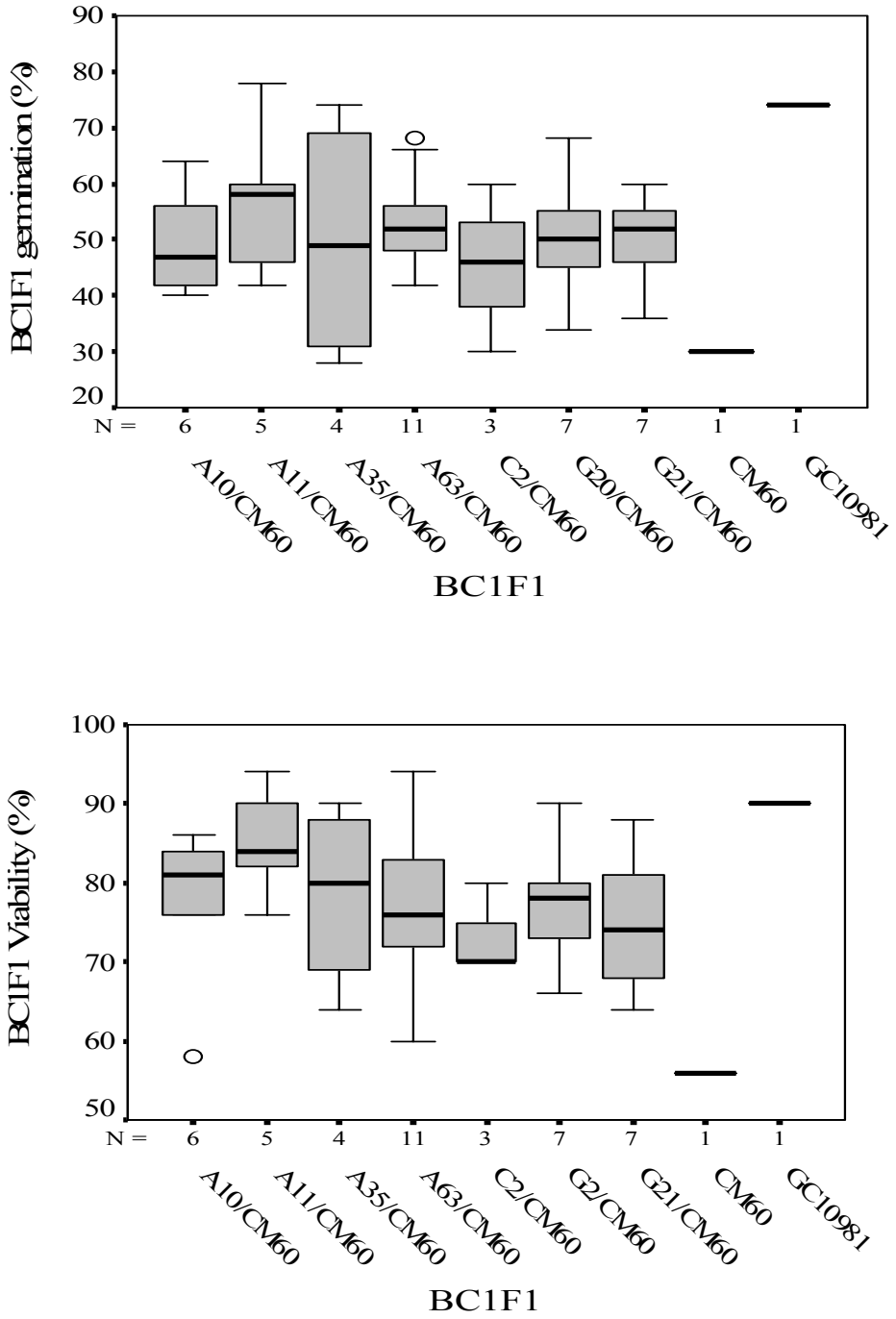

Figure 9 Boxplot comparison of the germination and viability of the seeds of different BC_1F_1 family after being subjected to controlled deterioration treatment.

