# Genetic analyses of fatty acid composition and

# pregermination flooding tolerance in soybean seeds

(ダイズ種子の脂肪酸組成および発芽前冠水抵抗性に関する遺伝分析)

**2002**

**Goro ISHIKAWA**

# Genetic analyses of fatty acid composition and pregermination flooding tolerance in soybean seeds

(ダイズ種子の脂肪酸組成および発芽前冠水抵抗性に関する遺伝分析)

2002

**Goro ISHIKAWA**

# Contents

**Part II. Genetic Analysis of Pregermination Flooding Tolerance of Soybean Seeds**

# General Introduction

Soybean (*Glycine max* (L.) Merr.) is an important crop extensively grown in the world, especially in Asia and America, because of its excellent source of seed oil and seed protein. In the USA, modern soybean breeding started in the late 1930s, and the first cultivar 'Pagoda' produced by crossbreeding was released in 1939 (Bernard et al. 1988). During the first few decades of breeding, the main objective was to increase grain yield; consequently, a 16.1 kg/ha annual gain on average was successfully achieved (Luedders 1977). In the next few decades, resistance or tolerance to biological stresses such as disease and insect was focused on; the first cultivar 'Pickett' resistant to some races of soybean cyst nematode was released in 1965 (Brim and Ross 1966). Thus until the 1970s, the main objective of soybean breeding was to develop high-yielding and/or stress resistant (or tolerant) cultivars. In the last few decades, however, people have become to prefer epicurean and/or health foods. In parallel with this, improving seed quality has become one of the important breeding objectives. In Japan, soybean breeding began more than one hundred years ago, and up to now, the main objective, like that in the USA, has been changed from high-yielding to high quality.

The primary breeding for seed quality aimed at increasing the content of seed oil or seed protein; consequently, genetic improvements for these traits were smoothly achieved because of their high heritabilities (Smith and Weber 1968). In the late 1970s, however, components of seed oil and seed protein became to public attention; many attempts to alter these two traits were made. As a result, genetic

mechanisms of protein and fatty acid composition were partly verified. Most recently, genetic manipulations made it possible to increase rare components such as isoflavone and tocopherol and to introduce novel components such as artificially modified globulin. These activities provide new niches for soybean.

Thus the genetic improvement of soybean seed quality seems to have been smoothly conducted. However, there are still many subjects to be solved for the further improvement. Soybean is one of the most susceptible crops to environmental conditions: both of seed component content and grain yield fluctuate dramatically with cropping season, location, and year. Therefore, breeding of cultivars that achieve stable and high production of high quality seeds has been required in various countries.

Nowadays, improving fatty acid composition, as well as protein composition, is an important objective in many countries including Japan. The fatty acyl component affects the chemical and physical properties of vegetable oil and their potentials for food or industrial usage (Ohlrogge 1994). For the fatty acid composition of soybean seeds, there is a great intervarietal and interspecific variation, suggesting the possibility of genetic improvement of soybean seed oil quality (Hawkins et al. 1983, Rebetzke et al. 1997). Using such genetic resources, breeders have successfully enhanced the oxidative stability of soybean seed oil, resulting from decreasing the polyunsaturated fatty acid content. Recently, many induced mutants with extreme fatty acid composition have been developed, and the improvement of oil quality by genetic engineering has been put into practice (Lühs and Friedt 1994, Yadav 1996, Liu 1999). However, the stable production of desirable fatty acid composition of

2

seeds is still difficult, especially in extreme fatty acid composition, due to environmental fluctuations. This indicates the significance of further investigation on the genetic mechanisms and environmental effects on fatty acid composition.

In soybean cultivation, excessive water before germination causes severe seed deterioration, resulting in decreased grain yield at maturity. Therefore, breeding of pregermination flooding tolerant cultivars has been required in the countries with much rainfall during sowing time. Several studies aiming at efficient breeding of tolerant soybean cultivars, such as exploiting useful screening methods and analyzing intervarietal differences, so far have been conducted (Hou and Thseng 1991, Hou and Thseng 1992, Hou et al 1995). Furthermore, physiological factors responsible for pregermination flooding tolerance, such as water uptake rate of seed, elongation ability of seedling, and seed storability, have been investigated (Thseng et al 1996a, 1996b). According to Fagerstedt and Crawford (1987), flooding tolerance of soybean plant was tightly related with the anoxia respiration ability of root. However, seed damages by excessive water might be caused not only by lowering of the respiration ability under anoxia conditions but also by rapid water absorption that disrupts the cell membrane, resulting in the leakage of electrolytes, sugar, and amino acids (Powell and Matthews 1978, Egley et al 1983, Pereira and Andrews 1985). Such complex nature of seed damages due to excessive water makes it difficult to elucidate the physiological and genetic mechanisms of pregermination flooding tolerance. Therefore, further genetic investigations on pregermination flooding tolerance should be done considering various physical and physiological factors.

In this thesis, the author dealt with two important traits, fatty acid composition

3

and pregermination flooding tolerance to obtain fundamental information essential

for developing stable and high-yielding cultivars with superior oil quality. This thesis

consists of two parts; the first part describes fatty acid composition in soybean seeds,

and the second part describes pregermination flooding tolerance of soybean seeds.

# Part I. Genetic Analysis of Fatty Acid Composition of Soybean Seeds

## Introduction

Soybean oil contains five commercially important fatty acids: palmitic (16:0), stearic (18:0), oleic (18:1), linoleic (18:2), and α-linolenic (18:3) acid. Traditional soybean oil with large proportions of linoleic and α-linolenic acids is used not only for food industry purposes but also for non-food uses, such as diesel fuel, lubricating oils, surface coatings or drying oils (Endres 1992).

This part was focused on genetic analyses of fatty acid composition not only in mature seeds but also in developing seeds to provide fundamental information useful for developing the cultivars that enable stable production of high quality oil. This part consists of the following four chapters: (1) genetic variation in fatty acid composition of mature seeds; (2) fatty acid accumulation pattern in developing seeds; (3) expression of fatty acid desaturase genes; and (4) algorithmic analysis of fatty acid flux in developing seeds.

# Chapter 1. Genetic Variation in Fatty Acid Composition of Mature Seeds

## 1.1 Introduction

It is known that the fatty acid composition of mature seeds is modified by the duration of growth period (Takagi et al. 1979). In this sense, the genetic analysis of fatty acid composition should be conducted taking account of the duration of growth period, especially of the duration of the seed filling period. But there are few reports describing the effect of the duration of the seed filling period on the fatty acid composition of mature seeds.

The objective of this chapter was to investigate the magnitude of genetic variation of fatty acid composition among 60 commercial and native soybean cultivars when considering the duration of the seed filling period.

## 1.2 Materials and Methods

**Plant materials and cultivations**

Sixty soybean cultivars selected from Japanese and American commercial cultivars and Japanese native cultivars were grown on an experimental field station at Kyoto Univ., Kyoto, Japan, in 1997. Seeds were sown in pots containing field soil on June 20, and seedlings were transplanted on July 22, with a randomized complete block design with two replications (15 plants/plot). Planting density was $70 \times 15$ cm. Three agronomic traits, number of days from sowing to flowering, number of days from flowering to maturity, and 100 air-dried seed weight, were measured for each

plant. Flowering date was recorded when an open flower was found on the main stem, and maturing date was recorded when 95% of normal pods on the main stem showed mature pod color.

**Fatty acid analysis**

Seeds of five plants per plot were used for the analysis of fatty acid composition using the method of Takagi et al. (1989). In the analysis, 10 seeds from each plant were crushed and crude oil was extracted with diethyl ether. Methylesters of fatty acids were prepared by intereseterification using sodium methoxide, and were separated into five fatty acids with a gas chromatograph GC-17A (Shimadzu, Japan). The fatty acid composition was expressed by the weight ratio of each fatty acid to the total fatty acid.

**Statistical analysis**

Analysis of variance (ANOVA) for fatty acid composition was performed for each maturity group. To obtain an integrated figure of the total variation of fatty acid composition among 60 cultivars, a principal component analysis with correlation matrix was conducted using the SAS microcomputer package (SAS Institute Inc., USA).

## 1.3 Results

**Flowering and maturity habits**

According to Fukui and Arai (1951), soybean cultivars form nine maturity groups (Ia-Vc), where I-V indicates the classes based on number of days from sowing to flowering, and a-c indicates the classes based on number of days from

flowering to maturity. The 60 cultivars used were classified into five maturity groups (IIb, IIc, IIIc, IVc, and Vc) (Table 1.1). There were no cultivars belonging to Ia, Ib, IIa, and IIIb in the present study. This may be due to that we omitted too early flowering cultivars. Such cultivars would not be available for the analysis because of insufficient vegetative growth before flowering, resulting in a small number of seeds per plant.

Japanese commercial cultivars were distributed almost equally in the five maturity groups, while most American commercial and most Japanese native cultivars belonged to relatively early and late flowering groups (II and III), respectively. In the present study, we divided the growing period into number of days from sowing to flowering and number of days from flowering to maturity, because the periods before and after flowering were assumed to have different effects on fatty acid composition.

**Genetic variation in fatty acid composition**

In all the maturity groups, significant differences between cultivars were found for all the fatty acids (Table 1.2 and 1.3). However, the intervarietal differences of oleic, linoleic, palmitic, and stearic acids in Vc were not so great as those in other maturity groups. Among the five fatty acids, stearic and oleic acids showed large overall coefficients of variation (>20%): their values were about twice and three times larger than those of palmitic and linoleic acids, respectively. In each maturity group, the coefficients of variation of all the fatty acids, especially of stearic and oleic acids, tended to decrease with the increase of number of days from sowing to flowering and number of days from flowering to maturity.

8

**Table 1.1** Classification of 60 soybean cultivars used in Chapter 1 into
five maturity groups based on Fukui et al. (1952)

| Cultivar | II[†] | | III | IV | V |
|---|---|---|---|---|---|
| | b[¶] | c | c | c | c |
| Japanese commercial | 7 | 6 | 7 | 4 | 3 |
| Japanese native | 0 | 4 | 6 | 4 | 2 |
| American commercial | 1 | 11 | 3 | 2 | 0 |
| Total | 8 | 21 | 16 | 10 | 5 |

[†] I - V = Classes based on number of days from sowing to flowering: I = <30 or 30;
II = 40; III = 50; IV = 60; V = >70.

[¶] a - c = Classes based on number of days from flowering to maturity: a = <60;
b = 60-70; c = >70 in II and III or >80 in IV and V.

Sown on June 20, 1997.

**Table 1.2** Statistics for the fatty acid composition in each maturity group

| Mturity group | Statistic | Fatty acid (%) | | | | |
|---|---|---|---|---|---|---|
| | | Palmitic | Stearic | Oleic | Linoleic | Linolenic |
| IIb | Mean | 12.7 | 2.9 | 24.1 | 52.4 | 8.0 |
| | S.D.[†] | 1.4 | 0.8 | 4.4 | 4.1 | 1.1 |
| | C.V. | 11.2 | 26.7 | 18.5 | 7.8 | 13.7 |
| IIc | Mean | 11.0 | 3.0 | 23.5 | 54.2 | 8.4 |
| | S.D. | 1.0 | 0.7 | 6.2 | 4.7 | 1.4 |
| | C.V. | 9.5 | 24.8 | 26.4 | 8.7 | 16.7 |
| IIIc | Mean | 10.9 | 2.8 | 20.5 | 56.2 | 9.6 |
| | S.D. | 0.9 | 0.5 | 3.2 | 2.9 | 1.0 |
| | C.V. | 8.0 | 16.8 | 15.8 | 5.2 | 10.9 |
| IVc | Mean | 10.0 | 2.7 | 20.9 | 57.2 | 9.3 |
| | S.D. | 0.7 | 0.4 | 0.3 | 3.0 | 0.8 |
| | C.V. | 7.1 | 14.1 | 16.6 | 5.3 | 8.9 |
| Vc | Mean | 10.9 | 2.6 | 19.5 | 58.1 | 9.0 |
| | S.D. | 0.9 | 0.4 | 1.5 | 2.4 | 1.1 |
| | C.V. | 8.1 | 13.8 | 7.8 | 4.1 | 12.2 |
| Pooled | Mean | 11.0 | 2.8 | 22.0 | 55.3 | 8.8 |
| | S.D. | 1.2 | 0.6 | 4.8 | 4.1 | 1.3 |
| | C.V. | 11.0 | 21.3 | 21.7 | 7.4 | 14.4 |

[†] S.D. = standard deviation, C.V. = coefficient of variation.

**Table 1.3** Analysis of variance for the fatty acid composition in each maturity group

| Maturity group | Source | d.f. | Palmitic MS | | Stearic MS | | Oleic MS | | Linoleic MS | | Linolenic MS | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Cultivar | 7 | 4.05 | *** | 1.16 | *** | 39.54 | *** | 33.12 | *** | 2.41 | *** |
| IIb | Block | 1 | 0.09 | | 0.00 | | 2.38 | | 0.91 | | 0.05 | |
| | Error | 7 | 0.23 | | 0.03 | | 1.39 | | 0.87 | | 0.12 | |
| | Cultivar | 20 | 2.15 | *** | 1.10 | *** | 77.11 | *** | 44.50 | *** | 3.90 | *** |
| IIc | Block | 1 | 0.20 | | 0.00 | | 1.29 | | 0.00 | | 0.27 | |
| | Error | 20 | 0.05 | | 0.01 | | 3.98 | | 2.06 | | 0.22 | |
| | Cultivar | 15 | 1.54 | *** | 0.43 | *** | 20.88 | *** | 16.96 | *** | 2.16 | *** |
| IIIc | Block | 1 | 0.00 | | 0.21 | ** | 0.00 | | 0.08 | | 0.01 | |
| | Error | 15 | 0.07 | | 0.02 | | 0.74 | | 0.41 | | 0.12 | |
| | Cultivar | 9 | 1.00 | *** | 0.28 | *** | 24.00 | *** | 18.49 | *** | 1.37 | *** |
| IVc | Block | 1 | 0.01 | | 0.01 | | 0.10 | | 0.03 | | 0.12 | |
| | Error | 9 | 0.05 | | 0.02 | | 1.45 | | 1.08 | | 0.06 | |
| | Cultivar | 4 | 1.55 | * | 0.25 | * | 4.60 | ** | 11.19 | ** | 2.40 | *** |
| Vc | Block | 1 | 0.25 | | 0.05 | | 0.04 | | 0.01 | | 0.04 | |
| | Error | 4 | 0.10 | | 0.02 | | 0.17 | | 0.28 | | 0.04 | |
| | Cultivar | 59 | 2.96 | *** | 0.72 | *** | 45.61 | *** | 33.05 | *** | 3.23 | *** |
| Pooled | Block | 1 | 0.08 | | 0.11 | | 0.08 | | 0.01 | | 0.14 | |
| | Error | 59 | 0.09 | | 0.02 | | 2.00 | | 1.11 | | 0.14 | |

*, ** and *** Significant at 5, 1 and 0.1% levels, respectively.
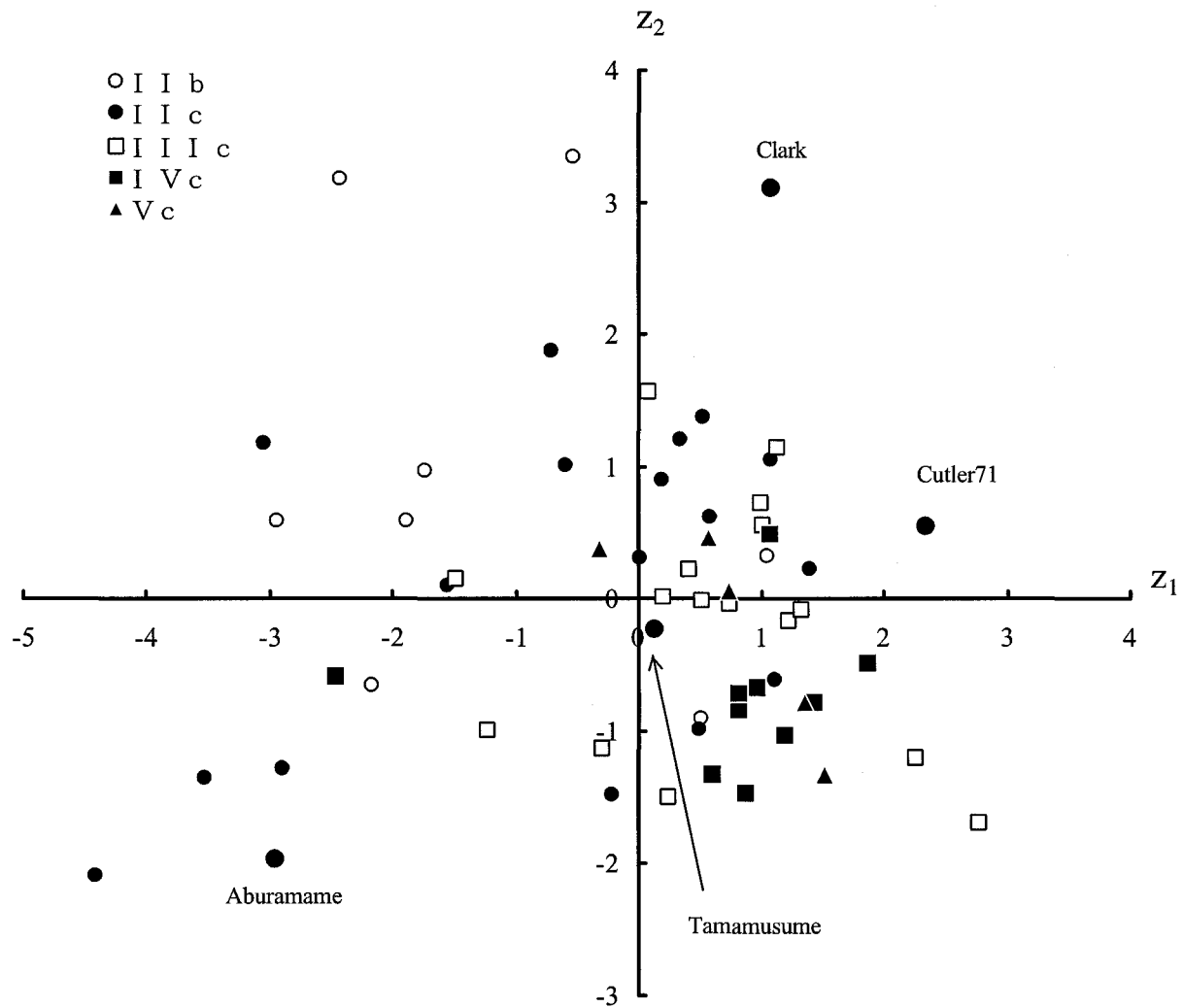
**Principal component analysis**

As a result of principal component analysis, about 80% of the total variation of fatty acid composition could safely be explained by the first two principal components ($Z_1$, $Z_2$). The $Z_1$ with a contribution ratio of 50.1% to the total variation represented the allometric phase of variation between high proportions of linoleic and $\alpha$-linolenic acids and a low proportion of oleic acid: the cultivar with a larger $Z_1$ score exhibited higher linoleic and $\alpha$-linolenic acid proportions and a lower oleic acid proportion. The $Z_2$ with a contribution ratio of 29.0% represented the isometric phase of variation of palmitic and stearic acid proportions: the cultivar with a larger $Z_2$ score exhibited higher proportions of these two fatty acids.

The scatter diagram of 60 cultivars according to $Z_1$ and $Z_2$ scores is shown in Fig. 1.1. About 50% of the cultivars used were deployed in the neighborhood of the origin in the first and fourth quadrants. The cultivars of IIb were mainly assembled in the second quadrant. Among the five maturity groups, IIc showed the largest variation involving several cultivars with extremely large negative $Z_1$ scores, which were not found in other maturity groups. In IIIc and IVc, three cultivars exhibited unique fatty acid composition: two in IIIc showed high linoleic and $\alpha$-linolenic acid composition and one in IVc showed high oleic acid composition.

## 1.4 Discussion

In breeding practice of soybean oils, wild *Glycine* germplasm (Palmer et al. 1996, Pantalone et al. 1997), induced mutations (compiled by Liu 1999), and transformants by genetic engineering (Kinney 1998, Kinney and Knowlton 1998)

12

**Fig. 1.1** Scatter diagram of 60 soybean cultivars according to $Z_1$ and $Z_2$ scores extracted from a principal component analysis for the fatty acid composition. IIb, IIc, IIIc, IVc, and Vc are maturity groups classified by number of days from sowing to flowering and number of days from flowering to maturity.

have been used as novel sources of fatty acid variation; consequently, new cultivars have been developed and tested with regard to their improved properties and added value in food industry (Specht and Graef 1996, List et al. 1996, Shen et al. 1997, Cole et al. 1998, Liu 1999). On the other hand, the material used for this investigation represents not a real core collection of soybean with all fatty acid variants. Therefore, we could not conclude about total genetic variation of fatty acid composition in this study due to the lack of fatty acid extreme types. Among the limited set of cultivars, however, we could found some genetic variation with regard to stearic and oleic acid contents, which have been targeted for modification of soybean oils.

Due to the lack of fatty acid extreme types in this study, the author is not able to consider stearic and oleic acid as major descriptors of total fatty acid variations. The mechanisms for expression of extreme fatty acid compositions, e.g. 50% oleic acid composition (Wilson et al. 1981), and 20% stearic acid composition (Hammond and Fehr 1983), can be considered due to the deficiency of gene(s) controlling fatty acid synthesis, or high suppression of gene transcription. Contrary, the intervarietal differences in stearic and oleic acid composition observed in this study, which was not so extreme, may be caused by the differences of amounts of gene(s) expression and/or enzyme(s) activities related to these two fatty acid syntheses.

14

# Chapter 2. Fatty Acid Accumulation Pattern in Developing Seeds

## 2.1 Introduction

The fatty acid composition of mature soybean seeds is determined by the accumulation amount of each fatty acid in its biosynthetic pathway during the seed filling period. Relevant enzymes are involved in each step of the pathway, and one or more specific genes regulate the activity of each enzyme (Murphy 1995, Harwood 1996, Ohlrogge and Jaworski 1997). For the efficient improvement of oil quality, the accumulation pattern of each fatty acid during the seed filling period should be investigated.

There are several reports describing the change of fatty acid composition during the seed filling period (Cherry et al. 1984, Sangwan et al. 1986, Dornbos and McDonald 1986). In these reports, two different indices were used to express the seed filling stages: Cherry et al. (1984) and Sangwan et al. (1986) used number of days after flowering, while Dornbos and McDonald (1986) used pod or seed size (Growth stage) according to Fehr et al. (1971). Since late maturing seeds develop consistently faster than early ones (Gbikpi and Crookston 1981), number of days after flowering seems not a good index of seed filling stage. Contrary, fresh weight per seed directly shows the seed filling stage.

The objectives of this chapter were to investigate the genetic variation of fatty acid accumulation pattern using the unique cultivars and fresh weight per seed as plant materials and a parameter of seed filling stage, respectively.

15

## 2.2 Materials and Methods

**Plant materials, cultivations and sampling methods**

Four cultivars, 'Cutler71', 'Clark', 'Aburamame', and 'Tamamusume', selected based on the results of Chapter 1, were grown in an experimental field at Kyoto Univ. using three cropping seasons (Early, Middle, and Late). These cultivars all belonged to the maturity group IIc of Fukui and Arai (1951). The first three cultivars exhibited unique fatty acid composition: 'Cutler71', high linoleic acid; 'Clark', high stearic acid; 'Aburamame', high oleic acid proportion. Contrary, 'Tamamusume' represents the standard fatty acid composition. 'Cutler71' and 'Clark' are American commercial cultivars with an indeterminate growth habit, and 'Aburamame' and 'Tamamusume' are Japanese native and Japanese commercial cultivars respectively, both of which have a determinate growth habit.

Seeds were sown in pots containing field soil on May 27, June 11, and June 26, 1998, for early, middle, and late cropping seasons respectively, and seedlings were transplanted on June 18, July 3, and July 17, 1998, respectively, with a randomized complete block design with two replications (10 plants/plot). Planting density was 50 × 20cm. Three agronomic traits, days from sowing to flowering, days from flowering to maturity, and seed weight were measured.

Immature seeds of 'Cutler71' and 'Clark' were collected twice (30 and 50 days after flowering) from low, middle, and high nodes of the main stem, and those of 'Aburamame' and 'Tamamusume' were collected four times (20, 30, 40, and 50 days after flowering) from middle nodes of the main stem. According to Fehr et al. (1971),

16

the reproductive phase of soybean after flowering is divided into eight growth stages (R1-R8) and the seed filling period corresponds to the period from growth stage R5 to R6. Since seed mass rapidly increases during this period, we classified immature seeds into the following eight filling stages based on fresh weight per seed: Filling stage 1=<20; 2=20-50; 3=50-100; 4=100-200; 5=200-300; 6=300-400; 7=400-500; 8=>500 mg. All the seeds collected were frozen in liquid nitrogen and were stored at -80℃. The filling stage 2 and 8 were approximately consistent with growth stage R5 and R6, respectively. Mature seeds were also harvested.

**Fatty acid analysis**

The immature seeds at eight filling stages and mature seeds were subjected to a fatty acid analysis using the method of Takagi et al. (1989) with minor modifications. Freeze-dried seeds at each filling stage were ground in a mortar with a pestle, and 100mg of soybean powder were prepared (two replications). Before oil extraction, soybean powder was boiled in 99.5% 2-propanol to ensure the inactivation of lipolytic enzymes. The oil was extracted in diethyl ether with the same method as Chapter 1 with an exception of adding triheptadecanoin as an internal standard before interesterification. Each fatty acid was calibrated by weight using authentic fatty acid methyl ester (Sigma, USA). Fatty acid contents were measured three times for each replication.

**Statistical analysis**

The multiple comparison analysis of fatty acid content among the four cultivars was conducted using Fisher's PLSD (Protected least significant differences) method by the microcomputer package StatView 5.0 (SAS Institute Inc., USA).

## 2.3 Results

**Flowering and maturity habits**

Four cultivars, 'Cutler71', 'Clark', 'Aburamame', and 'Tamamusume' being selected based on the results of Chapter 1, were investigated for the accumulation patterns of crude oil and fatty acids in developing seeds. These cultivars showed similar 100 air-dried seed weights (ca. 22g) at maturity.

For all the cultivars, it was found that both number of days from sowing to flowering and number of days from flowering to maturity tended to decrease with the delay of cropping season, but the effect of cropping season on seed weight was almost negligible (Table 2.1). The effect of cropping season on fatty acid composition was recognized, but it was obviously smaller than that of cultivar (Table 2.1).

**Analysis of developing seeds**

The accumulation amount of crude oil gradually increased up to filling stage 3, and then rapidly increased up to maturity, excepting that 'Aburamame' showed a gradual increase during the period from filling stage 4 to 6 (Fig. 2.1a). The accumulation pattern of crude oil thus showed a sigmoidal increase during the seed filling stage in all the cropping seasons, although a little intervarietal difference was found. The accumulation pattern of the total fatty acid content was similar to that of crude oil: all the cultivars except 'Aburamame' showed a rapid increase during the period from filling stage 4 to 6 in all the cropping seasons (Table 2.2).