CONCLUSIONS

The field weathering resistance of *CM60* and *GC10981* were evaluated by 7 treatments; the results confirmed that the field weathering resistance of *CM60* and *GC10981* was obviously different. *GC10981* was more resistant to field weathering than *CM60*. The modified incubator weathering (pods at physiological maturity were incubated at 30℃ and 90-100% RH for 7 days) and controlled deterioration (dry seeds were soaked in water for 60 minutes and then incubated at 41℃ and 90-100% RH for 3 days) methods used in this study could distinguish this difference efficiently. The distribution of the germination and viability of the F_2 progenies revealed that field weathering resistance was controlled by polygene. The genetic polymorphism between *CM60* and *GC10981* was low. In this study, only 5 field weathering resistance linked polymorphic DNA bands were identified by 198 RAPD primers and 64 pairs of AFLP primer combinations. The AFLP markers were more efficient to detect this polymorphism than the RAPD markers. The AFLP combining with BSA could be used for fast identifying the field weathering resistance genes. By transferring the AFLP markers to SCAR markers could also simplify the genetic mapping procedure of AFLP markers. The SCAR markers developed in this study (Eact/Mctt-157 and Eaag/Mcac-233) were successfully used to identify a major QTL controlling the field weathering resistance of soybean. The QTL explained 29.7% of the variation in field weathering resistance. By marker assisted selection and backcrossing. The field weathering resistance of the backcross progenies (BC_1F_1) also showed that the markers developed in this study could be used for marker assisted selection efficiently. However, the mapping position of these markers on the soybean genome was still unknown. This approach will be reached by further genetic mapping and genomic sequencing of soybean. The present study is the first attempt to identify the genes controlling the field weathering resistance of soybean, detailed genetic mechanism of the field weathering resistance of soybean was not verified for the limited time and available markers. Further studies on this topic should be proposed to develop more markers on genetic mapping and QTL analysis of this trait.

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APPENDIX

Appendix Table A Some agronomic characters, germination, viability and marker segregation of the F_2 progenies

F_2 plant no.	Plant height (cm)	100 seeds weight (g)	Incubator weathering		Controlled deterioration		Marker Eaag/ Mcac -233*	Marker Eact/ Mctt -157
			Germination (%)	Viability (%)	Germination (%)	Viability (%)		
A1	30	13.60	63.6	75.0	66	78	1	1
2	26	14.80	36.7	65.3	56	70	1	1
3	29	14.76	53.7	75.6	54	72	0	0
4	36	16.08	53.8	74.4	56	68	1	1
5	31	18.20	42.5	55.0	66	78	1	0
6	24	13.72	63.0	73.9	68	74	1	0
7	31	14.52	54.5	72.7	58	70	1	1
8	32	16.52	25.7	54.3	22	44	1	1
9	37	13.72	60.8	72.5	66	76	1	1
10	37	15.16	67.7	80.6	82	90	0	0
11	42	15.32	72.7	90.9	74	86	0	0
12	39	10.52	32.3	54.8	34	58	1	1
13	28	14.28	52.5	72.5	34	70	1	1
14	36	14.24	28.2	64.1	32	44	1	1
15	41	14.00	57.8	71.1	52	70	1	1
16	32	16.44	26.7	60.0	26	48	1	1
17	30	15.52	41.3	60.9	38	58	1	1
18	32	13.36	47.9	68.8	50	74	0	1
19	33	13.12	54.9	74.5	58	66	1	1
20	30	14.92	72.5	85.0	72	78	0	1
21	27	14.92	57.8	75.6	52	72	1	1
22	22	15.28	61.8	70.6	66	78	1	1
23	28	13.40	48.8	65.1	46	62	1	1
24	30	14.60	41.2	70.6	62	74	1	1
25	35	15.12	55.1	69.4	62	76	1	1
26	33	12.84	54.9	72.5	40	54	1	1
27	38	17.48	52.5	65.0	54	66	1	1
28	31	11.92	61.4	72.7	62	70	1	1
29	37	15.20	45.7	60.0	54	68	1	1
30	41	14.68	43.9	70.7	54	70	1	1

Appendix Table A (Continued)

F_2 plant no.	Plant height (cm)	100 seeds weight (g)	Incubator weathering		Controlled deterioration		Marker Eaag/ Mcac -233	Marker Eact/ Mctt -157
			Germination (%)	Viability (%)	Germination (%)	Viability (%)		
31	38	13.48	65.1	74.4	34	62	1	1
32	38	13.84	72.5	85.0	72	78	0	1
33	29	12.12	52.1	68.8	64	72	1	1
34	25	15.08	66.7	76.2	52	66	1	1
35	33	14.84	75.0	93.2	76	90	0	0
36	24	15.44	63.4	78.0	56	66	1	0
37	35	14.72	42.2	66.7	66	76	1	1
38	25	14.60	32.3	58.1	32	68	0	1
39	26	13.12	44.4	58.3	38	54	0	0
40	27	15.04	63.8	74.5	62	70	1	1
41	33	13.72	68.9	77.8	60	78	1	0
42	31	14.28	48.7	69.2	56	72	1	1
43	27	17.24	51.4	62.9	46	62	1	1
44	31	12.80	66.7	76.5	66	74	1	1
45	25	11.16	40.5	51.4	60	74	1	0
46	31	12.08	46.5	69.8	58	70	1	1
47	32	17.68	46.3	58.5	24	48	1	1
48	28	11.88	46.3	68.5	72	82	0	1
49	34	12.44	29.2	62.5	76	86	0	0
50	35	13.84	39.0	75.6	74	82	0	0
51	34	11.40	34.9	55.8	46	66	1	1
52	34	12.80	39.1	67.4	54	72	1	1
53	22	14.60	43.9	61.0	54	76	1	1
54	36	10.08	36.1	66.7	66	74	1	1
55	32	16.96	35.3	55.9	48	78	1	0
56	26	12.76	50.0	70.5	68	82	1	0
57	35	14.36	59.6	70.2	32	62	1	1
58	28	12.92	56.3	72.9	66	74	1	1
59	38	14.08	26.3	63.2	70	82	0	1
60	30	12.28	42.0	52.0	66	76	1	1