There were minor intervarietal differences in accumulation patterns of palmitic,

18

**Table 2.1** Number of days from sowing to flowering (DF), number of days from flowering to maturity (DM), 100 air-dried seed weight, and fatty acid composition of mature seeds of four soybean cultivars in three cropping seasons

| Cultivar | Sowing date | DF | DM | Seed weight (g) | Fatty acid (%) | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | Palmitic | Stearic | Oleic | Linoleic | Linolenic |
| Cutler71 | May 27 | 42 | 101 | 19.6 | 9.9 | 6.4 | 17.1 | 58.7 | 7.9 |
| | June 11 | 40 | 96 | 23.0 | 10.3 | 5.8 | 16.2 | 58.9 | 8.8 |
| | June 26 | 32 | 84 | 21.5 | 10.3 | 5.3 | 17.0 | 58.9 | 8.5 |
| Clark | May 27 | 39 | 104 | 20.2 | 10.1 | 6.9 | 16.8 | 58.5 | 7.7 |
| | June 11 | 39 | 92 | 20.2 | 10.4 | 5.7 | 17.9 | 57.3 | 8.7 |
| | June 26 | 33 | 81 | 21.5 | 10.4 | 4.4 | 14.4 | 61.8 | 9.0 |
| Aburamame | May 27 | 49 | 82 | 26.7 | 9.7 | 3.7 | 36.8 | 42.9 | 6.9 |
| | June 11 | 44 | 74 | 22.8 | 10.3 | 3.9 | 30.0 | 47.3 | 8.5 |
| | June 26 | 38 | 70 | 24.1 | 10.4 | 2.9 | 22.6 | 54.8 | 9.3 |
| Tamamusume | May 27 | 46 | 70 | 22.0 | 10.9 | 3.7 | 24.2 | 52.3 | 8.9 |
| | June 11 | 43 | 69 | 18.3 | 11.5 | 3.9 | 17.9 | 56.8 | 9.9 |
| | June 26 | 34 | 66 | 20.3 | 10.7 | 3.3 | 19.8 | 57.1 | 9.1 |
| $LSD_{0.05}$ | | 1 | 2 | 3.9 | 0.6 | 0.7 | 1.5 | 2.3 | 3.6 |
| $LSD_{0.01}$ | | 1 | 2 | 5.6 | 0.8 | 0.9 | 2.0 | 3.1 | 4.7 |
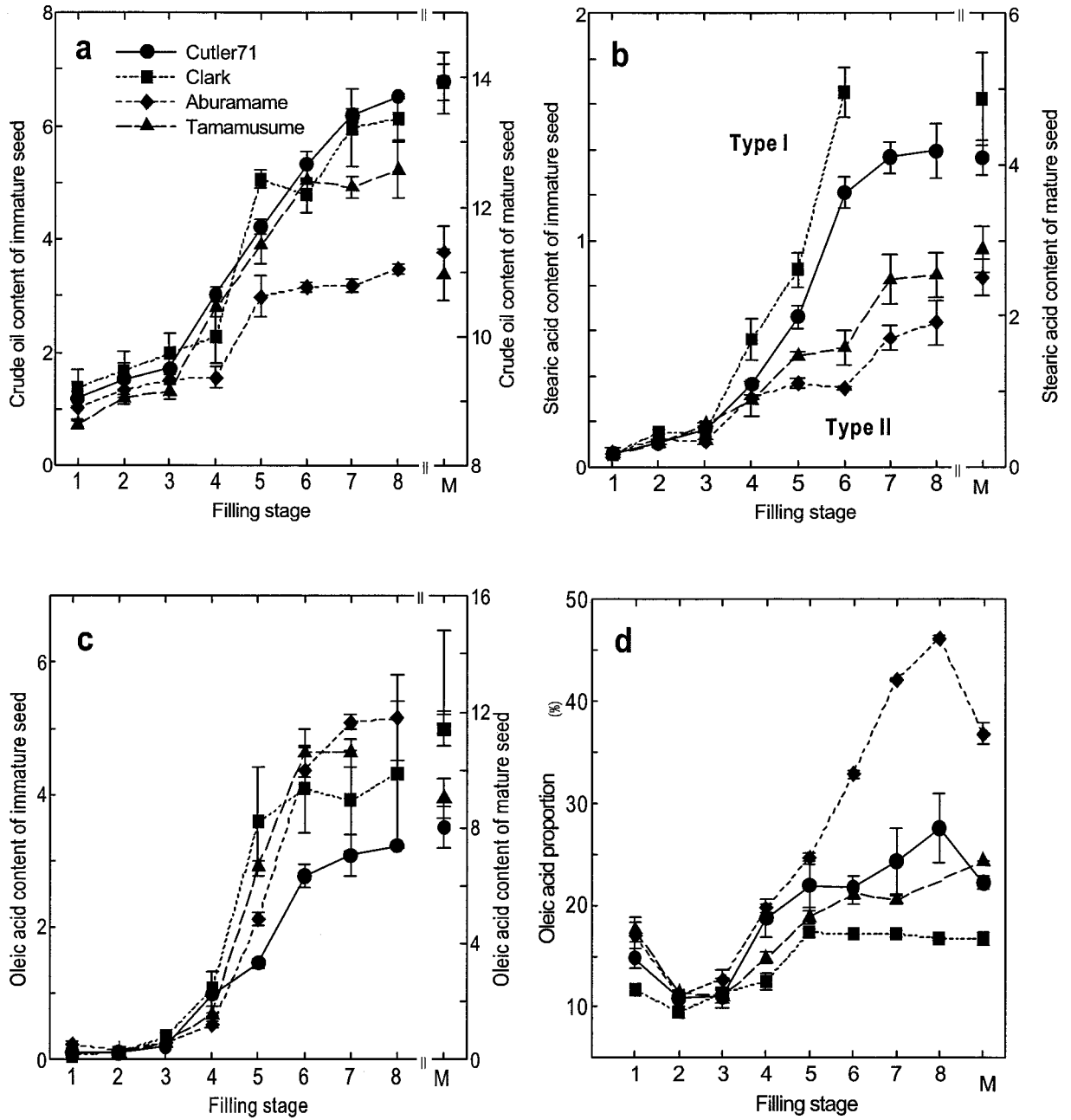
**Fig. 2.1** a-c. Changes of crude oil and fatty acid contents during the seed filling period in four cultivars, 'Culter71', 'Clark', 'Aburamame', and 'Tamamusume': (a) crude oil content in late cropping season, (b) stearic acid content in middle cropping season, and (c) oleic acid content in early cropping season. The crude oil and fatty acid contents were expressed by mg and imol in 100mg soybean powder, respectively. d. Change of oleic acid proportion during the seed filling period in four cultivars, 'Cutler71', 'Clark', 'Aburamame', and 'Tamamusume'. Vertical bars indicate standard errors at each filling stage. Filling stages are 1=<20; 2=20-50; 3=50-100; 4=100-200; 5=200-300; 6=300-400; 7=400-500; 8=>500 mg/seed respectively, and 'M' indicates mature seeds.

20

**Table 2.2** Change of total fatty acid content during the seed filling period in four cultivars

| Sowing date | Cultivar | Fatty acid content (mg/100mg seed) | | |
|---|---|---|---|---|
| | | 1-4[†] | 4-6 | 6-8 |
| May 27 | Cutler71 | 1.81 a[¶] | 3.08 a | -0.13 a |
| | Clark | 1.00 b | 3.49 b | 0.60 a |
| | Aburamame | 0.99 b | 2.22 c | 0.97 a |
| | Tamamusume | 1.55 c | 3.33 ab | -0.26 a |
| June 11 | Cutler71 | 1.83 a | 3.29 a | -0.34 a |
| | Clark | 1.59 ab | 2.55 a | 1.84 b |
| | Aburamame | 1.18 c | 0.46 b | 2.47 b |
| | Tamamusume | 1.27 bc | 3.76 a | 2.49 b |
| June 26 | Cutler71 | 1.44 a | 4.67 a | 0.01 a |
| | Clark | 1.46 a | 3.07 b | 0.60 a |
| | Aburamame | 1.24 a | 0.65 c | 0.97 b |
| | Tamamusume | 3.35 b | 4.55 a | -0.26 c |

[†] Filling stage: 1= <20; 2 = 20-50; 3 = 50-100; 4 = 100-200; 5 = 200-300; 6 = 300-400; 7: 400-500, 8: >500 mg/seed.

[¶] Values followed by the same letter are not significantly different at 1% level.

linoleic, and α-linolenic acids, which were similar to those of their respective crude oil and total fatty acid in all cropping seasons. For the accumulation pattern of stearic acid, however, the four cultivars formed two distinct groups, high accumulation type (Type I: 'Cutler71' and 'Clark') and low accumulation type (Type II: 'Aburamame' and 'Tamamusume') after filling stage 5 (Fig. 2.1b), which significantly differed also in stearic acid content in mature seeds ($P<0.01$). Cropping season did not affect the stearic acid content.

**High oleic acid composition of 'Aburamame'**

Changes in the content and proportion of oleic acid during the seed filling period in early cropping season are shown in Fig. 2.1c and d, respectively. The four cultivars did not much differ about contents. After filling stage 5, however, 'Aburamame' showed a considerably higher oleic acid proportion than other cultivars: its increasing rate during filling stage 4 to 6 was 2.5 times higher than those of other cultivars. For the other fatty acids, 'Aburamame' showed a lower increasing rate during filling stage 4 to 6, which was observed in all the cropping seasons; therefore, the high proportion of oleic acid in 'Aburamame' was considered due to the lower accumulations of other fatty acids.

## 2.4 Discussion

In the present study, environmental fluctuations in fatty acid composition were observed. The cultivar 'Cutler71' and 'Clark' were considered unique in fatty acid composition in Chapter 1: the former showed a high linoleic acid composition, while the latter, high stearic acid composition (Table 2.1). In Chapter 2, however, there was

no significant difference in fatty acid composition between the two cultivars when grown in the same cropping season as Chapter 1. This was due to that 'Cutler71' showed higher stearic and lower linoleic acid composition in Chapter 2, while 'Clark', as well as 'Aburamame' and 'Tamamusume', showed stable fatty acid composition. This indicates the presence of genotype $\times$ environment interaction on fatty acid composition, as reported by Burton et al. (1983), Hawkins et al. (1983), Carmer and Beversdorf (1984) and Carver et al. (1986). In the present study, all the fatty acids showed different responses to cropping season from each other (Table 2.1). Among them, oleic acid composition showed the greatest fluctuation. The environmental fluctuation of oleic acid composition was greater than those of four other fatty acids because of its low heritability (Burton et al. 1983, Liu 1999). In 'Aburamame', we found only oleic acid composition to be fluctuated among cropping seasons. This fluctuation was greater than those of other cultivars with lower oleic acid composition.

Analysis of accumulation patterns in developing seeds could clarify the differences of the magnitude of fatty acid synthesis. In the present study, two types of stearic acid accumulation patterns were found. The content and proportion of stearic acid in mature seeds of Type I were both about twice higher than those of Type II respectively, which reflected well the difference in accumulation pattern. There are many reports identifying mutant gene(s) responsible for the fatty acid composition of soybean seeds: the high stearic acid proportions of three mutants induced by sodium azide or ethylmethane sulfonate treatment were found to be controlled by different recessive alleles at the same locus (Fas) (Graef et al. 1985a); and those of two

mutants induced by X-ray irradiation were controlled by recessive alleles at different

loci ($St_1$ and $St_2$) (Rahman et al. 1997). These mutants all showed twice to seven

times higher stearic acid proportion than their respective original cultivars.

Furthermore, Graef et al. (1985b) investigated the fatty acid composition of a

high-stearic mutant 'A6' during seed development, and suggested that its mutant

allele inhibits stearate desaturation or alters the substrate specificity for acyl-ACP

hydrolase. In the present study, the differences in stearic acid accumulation pattern

between Type I and Type II were observed from the middle of seed filling period

(seed weight: 300mg). This indicates that the gene expression and/or enzyme activity

controlling the stearic acid synthesis become remarkable at this stage.

The accumulation pattern of oleic acid was not different among the four

cultivars. For its proportion, 'Aburamame' showed a changing pattern different from

those of other cultivars. This indicates that the high oleic acid proportion in

'Aburamame' is caused by lower accumulation of other fatty acids: the enzymes

responsible for the syntheses of fatty acids except oleic acid may be fewer in amount

and/or less in activation in 'Aburamame'. So far, the conventional breeding methods

like recurrent selection (Wilson et al. 1981) and genetic engineering utilizing

co-suppression of *FAD2* (Fatty Acid Desaturase - 2) gene and its antisense RNA

(Heppard et al. 1996, Kinney 1998, Kinney and Knowlton 1998) have led to

extremely high oleic acid proportions in soybean oil (51% and more than 80%,

respectively). Compared to these lines, the oleic acid in 'Aburamame' is not

necessarily high in proportion. Nevertheless, this cultivar would be useful for

investigating the genetic factor(s) controlling the oleic acid composition for the

following reason.

Rahman et al. (1996) showed that the high oleic acid proportions of two mutants induced by X-ray irradiation were conferred by different alleles at the same locus (Ol), and that these alleles also controlled linoleic acid content by blocking the desaturation of oleic acid. For the mechanism responsible for the change in fatty acid, several biochemical steps and glycerolipid biosynthesis may be influenced by genetic manipulation in developing seed (Roehm and Privett 1970, Wilson and Rinne 1978, Wilson 1987, Zanakis et al. 1994, Nielsen 1996). The present study showed that the high proportion of oleic acid in 'Aburamame' might be attributed to the promotion of the desaturation step from stearic to oleic acid rather than the blocking of the desaturation step from oleic to linoleic acid. This suggests the existence of one or more gene(s) in 'Aburamame' enhancing the activity of enzyme(s) responsible for the desaturation step from stearic to oleic acid.

With emphasis on the accumulation pattern in developing seeds, we found genetically elevated stearic and oleic acid profiles, which have been targeted to improve soybean oils due to high nutritional and oxidative stability values. Most of the genetic analyses of seed fatty acids in soybean have been performed as to their proportions in mature seeds. But the present study showed that the analysis on fatty acid content in developing soybean seeds explains the latent genetic variations of fatty acids, and eventually could afford a clue to elucidate the regulation system of enzymes involved in the elongation and desaturation of fatty acids, which would be useful for quantitative and qualitative improvement not only of soybean oil but also of other crop oils.

# Chapter 3. Expression of Fatty Acid Desaturase Genes

## 3.1 Introduction

In most oilseed crops, such as soybean, canola, sunflower and safflower, oils predominantly containing 18-carbon unsaturated fatty acids are stored mainly in oil bodies. The synthetic pathways of triacylglycerol (TAG) in *Arabidopsis* are summarized in Fig 3.1. These pathways are considered to be present also in other oilseed crops; therefore, they could provide an excellent framework for discussing lipid metabolisms in many different species (Browse and Somerville 1991, Miquel and Browse 1994).

The first gene cloned with chromosome walking (map-based cloning) in higher plants is *Arabidopsis FAD3*, which encodes the endoplasmic reticulum 18:2 desaturase (Arondel et al. 1992). *Arabidopsis FAD3* gene was also cloned by T-DNA tagging. Soybean *FAD3* gene was isolated by screening of soybean cDNA library using *Arabidopsis FAD3* as a probe (Yadav et al. 1993). Chen et al. (1995) isolated a cDNA clone of soybean stearoyl-ACP desaturase (*SACPD*) by low stringency hybridization screening of cDNA library prepared from mRNA of soybean seedlings. The probe used for the hybridization screening was stearoyl-ACP desaturase cDNA of castor bean (Shanklin and Somerville 1991). *SACPD* encodes a principle enzyme necessary for the conversion of saturated fatty acids to unsaturated fatty acids. On the other hand, *Arabidopsis FAD2* gene was cloned by a T-DNA insertional mutagenesis approach (Okuley et al. 1994). Using *Arabidopsis FAD2* cDNA as a probe, Heppard et al. (1996) isolated two soybean *FAD2* genes (*FAD2-1* and *FAD2-2*), which are

Plastid

C16:0-ACP $\longrightarrow$ C18:0-ACP $\boxed{SACPD}$ C18:1-ACP

C16:0-CoA ( Palmitic acid)   C18:0-CoA(Stearic acid)   C18:1-CoA ( Oleic acid)

Endoplasmic
reticulum

Acyl-CoA Pool

Desaturation pathway                                    Kennedy pathway

C18:1-CoA ( Oleic acid)                                  Lysophosphatidic acid

C18:1-PdtC                                               Phosphatidic acid

$\boxed{FAD2}$

C18:2-PdtC $\longrightarrow$ C18:2-CoA                    Diacylglycerol
( Linoleic acid)

$\boxed{FAD3}$                                            $\boxed{DGAT}$

C18:3-PdtC $\longrightarrow$ C18:3-CoA                    Triacylglycerol
( Linolenic acid)

Fig. 3.1 Triacylglycerol biosynthesis in the developing soybean seeds. ACP: acyl carrier protein, CoA: coenzyme A, PdtC: phosphatidylcholine. SACPD: Stearoyl-ACP desaturase, FAD2: Fatty acid desaturase-2, FAD3: Fatty acid desaturase-3, DGAT: Diacylglycerol acyltransferase. Kinney (1997).

nonallelic to each other, by screening of soybean cDNA library. These isolated genes are known to encode enzymes related to fatty acid modification, and to determine the fatty acid composition of soybean seeds.

In this chapter, mRNA transcription levels of *SACPD*, *FAD2-1*, *FAD2-2* and *FAD3*, and genomic polymorphisms at the *FAD2-1* and *FAD2-2* loci were investigated using three cultivars with specific fatty acid composition.


## 3.2 Materials and Methods

**Plant materials and sampling methods**

Three cultivars with specific fatty acid composition, 'Cutler71', 'Clark' and 'Aburamame', and one cultivar with the standard fatty acid composition 'Tamamusume' were used. Experimental design followed Chapter 2. One month after sowing, young leaves of each cultivar were harvested for DNA isolation. For RNA isolation, developing seeds of 'Cutler71' and 'Clark' were collected twice (30 and 50 days after flowering) from low, middle, and high nodes of the main stem, and those of 'Aburamame' and 'Tamamusume' were collected four times (20, 30, 40 and 50 days after flowering) from middle nodes of the main stem. Developing seeds were classified into three filling stages based on fresh weight per seed: 100, 300 and 500 mg/seed. All seeds collected were frozen in liquid nitrogen and were stored at -80℃. Mature seeds were also harvested.

**Northern analysis**

For northern analysis, total RNAs were extracted from developing seeds at three stages (100, 300 and 500 mg/seed) and mature seeds (M) using the modified

Phenol/SDS method. After electrophoresis on 1.0 % denatured agarose gel, total

RNAs were blotted onto a positively charged membrane (Hybond N+; Amercham,

UK) and were subjected to northern hybridization. Four probes, *SACPD* (Accession

No. L34346), *FAD2-1* (L43920), *FAD2-2* (L43921) and *FAD3* (P48625), which were

kindly provided by Saga University, Japan, were used. Labeling of probes and

northern hybridization procedures were performed using the DIG RNA labeling kit

and the DIG luminescent detection kit (Roche, Germany), respectively.

Hybridization was performed with the high SDS hybridization buffer.

**Southern analysis**

Total genomic DNAs were extracted from leaves using the CTAB method

(Murray and Thompson 1980) with slight modifications. Extracted genomic DNAs

were digested with two restriction enzymes, *Eco*RI and *Hind*III. The electrophoresis

was conducted on 1.0% agarose gel, and the DNAs were blotted onto a positively

charged membrane (Hybond N+; Amercham, UK) and were subjected to southern

hybridization. The same probes as those in the northern analysis were used. Labeling

of probes and southern hybridization procedures were performed using the AlkPhos

direct labeling kit and the AlkPhos detection system (Amersham, UK), respectively.

**PCR analysis**

A primer set for the PCR was constructed based on cDNA sequences of L43920,

which were registered as *FAD2-1* gene of *Glycine max* and included the nucleotide

sequences of 1,453bp from the cultivar 'Rye' (Heppard et al. 1996). PCR procedures,

using a pair of specific primers, were performed using rTaq DNA polymerase

(Toyobo Inc., Japan) as an enzyme for amplifying DNA segments. In PCR

procedures, the TP 2000 thermal cycler (Takara Biomedicals, Japan) was used. The PCR products were cut out of the gel after electrophoresis, and directly sequenced using an ABI PRISM Cycle Sequencing Kit and ABI 310 sequencer (Applied Biosystems, USA). To link up sequences, additional primers were designed on the basis of the DNA sequences obtained, and the same sequencing procedures as above were performed. Nucleotide sequences of each primer used in this study are shown in the Results.

## 3.3 Results

**Transcription of genes responsible for fatty acid modification**

The transcription levels of mRNAs of *SACPD*, *FAD2-1* and *FAD3* gradually increased with the development of seeds, and declined at maturity (Fig. 3.2), while that of *FAD2-2* appeared to increase slightly with the development of seeds. The transcription of *FAD2-2* was also found in mature seeds. There was no intervarietal difference in the transcription level of *SACPD*, and that of *FAD3* was not enough to detect an intervarietal variation (data not shown). Contrary, the transcription level of *FAD2-1* in 'Cutler71' was much lower than those of other cultivars during the seed filling period (Fig. 3.3). For *FAD2-2*, 'Aburamame' showed about a 50% lower transcription level during the seed filling stages from 300 to 500 mg/seed than three other cultivars.

**Southern analysis of genes responsible for fatty acid modification**

In the southern analysis, two distinct bands appeared when the genomic DNAs digested with *Hind*III and *Eco*RI were hybridized with *SACPD* and *FAD2-1* (data not
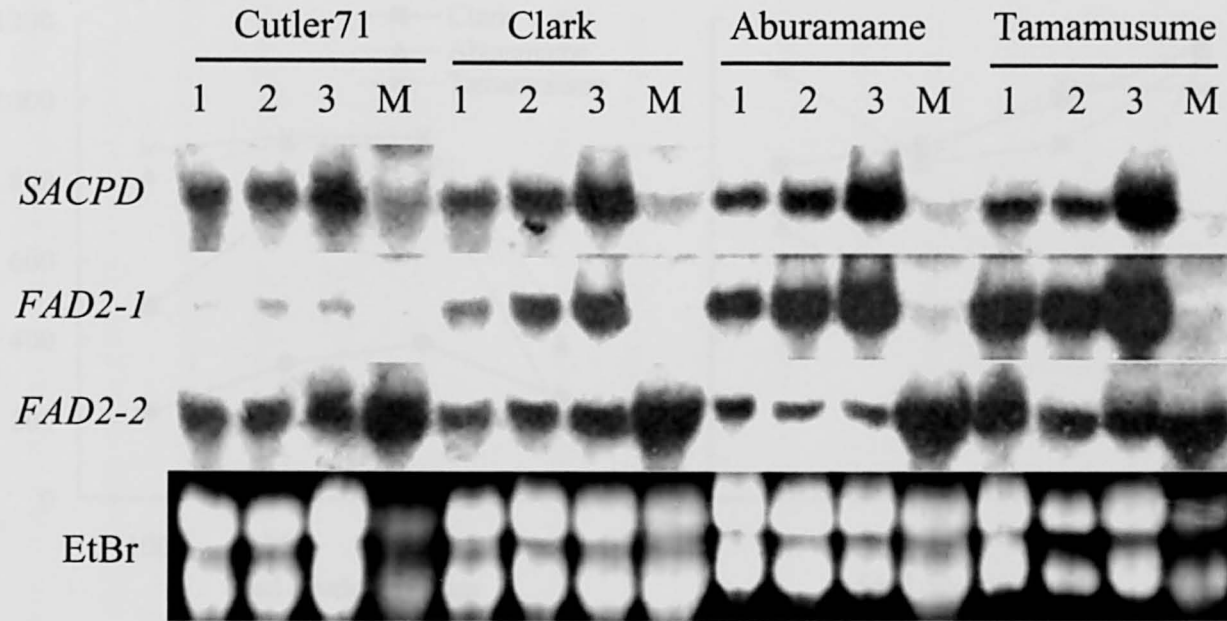
**Fig. 3.2** Northern blot analysis of the transcription of *SACPD*, *FAD2-1* and *FAD2-2* mRNAs in developing soybean seeds. EtBr: ethidium bromide staining of rRNA. 1 = 100 mg/seed stage: 2 = 300 mg/seed stage; 3 = 500 mg/seed stage; and M = dry mature seeds.
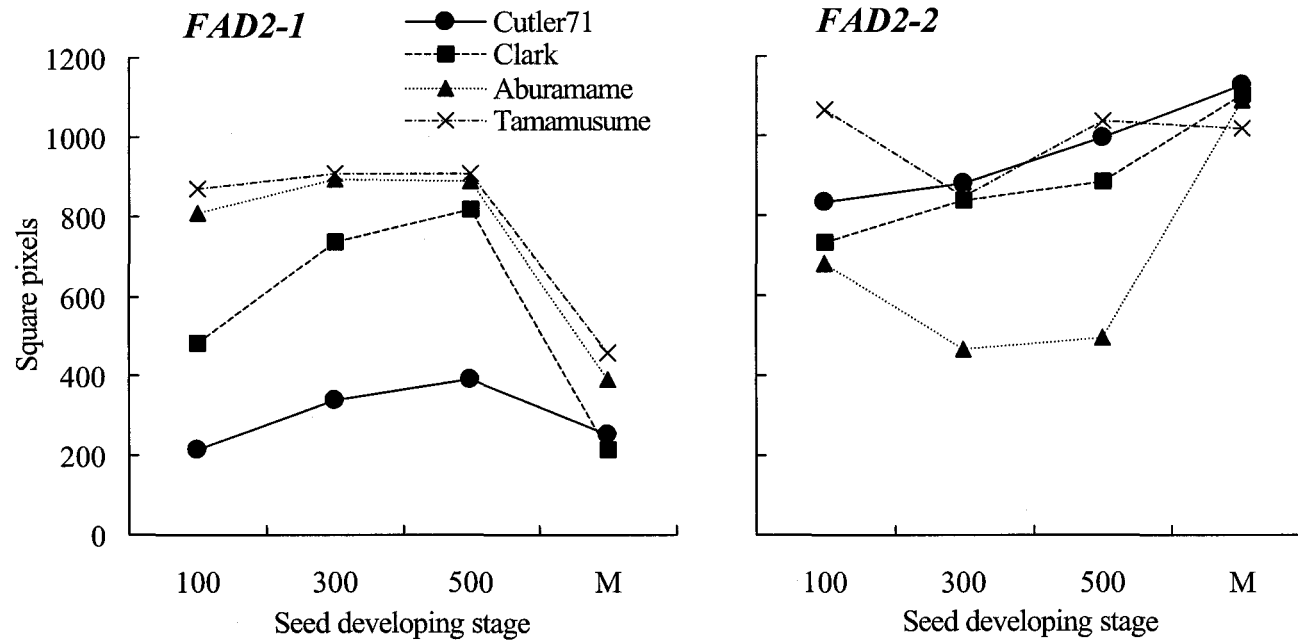
**Fig. 3.3** Intervarietal defferences of the transcription levels of *FAD2-1* and *FAD2-2* in developing soybean seeds. 100 = 100 mg/seed stage; 300 = 300 mg/seed stage; 500 = 500mg/seed stage; and M = dry mature seeds.
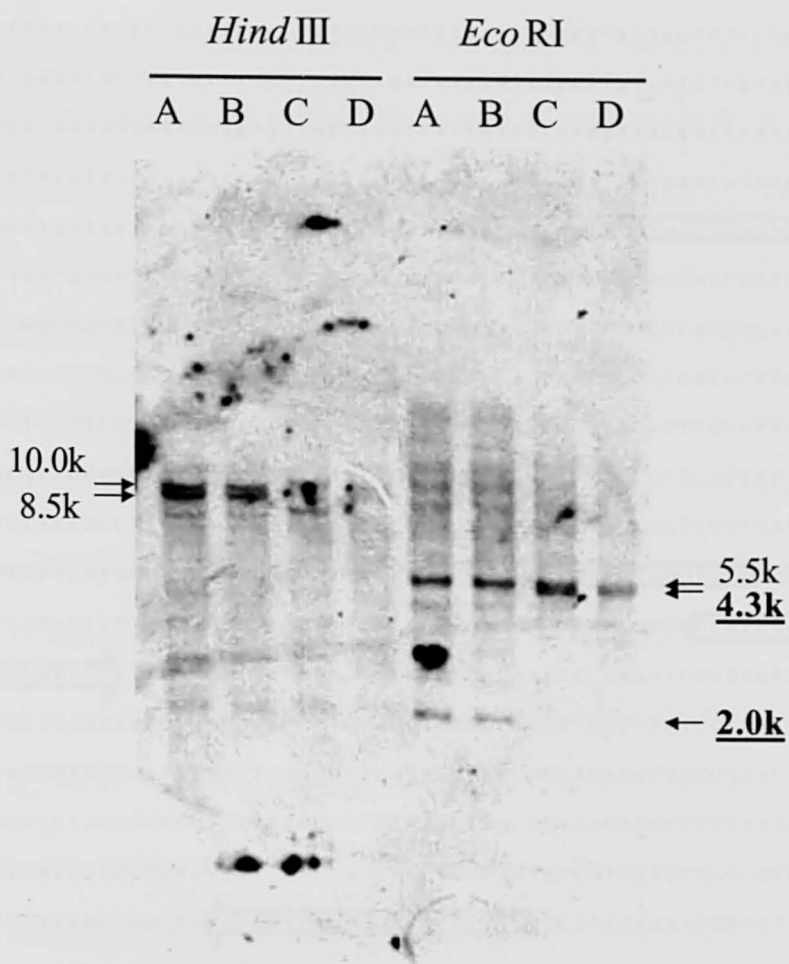
shown). When *Eco*RI-digested DNA was hybridized with *FAD2-1*, one of the two bands showed polymorphism among the four cultivars (Fig. 3.4): the 2.0 kb band was observed in the cultivar 'Cutler71' and 'Clark', while 4.3 kb band appeared in 'Aburamame' and 'Tamamusume'. The *Hind*III-digested DNA seemed to generate six bands when using *FAD2-2* and *FAD3* as probes. The *Eco*RI-digested DNA generated eight and four hybridizing bands when using *FAD2-2* and *FAD3* as probes, respectively. Some of the weakly hybridizing bands are considered due to incomplete DNA digestion and/or nonspecific hybridizations of probes.