Appendix Table A (Continued)

F_2 plant no.	Plant height (cm)	100 seeds weight (g)	Incubator weathering		Controlled deterioration		Marker Eaag/ Mcac -233	Marker Eact/ Mctt -157
			Germination (%)	Viability (%)	Germination (%)	Viability (%)		
61	33	16.84	54.3	71.4	54	66	1	1
62	28	13.64	51.2	68.3	42	66	1	1
63	25	14.52	77.1	85.4	72	90	0	0
C1	32	14.08	31.7	70.7	54	62	1	1
2	43	16	70.4	90.7	76	90	0	0
3	20	12.64	58.3	72.9	46	78	0	0
4	34	16.16	41.3	47.8	58	74	1	1
5	22	11.64	60.4	72.9	66	74	1	1
6	42	12.88	39.2	58.8	42	62	1	1
7	41	13.88	56.4	74.4	62	70	1	1
8	21	13.08	50.0	69.6	24	62	1	1
9	32	12.52	50.0	69.0	74	80	0	0
10	31	12.64	53.1	65.6	58	74	1	1
11	40	12.96	52.4	66.7	52	68	1	1
12	38	15	45.8	64.6	44	62	1	1
13	41	12.64	40.7	69.5	54	70	1	1
14	29	13.6	52.9	64.7	66	74	1	1
15	48	14.16	60.5	76.7	62	76	0	0
16	43	12.24	55.3	74.5	48	70	1	1
17	44	14.12	55.8	73.1	54	74	1	1
18	33	10.76	52.4	71.4	50	70	1	1
19	23	11.12	57.4	87.2	66	74	0	0
20	34	15.56	52.5	72.5	48	58	1	1
21	40	13.16	35.6	60.0	66	74	1	1
22	39	13.64	47.5	67.5	72	82	0	1
23	46	11.44	34.1	80.5	32	70	1	0
24	26	11.6	46.3	68.3	42	52	1	1
25	31	16.24	51.4	73.0	26	70	1	0
26	27	14.48	27.3	48.5	20	54	1	1
27	26	17.12	44.7	68.4	26	62	1	1
28	31	15.28	59.1	68.2	28	56	1	1
29	36	10.4	75.8	84.8	46	76	0	0
30	34	14.08	51.2	68.3	46	66	1	1

Appendix Table A (Continued)

F_2 plant no.	Plant height (cm)	100 seeds weight (g)	Incubator weathering		Controlled deterioration		Marker Eaag/ Mcac -233	Marker Eact/ Mctt -157
			Germination (%)	Viability (%)	Germination (%)	Viability (%)		
C 31	37	13.36	48.0	72.0	52	66	1	1
32	34	15.64	59.5	71.4	32	64	1	1
33	37	12.48	52.5	77.5	62	68	0	0
34	37	12.84	43.8	64.6	54	66	1	1
35	49	14.32	37.2	74.4	64	74	1	1
36	44	14.56	44.7	83.0	70	80	0	0
37	33	14.76	21.3	61.7	28	66	1	1
38	32	12.08	33.3	68.9	62	74	1	1
39	36	12.72	44.4	68.9	42	68	1	1
40	37	12.4	36.5	61.5	56	78	0	1
41	38	11.2	52.4	71.4	56	74	1	1
42	32	15.24	68.8	79.2	40	54	1	0
43	35	15.56	27.7	66.0	34	66	1	1
44	22	14.24	37.2	72.1	46	70	1	1
45	36	13.72	31.8	75.0	62	74	0	0
46	31	13.64	35.6	60.0	28	58	1	1
G1	39	17.2	39.6	62.5	42	66	1	1
2	36	14.12	69.4	77.6	40	70	0	1
3	38	13.12	62.7	82.4	72	82	1	0
4	27	13.52	29.8	74.5	28	64	1	1
5	38	12.2	37.5	66.7	54	62	1	1
6	43	14.2	57.4	72.3	64	74	1	1
7	44	14.12	51.0	69.4	54	70	1	1
8	47	14.68	60.5	72.1	36	64	1	1
9	32	15.8	51.2	68.3	62	74	1	1
10	27	13.56	48.8	68.3	56	70	0	1
11	40	13.64	44.7	66.0	36	64	1	1
12	36	14.16	43.8	77.1	46	74	1	0
13	41	12.76	40.0	67.5	34	64	1	1
14	41	12.04	61.7	76.6	64	70	1	0
15	43	14.52	56.1	82.9	44	76	1	0