**Genomic construction of *FAD2-1***

Fig. 3.5 shows the nucleotide sequence of *FAD2-1* in the cultivar 'Tamamusume'. The *FAD2-1* gene consisted of one intron (424bp), which existed just after the start codon, and two exons. An alignment of *FAD2-1* sequences of four cultivars revealed that there was no sequence polymorphism in both open reading frame and untranslated region.

# 3.4 Discussion

For fatty acid composition of soybean seeds, the genetic variations would be caused by the variations of the enzymes related to the fatty acid synthesis: the variations of enzymes could be expressed as the differences in their amounts and/or activities (Kinney 1998). One of the factors that determine the amounts of these enzymes would be the mRNA transcription level of related genes. Therefore, a northern analysis was first conducted to investigate the transcription levels of four genes, *SACPD*, *FAD2-1*, *FAD2-2* and *FAD3*, which play important roles for fatty acid

**Fig. 3.4** Genomic southern analysis of four soybean cultivars using *FAD2-1* as a probe. A = 'Cutler71'; B = 'Clark'; C = 'Aburamame'; and D = 'Tamamusume'.

1 CCATATACTAATATTTGCTTG[TATTGATAGCCCCTCCGTTCCC]AGAGTATAAAACTGCA
                          U1

61 TCGAATAATACAAGCCACTAGGC[ATG]gtaaattaaattgtgcctgcacctcgggatattt
                       Start

121 catgtggggttcatcatatttgttgaggaaaagaaactcccgaaattgaattatgcattt

181 atatatcctttttcatttctagatttcctgaaggcttaggtgtaggcacctagctagtag

241 ctacaatatcagcacttctctctattgataaacaattggctgtaatgccgcagtagagga

301 cgatcacaacatttccgtgctggttactttttgttttatggtcatgatttcactctctct

361 aatctctccattcattttgtagttgtcattatctttagatttttcactacctggctttaa

421 aattgagggattgtagttccggttggtacat[attacacattcagccaaacaac]gaaact
                                 U3

481 caactgaacttgtttatactttgacacagGGTCTAGCAAAGGAAACAACAATGGGAGGTA

541 GAGGTCGTGTGGCCAAAGTGGAAGTTCAAGGGAAGAAGCCTCTCTCAAGGGTTCCAAACA

601 CAAAGCCACCATTCACTGTTGGCCAACTCAAGAAAGCAATTCCACCACACTGCTTTCAGC

661 GCTCCCTCCTCACTTCATTCTCCTATGTTGTTTATGACCTTTCATTTGCCTTCATTTTCT

721 ACATTGCCACCACCTACTTCCACCTCCTTCCTCAACCCTTTTCCCTCATTGCATGGCCAA

781 TCTATTGGGTTCTCCAAGGTTGCCTTCTCACTGGTGTGTGGGTGATTGCTCACGAGTGTG

841 GTCACCATGCCTTCAGCAAGTACCAATC[GGTTGATGATGTTGTGGGTTTG]ACCCTTCACT
                                L3

901 CAACACTTTTAGTCCCTTATTTCTCATGGAAAATAAGCCATCGCCG[CCATCACTCCAACA
                                               U2

961 CAGGTTCC]TTGACCGTGATGAAGTGTTTGTCCCAAAACCAAAATCCAAAGTTGCATGGT

1021 TTTCCAAGTACTTAAACAACCCTCTAGGAAGGGCTGTTTCTCTTCTCGTCACACTCACAA

1081 TAGGGTGGCCTATGTATTTAGCCTTCAATGTCTCTGGTAGACCCTATGATAGTTTTGCAA

1141 GCCACTACCACCCTTATGCTCCCATATATTCTAACCGTGAGAGGCTTCTGATCTATGTCT

1201 CTGATGTTGCTTTGTTTTCTGTGACTTACTCTCTCTACCGTGTTGCAACCCTGAAAGGGT

1261 TGGTTTGGCTGCTA[TGTGTTTATGGGGTGCCTTTG]CTCATTGTGAACGGTTTTCTTGTGA
                               L2

1321 CTATCACATATTTGCAGCACACACACTTTGCCTTGCCTCATTACGATTCATCAGAATGGG

1381 ACTGGCTGAAGGGAGCTTTGGCAACTATGGACAGAGATTATGGGATTCTGAACAAGGTGT

1441 TTCATCACATAACTGATACTCATGTGGCTCACCATCTCTTCTCTACAATGCCACATTACC

1501 ATGCAATGGAGGCAACCAATGCAATCAAGCCAATATTGGGTGAGTACTACCAATTTGATG

1561 ACACACCATTTTACAAGGCACTGTGGAGAGAAGCGAGAGAGTGCCTCTATGTGGAGCCAG

1621 ATGAAGGAACATCCGAGAAGGGCGTGTATTGGTACAGGAACAAGTATT[GAT]GGAGCAACC
                                                       Stop

1681 AATGGGCCA[AGTGGGAGTTATGGAAGTTTTG]TCATGTATTAGTACATAATTAGTAGAAT
                             L1

1741 GTTATAAATAAGTGGATTTGCCGCGTAATGACTTTGTGTGTATTGTGAAACAGCTTGTTG

1801 CGATCATGGTTATAATGTAAAAATAATTCTGGTATTAATTACATGTGGAAAGTGTTCTGC

1861 TTATAGCTTTCTGCCT

Fig. 3.5 Genomic sequence of *FAD2-1* of soybean cv. 'Tamamusume'. Arrows
indicate the primers used. Intron was expresed as lower case.

formation in developing soybean seeds.

*FAD2* gene encodes ω-6 fatty acid desaturase, which synthesize linoleic acid by desaturation of oleic acid in the endoplasmic reticulum. Therefore, *FAD2* would have an important role to determine oleic and linoleic acid compositions. Experimental results showed that the low expression level of *FAD2-2* possibly causes high oleic acid composition in 'Aburamame'. The low amount of ω-6 desaturase, which results from low transcription of *FAD2-2*, may prevent the synthetic step from oleic to linoleic acid. However, the low transcription level of *FAD2-1* was observed in 'Cutler71' that exhibited high linoleic acid composition. This discrepancy may be caused by the difference of enzyme activities of ω-6 desaturase encoded by *FAD2-1*. To confirm this aspect, it is necessary to detect isozymes different in ω-6 desaturase activities. For this purpose, the *FAD2-1* nucleotide sequences of four cultivars were determined. However, all the four cultivars showed the same sequence, indicating that there were no isozymes for *FAD2-1* and that polymorphic band detected by southern analysis, when *Eco*RI-digested DNA was hybridized with *FAD2-1*, would be caused by the hybridization of *FAD2-1* probe with other similar sequence. Thus, there was no evidence that the high linoleic acid in 'Cutler71' depended on the intensive action of *FAD2-1*.

Since the transcription level of mRNA was not necessarily consistent with the amount of enzyme encoded, investigation of the amounts and activities of the enzymes related to fatty acid syntheses should be needed. In most of higher plant cells, fatty acid desaturation processes take place on glycerolipid as substrate of desaturase, while in animal cells they take place on acyl-CoA as substrate. In most

cases, fatty acid desaturase related to glycerolipid exists in the membranes of organelles, and is difficult to be solubilized. Therefore, *in vitro* methods measuring the activity of plant fatty acid desaturase have not been established as yet. There are some reports describing the fatty acid desaturase activity in higher plants (Ohnishi and Yamada 1980, Stobart and Styme 1985), but preparing glycerolipid as substrate of desaturase is a laborious and time-consuming work.

Molecular approaches in this study revealed that there are some factors, other than desaturase examined, causing the genetic variation of fatty acid composition in soybean seeds. One of the most reliable factors is diacylglycerol acyltransferase (DGAT), which catalyzes the formation of triacylglycerol (see Fig. 3.1). Zou et al. (1999) reported that an *Arabidopsis* mutant line AS11 with reduced DGAT activity showed the fatty acid composition rich in 18:3 but poor in 20:1 and 18:1 and an elevated diacylglycerol level. This implies that diacylglycerol accumulates without DGAT activity and has more time in equilibrating with phosphatidylcholine. This could allow an enrichment in conversion of 18:1 to polyunsaturated 18-carbon fatty acids while esterified to phosphatidylcholine. Partial purification of DGAT from cotyledons of germinating soybean seeds has been reported (Kwanyuan and Wilson 1986), but the detailed molecular characterization of this enzyme has not been conducted as yet.

The establishment of the procedure of measuring activities responsible for fatty acid syntheses is urgent necessity to understand the perspective of fatty acid syntheses. This perspective would help us understand the genetic factors responsible for the great intervarietal variation in fatty acid composition.

# Chapter 4. Algorithmic Analysis of Fatty Acid Flux in Developing Seeds

## 4.1 Introduction

It is well documented that temperatures during the seed-filling period influence the contents of fatty acids, particularly of the unsaturated fatty acids. Kane et al. (1997) reported that high temperatures during the seed-filling period increased the contents of total oil and oleic acid, although reducing that of $\alpha$-linolenic acid. Furthermore, it is known that the contents of some of the fatty acids are influenced by genotype $\times$ environment (GE) interactions, such as genotype $\times$ location, genotype $\times$ year and genotype $\times$ location $\times$ year (Hawkins et al. 1983). Thus many studies have indicated the influences of environments and GE interactions on fatty acid composition, but little is known about their biochemical mechanism(s).

Kinney (1997) showed a biosynthetic pathway of triacylglycerol, a storage form of fatty acid, in developing soybean seeds as follows: first, palmitoyl-ACP is produced by the combined activity of acetyl-CoA carboxylase with fatty acid synthase in the plastid; subsequently, palmitoyl-ACP is converted into stearoyl-ACP by elongase, which is mostly modified into oleoyl-ACP by desaturase; further modification of oleic acid, combined with phosphatidylcholine, advances in the endoplasmic reticulum, and thereby linoleic acid and subsequently $\alpha$-linolenic acid are synthesized in seeds. Such complex nature of fatty acid synthetic pathway makes it difficult to investigate the biochemical effects of environments and GE interactions on fatty acid composition. In this sense, a dynamic analysis of fatty acid flux at each

biosynthetic step from palmitic to α-linolenic acid during the seed-filling period under different environments seems to provide plenty of useful information about the environmental effects and GE interactions on the biochemical pathway of fatty acid synthesis.

In the present study, effects of environments, temperature, amount of solar radiation and precipitation, on fatty acid composition in mature seeds were first investigated using three cultivars with specific fatty acid composition and one cultivar with the standard composition. Subsequently, simple novel algorithm was devised and applied to estimate the fatty acid fluxes in developing seeds. Based on the results, environmental and genetic effects on the fatty acid biosynthetic pathway were discussed.

## 4.2 Materials and Methods

**Plant materials and cultivation**

Four soybean cultivars, 'Cutler71', 'Clark', 'Aburamame' and 'Tamamusume', were used. The first three cultivars exhibit unique fatty acid composition: 'Cutler71', high linoleic acid; 'Clark', high stearic acid; 'Aburamame', high oleic acid composition. In contrast, 'Tamamusume' exhibits the standard fatty acid composition. 'Cutler71' and 'Clark' are American commercial cultivars with an indeterminate growth habit, and 'Aburamame' and 'Tamamusume' are Japanese native and Japanese commercial cultivars respectively, both of which have a determinate growth habit.

These four cultivars were grown in an experimental field at Kyoto Univ., Kyoto, Japan, in 1998 and 1999. Seeds were sown in pots containing field soil on May 27,

39

June 11 and June 26, 1998, for early, middle and late cropping seasons respectively, and seedlings were transplanted on June 18, July 3 and July 17, 1998, respectively. In 1999, seeds were sown on May 14, June 11 and July 8, for early, middle and late cropping seasons respectively, and seedlings were transplanted on June 5, July 6 and July 26, respectively. In both years, experiment was conducted using a randomized complete block design with two replications (10 plants/plot), and planting density was $50 \times 20$cm. Three agronomic traits, number of days from sowing to flowering (DF), number of days from flowering to maturity (DM) and 100 air-dried seed weight, were measured for each plant. Flowering date was recorded when an open flower was found on the main stem, and maturing date was recorded when 95% of normal pods on the main stem showed mature pod color. Meteorological data (temperature, amount of solar radiation and precipitation) in the experimental field were kindly given by Laboratory of Irrigation, Drainage and Hydrological Environment Engineering, Kyoto Univ.

**Sampling methods**

In each cropping season, immature seeds were collected four times (20, 30, 40 and 50 days after flowering) from middle nodes of the main stem. According to Fehr et al. (1971), the reproductive phase of soybean after flowering is divided into eight growth stages (R1-R8) and the seed-filling period corresponds to the period from R5 to R6. Since seed mass rapidly increases during this period, we classified immature seeds into the following seed-filling stages based on fresh weight per seed: <20, 20-50, 50-100, 100-200, 200-300, 300-400, 400-500, >500 mg/seed stages in 1998, and 50-100, 100-150, 150-200, 200-250, 250-300, 300-350, 350-400, 400-450,

40

450-500 mg/seed stages in 1999. All the seeds collected were frozen in liquid nitrogen and were stored at -80°C. The seed-filling stages ~50 and ~500 mg/seed were approximately consistent with growth stages R5 and R6, respectively. Mature seeds were also harvested.

**Fatty acid analysis**

Immature seeds at each seed-filling stage and mature seeds were subjected to a fatty acid analysis using the method of Takagi et al. (1989) with minor modifications. Freeze-dried seeds at each seed-filling stage were ground in a mortar with a pestle, and 100mg of soybean powder were prepared (two replications). Before oil extraction, soybean powder was boiled in 99.5% 2-propanol to ensure the inactivation of lipolytic enzymes. Methylesters of fatty acids were prepared by intereseterification using sodium methoxide with adding triheptadecanoin (17:0) as an internal standard before esterification, and were separated into five fatty acids with a gas chromatograph GC-17A (Shimadzu, Japan). Each fatty acid was calibrated by micromole using authentic fatty acid methyl ester (Sigma, USA). Fatty acid contents were measured three times for each replication.