Appendix Table A (Continued)

F_2 plant no.	Plant height (cm)	100 seeds weight (g)	Incubator weathering		Controlled deterioration		Marker Eaag/ Mcac -233	Marker Eact/ Mctt -157
			Germination (%)	Viability (%)	Germination (%)	Viability (%)		
16	37	15.32	32.6	63.0	44	74	0	1
17	33	12.08	51.0	60.8	50	66	1	1
18	42	14.96	66.7	81.0	46	66	1	1
19	45	13.72	63.2	81.6	60	76	1	0
20	52	12.72	81.6	94.7	70	82	0	0
21	46	14.88	70.0	82.0	62	86	0	0
22	32	14.72	52.5	72.5	72	82	1	0
23	42	15.8	80.0	95.6	38	64	0	0
24	23	13.6	38.7	64.5	42	66	1	1
25	34	17.44	31.4	62.9	30	64	1	1
26	28	11.6	62.3	79.2	52	80	1	1
27	33	14.56	62.7	74.5	34	66	1	1
28	28	15.44	60.9	71.7	46	68	1	1
29	34	13.64	53.2	72.3	56	78	1	0
30	36	14.16	31.7	65.9	62	74	1	1
CM 60	52	19.24	34.7	59.7	32	54	1	1
GC1 0981	18	11.6	75	93.4	72	94	0	0

* 1 = showed band; 0 = no band.

Appendix Table B Germination and viability of the BC_1F_1 plants by controlled deterioration test.

Plant no.	BC1F1 (F3/CM60)	Normal seedlings	Abnormal seedlings	Hard seeds	Dead seeds	Germination (%)	Viability (%)
1	A10/CM60-1	32	10	0	8	64	84
2	2	25	18	0	7	50	86
3	3	28	10	0	12	56	76
4	4	17	21	4	8	42	84
5	5	20	19	0	11	40	78
6	6	22	7	0	21	44	58
7	A11/CM60-1	21	16	8	5	58	90
8	2	21	17	0	12	42	76
9	3	37	8	2	3	78	94
10	4	30	11	0	9	60	82
11	5	23	19	0	8	46	84
12	A35/CM60-1	30	11	2	7	64	86
13	2	14	23	0	13	28	74
14	3	37	8	0	5	74	90
15	4	17	15	0	18	34	64
16	A63/CM60-1	23	12	0	15	46	70
17	2	21	17	0	12	42	76
18	3	27	14	0	9	54	82
19	4	25	10	0	15	50	70
20	5	28	13	0	9	56	82
21	6	34	10	0	6	68	88
22	7	31	14	2	3	66	94
23	8	26	2	2	20	56	60
24	9	25	13	0	12	50	76
25	10	22	15	0	13	44	74
26	11	24	16	2	8	52	84
27	C2/CM60-1	30	5	0	15	60	70
28	2	15	20	0	15	30	70
29	3	23	17	0	10	46	80

Appendix Table B (Continued)

Plant no.	BC1F1 (F3/CM60)	Normal seedlings	Abnormal seedlings	Hard seeds	Dead seeds	Germination (%)	Viability (%)
30	G20/CM60-1	27	12	0	11	54	78
31	2	28	12	0	10	56	80
32	3	22	23	0	5	44	90
33	4	17	19	0	14	34	72
34	5	23	10	0	17	46	66
35	6	34	6	0	10	68	80
36	7	25	12	0	13	50	74
37	G21/CM60-1	26	6	0	18	52	64
38	2	30	13	0	7	60	86
39	3	26	18	0	6	52	88
40	4	20	12	0	18	40	64
41	5	18	20	0	12	36	76
42	6	25	11	1	13	52	74
43	7	27	7	2	14	58	72
P1	*CM60*	15	13	0	22	30	56
P2	*GC10981*	35	8	2	5	74	90