**Algorithmic analysis**

We estimated the amounts of fatty acid fluxes from palmitic to stearic acid ($c_n$), from stearic to oleic acid ($e_n$), from oleic to linoleic acid ($g_n$) and from linoleic to α-linolenic acid ($i_n$) during the time $t_n$ to $t_{n+1}$ using the following algorithm (cf. Fig. 4.1),

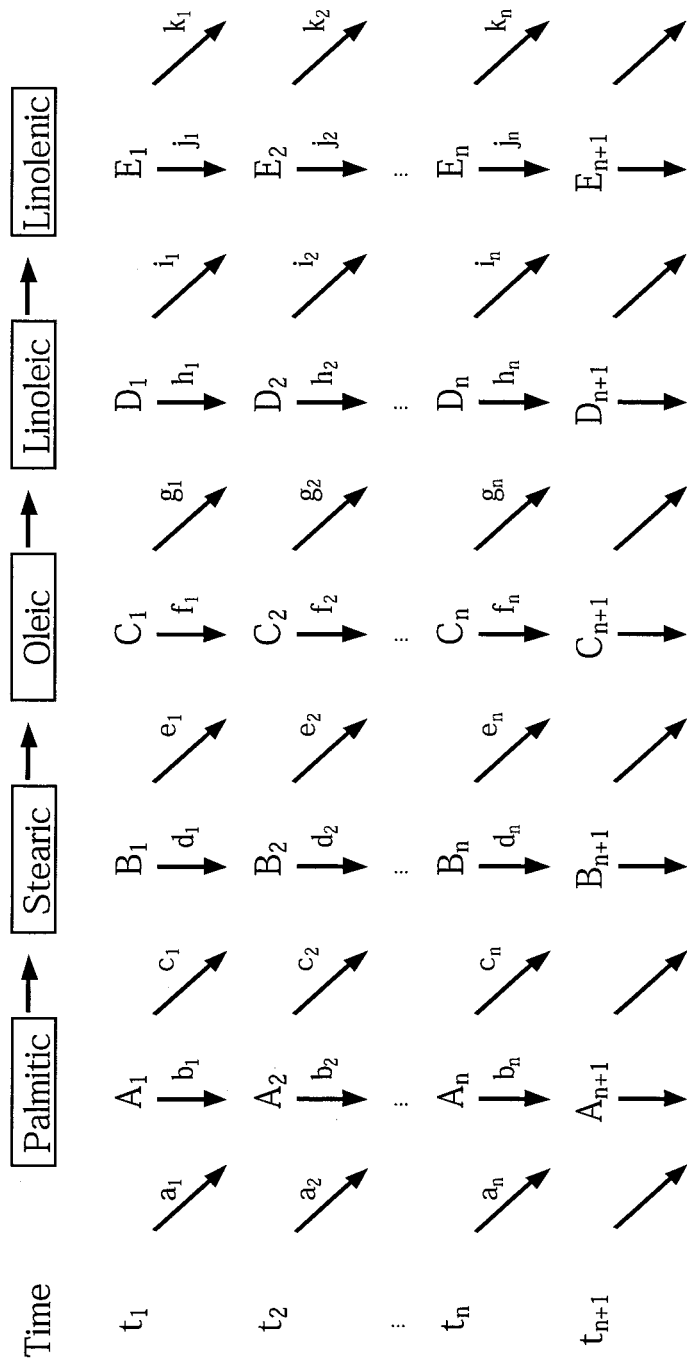$$c_n = a_n + A_n - A_{n+1}$$

$$e_n = c_n + B_n - B_{n+1}$$

**Fig. 4.1** Model of algorithm for fatty acid flux in developing soybean seeds.

$$g_n = e_n + C_n - C_{n+1}$$

$$i_n = g_n + D_n - D_{n+1}$$

where

$$A_n = b_n + c_n, \quad A_{n+1} = a_n + b_n$$

$$B_n = d_n + e_n, \quad B_{n+1} = c_n + d_n$$

$$C_n = f_n + g_n, \quad C_{n+1} = e_n + f_n$$

$$D_n = h_n + i_n, \quad D_{n+1} = g_n + h_n$$

$$E_n = j_n + k_n, \quad E_{n+1} = i_n + j_n$$

$A_n$, $B_n$, $C_n$, $D_n$ and $E_n$ indicate the contents of palmitic, stearic, oleic, linoleic and $\alpha$-linolenic acid at the time $t_n$ obtained from the fatty acid analysis, respectively; and $a_n$ indicates the content of palmitic acid modified from myristic acid (14:0); and $b_n$, $d_n$, $f_n$, $h_n$ and $j_n$ indicate the contents of fatty acids not modified to the next, respectively; and $k_n$ indicates the content of a fatty acid that was degraded. $c_n$, $e_n$, $g_n$ and $i_n$ can be uniquely determined when the amount of $a_n$ is given. In this study, $a_n$ was set at the difference of the total fatty acid content at the time $t_{n+1}$ from that of $t_n$, but when the total fatty acid content at $t_{n+1}$ was smaller than that at $t_n$, $a_n$ was set at 0.


## 4.3 Results

**Flowering and maturity habits of used cultivars**

All the four cultivars almost simultaneously flowered early, middle and late in July for early, middle and late cropping seasons, respectively, but their maturing dates did not differ much among cropping seasons. Similar tendency was observed in 1999: all the cultivars almost simultaneously flowered early in July, late in July and

middle in August for early, middle and late cropping seasons, respectively, and their maturing dates were similar among cropping seasons. In both years, not only DF but also DM decreased with the delay of cropping season (Table 4.1). The difference in DF was more remarkable between middle and late cropping season than between early and middle cropping season, while the difference in DM was clearer between early and middle cropping season than between middle and late cropping season.

**Fatty acid composition in mature seeds**

The contents of all the fatty acids showed great differences among cropping seasons and among years, and their contents were all higher in middle cropping season than in others in both years (Table 4.2). The contents of all the fatty acids, except palmitic acid, showed greater differences in percentage to the total fatty acid content among genotypes than among cropping seasons and among years (Table 4.3). The percentages of palmitic and stearic acids significantly differed among years. Significant differences among seasons and significant effect of year×cultivar were detected for the percentages of oleic, linoleic and α-linolenic acids.

**Environmental effects on fatty acid composition**

It is known that soybean seed components, such as protein and oil contents, are influenced markedly by the duration of the seed-filling period (Hanson 1992, Salados-Navarron et al. 1985). Therefore, to analyze the meteorological effect on fatty acid composition, we divided the whole developmental period of soybean into two phases, the pre-flowering developmental phase and the post-flowering developmental phase. Mean temperatures, amounts of solar radiation and precipitations during the two developmental phases in all the cropping seasons are

44

**Table 4.1** Flowering and maturity habits of accession cultivars used

| Cropping season | Sowing date | Cutler71 | | Clark | | Aburamame | | Tamamusume | |
|---|---|---|---|---|---|---|---|---|---|
| | | $DF^{\dagger}$ | DM | DF | DM | DF | DM | DF | DM |
| 1998 | | | | | | | | | |
| Early | May 27 | 41 | 107 | 39 | 109 | 48 | 82 | 46 | 70 |
| Middle | June 11 | 40 | 96 | 38 | 98 | 44 | 74 | 43 | 69 |
| Late | June 26 | 32 | 84 | 32 | 81 | 38 | 69 | 34 | 66 |
| 1999 | | | | | | | | | |
| Early | May 14 | 48 | 110 | 46 | 112 | 54 | 95 | 55 | 85 |
| Middle | June 11 | 46 | 84 | 44 | 86 | 48 | 73 | 46 | 66 |
| Late | July 8 | 34 | 75 | 34 | 69 | 35 | 63 | 34 | 64 |

$^{\dagger}$ DF = number of days to flowering after sowing, DM = number of days to maturity after flowering.

**Table 4.2** Fatty acid contents and percentages in mature soybean seeds

| Cultivar | Year | Cropping season | Fatty acid content [umol/g] | | | | | Fatty acid percentage | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Palmitic | Stearic | Oleic | Linoleic | Linolenic | Palmitic | Stearic | Oleic | Linoleic | Linolenic |
| Cutler71 | 1998 | Early | 58 | 34 | 92 | 314 | 43 | 10.7 | 6.3 | 17.0 | 58.1 | 7.9 |
| | | Middle | 82 | 42 | 120 | 432 | 65 | 11.1 | 5.6 | 16.2 | 58.3 | 8.8 |
| | | Late | 60 | 27 | 90 | 314 | 44 | 11.1 | 5.0 | 16.9 | 58.7 | 8.2 |
| | 1999 | Early | 101 | 24 | 116 | 293 | 42 | 17.5 | 4.2 | 20.1 | 50.9 | 7.3 |
| | | Middle | 123 | 32 | 132 | 360 | 63 | 17.4 | 4.5 | 18.6 | 50.7 | 8.9 |
| | | Late | 68 | 18 | 62 | 217 | 38 | 16.8 | 4.5 | 15.4 | 53.8 | 9.5 |
| Clark | 1998 | Early | 75 | 47 | 115 | 405 | 56 | 10.8 | 6.7 | 16.4 | 58.1 | 8.0 |
| | | Middle | 96 | 49 | 155 | 494 | 74 | 11.1 | 5.6 | 17.8 | 56.9 | 8.5 |
| | | Late | 58 | 22 | 73 | 316 | 46 | 11.2 | 4.3 | 14.2 | 61.3 | 9.0 |
| | 1999 | Early | 93 | 22 | 103 | 274 | 45 | 17.4 | 4.1 | 19.3 | 50.9 | 8.3 |
| | | Middle | 141 | 38 | 154 | 406 | 70 | 17.5 | 4.7 | 19.0 | 50.2 | 8.6 |
| | | Late | 75 | 21 | 66 | 221 | 39 | 17.8 | 4.9 | 15.7 | 52.4 | 9.3 |
| Aburamame | 1998 | Early | 48 | 16 | 164 | 198 | 32 | 10.5 | 3.5 | 35.7 | 43.2 | 7.0 |
| | | Middle | 72 | 24 | 193 | 299 | 53 | 11.2 | 3.7 | 30.1 | 46.6 | 8.3 |
| | | Late | 63 | 16 | 126 | 305 | 53 | 11.2 | 2.9 | 22.4 | 54.2 | 9.3 |
| | 1999 | Early | 84 | 13 | 245 | 167 | 31 | 15.6 | 2.4 | 45.4 | 31.0 | 5.7 |
| | | Middle | 118 | 19 | 318 | 256 | 49 | 15.6 | 2.5 | 41.8 | 33.7 | 6.4 |
| | | Late | 80 | 13 | 175 | 184 | 36 | 16.5 | 2.7 | 35.8 | 37.7 | 7.3 |
| Tamamusume | 1998 | Early | 44 | 14 | 90 | 195 | 33 | 11.7 | 3.7 | 23.9 | 51.8 | 8.9 |
| | | Middle | 94 | 28 | 134 | 423 | 75 | 12.5 | 3.7 | 17.8 | 56.1 | 10.0 |
| | | Late | 50 | 13 | 84 | 242 | 38 | 11.7 | 3.0 | 19.6 | 56.8 | 8.9 |
| | 1999 | Early | 118 | 20 | 121 | 333 | 64 | 18.0 | 3.0 | 18.5 | 50.7 | 9.8 |
| | | Middle | 125 | 17 | 159 | 322 | 60 | 18.3 | 2.5 | 23.3 | 47.2 | 8.8 |
| | | Late | 101 | 15 | 134 | 270 | 51 | 17.7 | 2.7 | 23.5 | 47.3 | 8.9 |

Table 4.3 Analysis of variance for fatty acid percentages in mature seeds

| Source | D.F. | Palmitic | | Stearic | | Oleic | | Linoleic | | Linolenic | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | MS | F | MS | F | MS | F | MS | F | MS | F |
| Cultivar | 3 | 9.34 | 12.06 ** | 15.32 | 25.76 ** | 1029.63 | 125.98 ** | 573.34 | 106.17 ** | 8.82 | 28.38 ** |
| Year | 1 | 17.66 | 22.81 ** | 2.74 | 4.61 * | 16.42 | 2.01 | 1.80 | 0.33 | 0.21 | 0.69 |
| Cropping season | 2 | 1.10 | 1.42 | 0.52 | 0.87 | 77.97 | 9.54 ** | 43.06 | 7.97 ** | 4.48 | 14.41 ** |
| Year × Cultivar | 3 | 0.17 | 0.22 | 0.05 | 0.08 | 37.10 | 4.54 * | 23.88 | 4.42 * | 1.21 | 3.89 * |
| Year × Cropping season | 2 | 0.85 | 1.10 | 1.48 | 2.49 | 11.52 | 1.41 | 1.93 | 0.36 | 0.70 | 2.25 |
| Cultivar × Cropping season | 6 | 0.95 | 1.23 | 0.10 | 0.16 | 12.91 | 1.58 | 7.09 | 1.31 | 0.47 | 1.51 |
| Year × Cultivar × Cropping season | 6 | 0.66 | 0.85 | 0.32 | 0.54 | 3.47 | 0.42 | 1.64 | 0.30 | 0.17 | 0.55 |
| Error | 24 | 0.77 | | 0.60 | | 8.17 | | 5.40 | | 0.31 | |

* and ** Significant at the 5 and 1% levels, respectively.

47

shown in Table 4.4. Palmitic and oleic acid percentages showed a significant positive correlation with mean temperature during the post-flowering phase (Fig. 4.2). In contrast, stearic and linoleic acid percentages showed a significant negative correlation with mean temperature. But $\alpha$-linolenic acid was not correlated with mean temperature. The amount of precipitation during the post-flowering phase positively affected only the percentage of stearic acid ($r = 0.593^{**}$). The relationship between fatty acid percentage and amount of solar radiation was not recognized in all the five fatty acids. The oleic acid percentage in 'Aburamame' showed a great variation among cropping seasons and among years. In 'Aburamame', the oleic acid percentage tended to increase with the increase of temperature.

**Algorithmic analysis of fatty acid flux**

As a result of the algorithmic analysis, 'Clark' was found to exhibit the most distinct pattern of fatty acid flux. The fatty acid fluxes in developing seeds of 'Clark' grown in middle cropping season, 1999 are illustrated in Fig. 4.3. The flux at the second fatty acid biosynthetic step, from stearic to oleic acid of the fatty acid, was almost synchronized with that of the first step, from palmitic to stearic acid. In these two steps, there were two active phases and one inactive phase. The inactive phase was at around 300-400 mg/seed stages. The flux pattern at the third step, from oleic to linoleic acid, was almost the same as those of the first two steps, although the amount was lower than those of the first two as a whole. At the last step, from linoleic to $\alpha$-linolenic acid, there was only one active phase at around the 300 mg/seed stage, and the total amount of flux was low.

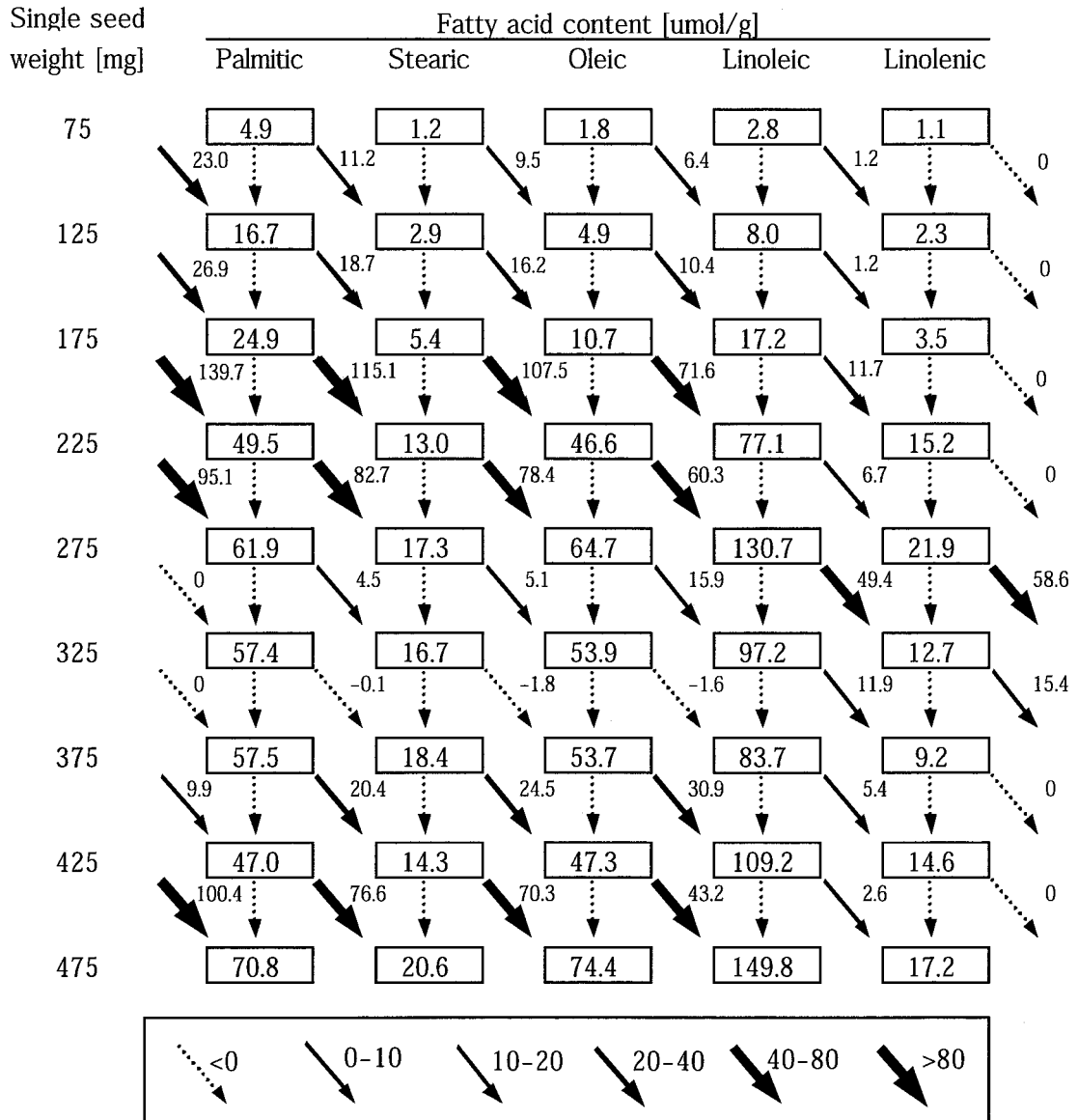**The fluctuations of fatty acid flux in developing seeds**

**Table 4.4** Meteorological data during cultivation of soybean in 1998 and 1999

| Year | Cropping Season | Cutler71 V[†] | R | Clark V | R | Aburamame V | R | Tamamusume V | R |
|---|---|---|---|---|---|---|---|---|---|
| | | Mean temperature [°C] | | | | | | | |
| 1998 | Early | 22.7 | 24.5 | 22.4 | 24.6 | 23.1 | 25.5 | 23.1 | 26.0 |
| | Middle | 24.4 | 24.2 | 24.4 | 24.2 | 24.6 | 25.4 | 24.6 | 25.8 |
| | Late | 26.0 | 24.5 | 26.0 | 24.7 | 26.3 | 24.8 | 26.1 | 25.5 |
| 1999 | Early | 21.0 | 26.3 | 21.0 | 26.3 | 21.1 | 27.3 | 21.2 | 26.1 |
| | Middle | 23.7 | 26.9 | 23.5 | 26.9 | 23.8 | 27.8 | 23.7 | 26.3 |
| | Late | 26.5 | 25.7 | 26.5 | 26.7 | 26.5 | 27.5 | 26.5 | 27.5 |
| | | Amount of solar radiation [MJ/m$^2$] | | | | | | | |
| 1998 | Early | 561 | 1252 | 530 | 1283 | 656 | 1028 | 620 | 967 |
| | Middle | 536 | 1086 | 518 | 1104 | 594 | 906 | 584 | 842 |
| | Late | 450 | 962 | 450 | 942 | 535 | 834 | 476 | 817 |
| 1999 | Early | 648 | 1337 | 641 | 1344 | 712 | 1212 | 733 | 1099 |
| | Middle | 575 | 975 | 544 | 1006 | 594 | 895 | 575 | 822 |
| | Late | 493 | 829 | 493 | 759 | 500 | 724 | 493 | 731 |
| | | Amount of precipitation [mm] | | | | | | | |
| 1998 | Early | 352 | 608 | 352 | 608 | 397 | 372 | 397 | 153 |
| | Middle | 318 | 542 | 318 | 542 | 318 | 371 | 318 | 344 |
| | Late | 99 | 520 | 99 | 493 | 104 | 358 | 102 | 333 |
| 1999 | Early | 432 | 545 | 346 | 631 | 465 | 500 | 465 | 446 |
| | Middle | 384 | 408 | 384 | 408 | 385 | 395 | 384 | 341 |
| | Late | 137 | 378 | 137 | 375 | 172 | 333 | 137 | 367 |

[†] V = pre-flowering developmental phase, R = post-flowering developmental phase.

49
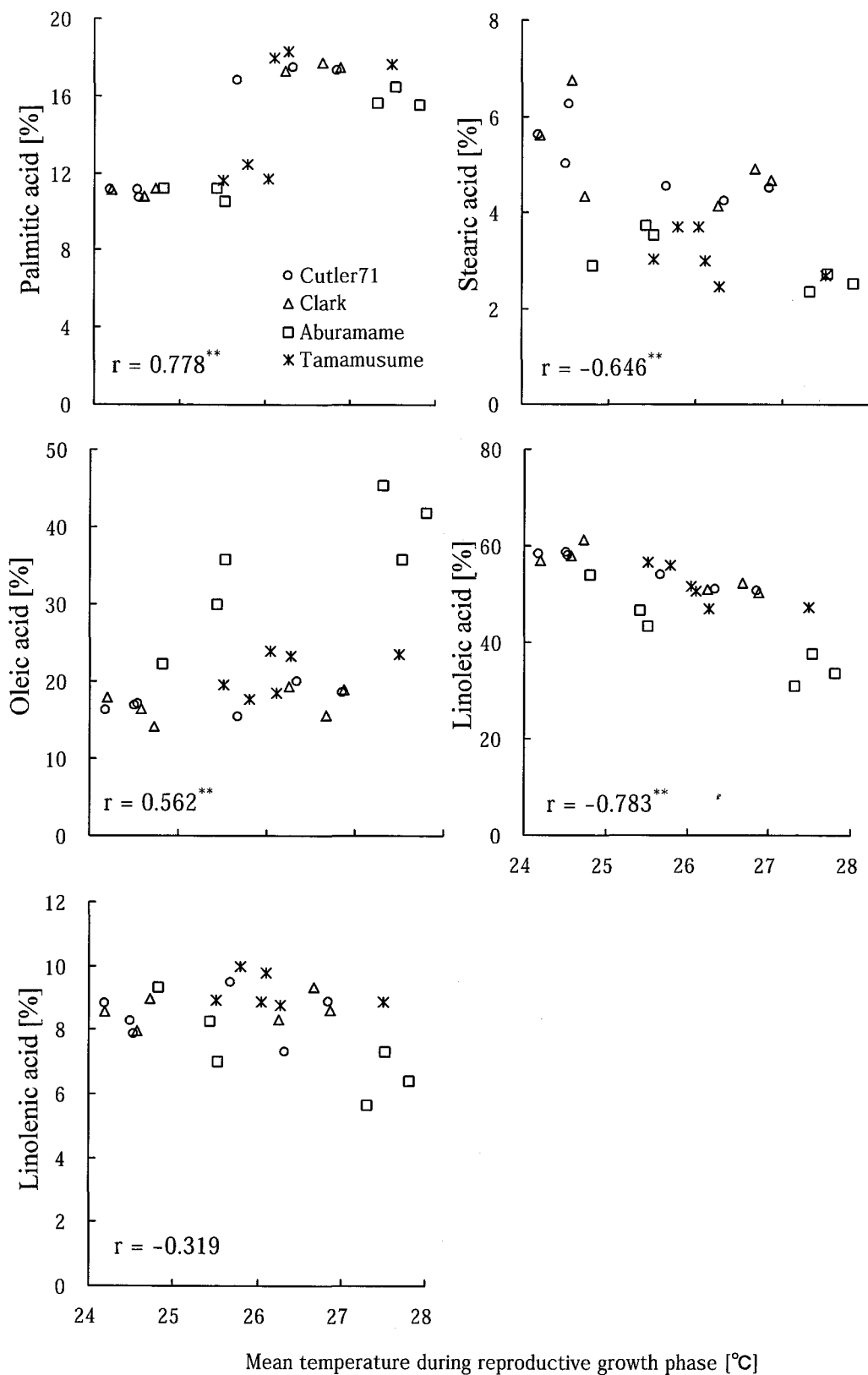
**Fig. 4.2** Relationships of fatty acid composition with mean temperature during the post-flowering developmental phase.

50

Single seed weight [mg] — Fatty acid content [umol/g]

| Single seed weight [mg] | Palmitic | Stearic | Oleic | Linoleic | Linolenic | |
|---|---|---|---|---|---|---|
| 75 | 4.9 | 1.2 | 1.8 | 2.8 | 1.1 | |
| | 23.0 | 11.2 | 9.5 | 6.4 | 1.2 | 0 |
| 125 | 16.7 | 2.9 | 4.9 | 8.0 | 2.3 | |
| | 26.9 | 18.7 | 16.2 | 10.4 | 1.2 | 0 |
| 175 | 24.9 | 5.4 | 10.7 | 17.2 | 3.5 | |
| | 139.7 | 115.1 | 107.5 | 71.6 | 11.7 | 0 |
| 225 | 49.5 | 13.0 | 46.6 | 77.1 | 15.2 | |
| | 95.1 | 82.7 | 78.4 | 60.3 | 6.7 | 0 |
| 275 | 61.9 | 17.3 | 64.7 | 130.7 | 21.9 | |
| | 0 | 4.5 | 5.1 | 15.9 | 49.4 | 58.6 |
| 325 | 57.4 | 16.7 | 53.9 | 97.2 | 12.7 | |
| | 0 | -0.1 | -1.8 | -1.6 | 11.9 | 15.4 |
| 375 | 57.5 | 18.4 | 53.7 | 83.7 | 9.2 | |
| | 9.9 | 20.4 | 24.5 | 30.9 | 5.4 | 0 |
| 425 | 47.0 | 14.3 | 47.3 | 109.2 | 14.6 | |
| | 100.4 | 76.6 | 70.3 | 43.2 | 2.6 | 0 |
| 475 | 70.8 | 20.6 | 74.4 | 149.8 | 17.2 | |

Legend: <0   0-10   10-20   20-40   40-80   >80

**Fig. 4.3** Schematic diagram of fatty acid flux in developing seeds of 'Clark' in middle cropping season in 1999.

51

Environmental and genetic variations in the amount and pattern of fatty acid flux were recognized at all the four steps (Fig. 4.4). Among the four steps, the first three showed similar patterns to each other within the same cultivar and the same cropping season, while the flux pattern at the step from linoleic to $\alpha$-linolenic acid fluctuated among cultivars and cropping seasons. At all the four steps, 'Clark' showed the most extreme changing pattern of flux. Although the responses of flux amount to environmental conditions were not the same among cultivars, the flux in early cropping season was low on the whole, and the active phase at the last step, from linoleic to $\alpha$-linolenic acid, tended to become late with the delay of cropping season in all the four cultivars. At the first three steps, the 'Cutler71' and 'Clark' showed similar patterns with a great fluctuation and two extremely active phases, while 'Aburamame' and 'Tamamusume' showed patterns with two ambiguously active phases. The amount of flux from linoleic to $\alpha$-linolenic was lower than those of the other three steps in all the cultivars and growing conditions, although genetic variation in flux pattern was recognized.

## 4.4 Discussion

Fatty acid composition in developing soybean seeds so far has been analyzed mostly using seeds at various seed-filling stages based on days after flowering (Cherry et al. 1984, Sangwan et al. 1986). In this study, however, the duration of the seed-filling period remarkably decreased with the delay of cropping season (Table 4.1), and all of the four cultivars used, without regard to cropping season, showed similar 100-seed weights (~22g) at maturity. Therefore, we investigated fatty acid

Palmitic -> Stearic  Stearic -> Oleic  Oleic -> Linoleic  Linoleic -> Linolenic

120 'Cutler71'

90

60

30

0

—⊖— Early
--⊟-- Middle
····△···· Late

120 'Clark'

90

60

30

0

Fatty acid flux [umol]

120 'Aburamame'

90

60

30

0

120 'Tamamusume'

90

60

30

0

75-  125- 175- 225- 275- 325- 375- 425-
125  175  225  275  325  375  425  475

Developing seed [mg/seed]
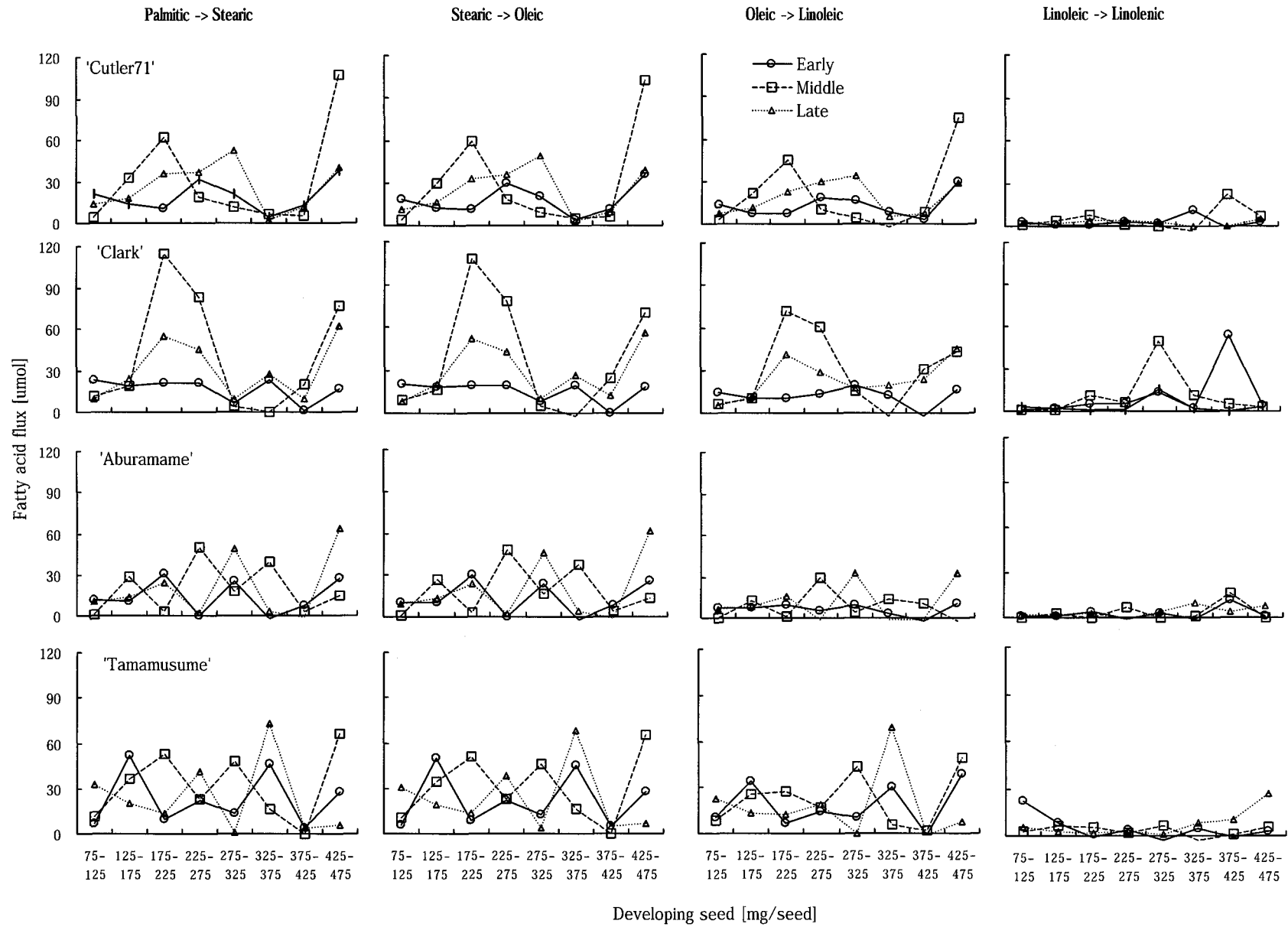
**Fig. 4.4** Differences of fatty acid flux in developing soybean seeds among genotype and among cropping seasons in 1999.

composition in developing seeds under different environmental and genetic conditions using the seed-filling stages based on seed weight.

In both years, all the cultivars showed the highest fatty acid content at maturity in the middle cropping season. Since the middle cropping season used is best for the commercial soybean production, this suggests that the right cropping season gives soybean plants appropriate durations of vegetative and reproductive growth phases, which brings about a good balance between sink and source. In this study, not only genetic and environmental effects but also GE interaction effects on fatty acid composition in mature seeds were detected. Palmitic and stearic acids showed great year-to-year variations, while oleic, linoleic, and $\alpha$-linolenic acids showed great seasonal variations. This indicates that the syntheses of the last three fatty acids are apt to be influenced by the duration of the seed-filling period, while those of the first two fatty acids are not affected by it. Burton et al. (1983) and Liu (1999) indicated that the amount of oleic acid exhibited a greater environmental variation than those of four others. The syntheses of linoleic and $\alpha$-linolenic acids depend on the amount of oleic acids. The GE interactions detected for the last three fatty acids result mainly from the marked environmental fluctuations of oleic acid content in 'Aburamame'.

Palmitic and oleic acid contents at maturity were positively correlated with mean temperature during the post-flowering developmental phase, while stearic and linoleic acid contents were negatively correlated with it (Fig. 4.2). This indicates that high temperatures during the post-flowering developmental phase enhance the syntheses of palmitic and oleic acids, while low temperatures enhance the syntheses of stearic and linoleic acids. According to previous reports, low temperatures during

the seed-filling period enhance the activity of an enzyme FAD2, which desaturates oleic acid to linoleic acid, even when the corresponding genes, *FAD2-1* and *FAD2-2*, normally manifest themselves (Harris and James 1969, Cheesbrough 1989, Heppard et al. 1996). Therefore, the negative correlation between the oleic acid content in mature seeds and temperature may be caused by the enhancing effect of low temperature on FAD2 activity. To date, there have been few reports describing the environmental fluctuation of stearic acid content. In this study, stearic acid content was found to be affected by temperature and precipitation during the post-flowering phase. Biochemical aspects of the enzyme responsible for the stearic acid synthesis are still unclear. Further investigation of the enzyme will help understand the mechanism of the enhanced synthesis of stearic acid under low temperature and high precipitation.

Simple algorithm was applied to verify the fatty acid flux during the seed-filling period. The changing pattern of the flux revealed the dynamics of fatty acid syntheses under different environmental and genetic conditions. The synchronized changes of two fluxes, from palmitic to stearic acid and from stearic to oleic acid, imply that the oleic acid is synthesized immediately after the synthesis of stearic acid, and that the flux from palmitic to linoleic acid infallibly ceases once at around the 350mg/seed stage and starts again at the last stage of seed development. The reason why the flux ceases once during the seed-filling period remains unknown. The delayed active-phase in the flux from linoleic to α-linolenic acid suggests that the synthesis of α-linolenic acid starts later than those of other fatty acids, and that the enzyme activity at this step was lower than those at other steps.

The fatty acid biosynthetic pathway is now well described with regard to related enzymes (Harwood 1996, Ohlrogge and Jaworski 1997); the three steps from stearic to oleic, from oleic to linoleic and from linoleic to $\alpha$-linolenic acid progress with stearoyl-ACP desaturase (SAD), $\omega$-6 fatty acid desaturase (FAD2), and $\omega$-3 fatty acid desaturase (FAD3), respectively. It is known that the activities of these desaturases are modified by several physiological factors, such as pH in the cell and substances that interact with the enzymes, and have optimal conditions (Cheesbrough 1990, Murphy and Piffanelli 1998). Experimental results showed that the active phase of SAD was consistent with that of FAD2 without regard to genotype, cropping season and year, and that the active phase of FAD3 was later than those of the other enzymes, including the unknown at the step from palmitic to stearic acid. It was also found that the activity of FAD3 was rather lower than those of the other enzymes. These results indicate that SAD and FAD2 require similar optimal conditions to be activated, while FAD3 requires optimal conditions different from those of the other enzymes. Furthermore, the time lags of active phases of SAD, FAD2 and FAD3 explain well the theoretical pathway of fatty acid synthesis: palmitic - stearic - oleic - linoleic - $\alpha$-linolenic acid (Kinney 1997). Therefore, the algorithm devised in this study is surely available for understanding the dynamics of fatty acid synthetic pathway.

The flux amount of fatty acid was lower in early cropping season than in other cropping seasons, which were common to both years. This indicates that low temperatures at early vegetative growth stage disturb sufficient growth of soybean plants. In addition, active phases of flux were different among cropping seasons,

indicating that the fatty acid synthesis is controlled not only by the developmental stage of seeds but also by environmental conditions such as temperature and amount of precipitation. In other words, the seed-filling stages optimal for related enzymes were different among cropping seasons. 'Cutler71' and 'Clark' showed similar fluxes to each other at all the steps. Thus the first two cultivars have almost the same genetic systems for fatty acid syntheses, while other cultivars have different systems.

Based on the accumulation patterns of fatty acids, Ishikawa et al. (2001) reported that the high oleic acid composition in 'Aburamame' might be attributed to the promotion of the desaturation step from stearic to oleic acid. Experimental results, however, showed that the FAD2 activity were lower in 'Aburamame' than in three other cultivars. It is therefore considered that the high oleic acid in 'Aburamame' is caused not by the promotion of stearic acid desaturation but by the blocking of the desaturation step from oleic to linoleic acid. A great environmental fluctuation of oleic acid percentage in 'Aburamame' might be caused by the blocking of FAD2, because the environmental fluctuations of palmitic and stearic acids will be inevitably added to that of oleic acid.

Recently, biochemical studies of *in vitro* fatty acid syntheses have rapidly progressed. However, *in vivo* biosyntheses of fatty acid in developing seeds are still unclear. As indicated by the present study, the application of flux model would help the total understanding of fatty acid synthesis in developing soybean seeds, which will provide much novel information about efficient breeding of commercial cultivars with improved oil composition.

# Part II. Genetic Analysis of Pregermination Flooding Tolerance of Soybean Seeds

## Introduction

Hou and Thseng (1992) proposed a screening method of pregermination flooding tolerance of soybean. The procedures of the method are as follows: 1) seeds were sterilized with 70% ethanol, and were soaked with distilled water at 25°C, 2) after air-dried on filter paper for about 6 h, seeds were sown on moist filter paper in a petri dish at 25°C. Using such a method, they found that there were great intervarietal differences in pregermination flooding tolerance, which was closely associated with seed coat coloration and storability, and was independent of water uptake rate of seed and elongation ability of shoot. They also indicated that a small number of genes control pregermination flooding tolerance.

In this part, genetic analysis of pregermination flooding tolerance was performed using a new index, which enabled to estimate physiological aspects of seed damages. Furthermore, seed damages due to excessive water were assessed in detail to understand genetic mechanisms of pregermination flooding tolerance. The goal of this study is to learn whether pregermination flooding tolerance can be introduced into commercial soybean cultivars without changing their commercial values. This part consists of the following two chapters: (5) inheritance of pregermination flooding tolerance and its relationship with seed characters; and (6) exploitation of new rating system of pregermination flooding tolerance.

# Chapter 5. Inheritance of Pregermination Flooding Tolerance and its Relationship with Seed Characters

## 5.1 Introduction

There are several reports on genetic analysis of pregermination flooding tolerance in maize, barley, and wheat (Fausey et al. 1985, Takeda and Fukuyama 1987, Ueno and Takahashi 1997), but there are few in soybean.

In this chapter, genetic analyses of pregermination flooding tolerance were performed using seeds of two $F_{2:3}$ populations from reciprocal crosses between tolerant and susceptible cultivars. Subsequently, the relationships of pregermination flooding tolerance with seed characters, such as seed size, seed shape, and seed coat coloration were investigated.

## 5.2 Materials and Methods

**Plant materials**

Reciprocal crosses were conducted between two soybean cultivars, 'Peking' and 'Tamahomare'. Seeds of 100 $F_2$ plants ($F_{2:3}$ lines) for each cross were used together with those of their parental cultivars. 'Peking' is a strong pregermination flooding tolerant cultivar with a black seed coat and a small seed size, while 'Tamahomare' is a weak pregermination flooding tolerant cultivar with a yellow seed coat and an intermediate seed size. All the seeds were harvested at the experimental field of Kyoto University in Kyoto in 1999. Harvested seeds were air-dried until their moisture contents fell below 15% and kept in storage at $4°C$. Seeds with damaged

coat were excluded.

**Flooding treatment**

One hundred $F_{2:3}$ lines for each cross were subjected to flooding treatment with two replications using the method of Hou and Thseng (1992) with slight modifications. For each line, ten seeds per replication were used. The flooding treatment was conducted in an incubator at 25℃ for 3 d, because the preliminary experiment showed that the variation in germination rate among parental cultivars and 10 randomly selected $F_{2:3}$ lines was greatest in this condition. Soon after flooding treatment, seeds were sown in pots with sterilized soil in a greenhouse at $25 \pm 5$℃. Germination rate and shoot length were measured 7 d after sowing. Flooding tolerance of each line was evaluated by two indices, germination rate (GR) and shoot growth rate (shoot length of treatment / that of control: SR).

**Seed characters examined**

In addition to GR and SR, seven seed characters, seed length, seed thickness, seed width, seed shape, 100 air-dried seed weight, seed coat ratio (seed coat weight/ whole seed weight), and seed coat coloration were measured. The first four characters were measured for each of 10 seeds per $F_{2:3}$ line with a hand-held micrometer. Seed shape was expressed by the ratio of thickness to width and that of width to length. Seed weight and seed coat ratio were measured for 20 and 10 seeds per $F_{2:3}$ line with two replications, respectively. Seed coat coloration, which was measured for each of 10 seeds per $F_{2:3}$ line, was directly scanned from seed surface with the computer equipped digital scanner GT-9000 (Seiko Epson Co., Japan). Relative intensities of three primary colors (red, green, and blue) and brightness were

estimated for each seed using the Photoshop 5.0 computer program (Adobe Systems Inc., USA), where the intensities of 'Peking' and 'Tamahomare' were all set at 0 and 100%, respectively. Visual seed coat coloration was also rated.

**Statistical methods**

To obtain an integrated figure of the total variation of three primary colors and brightness of seed coat in the two $F_{2:3}$ populations, a principal component analysis (PCA) based on variance-covariance matrix was conducted using the SAS microcomputer package (SAS Institute Inc., USA). Not fitting a normal distribution, the data of GR and SR were subjected to non-parametric statistical analyses using the microcomputer package StatView 5.0 (SAS Institute Inc., USA). Using this program, Kendall's correlation analysis and Scheffe's test were conducted in each seed coat coloration group classified by the PCA.

# 5.3 Results

**Inheritance of pregermination flooding tolerance**

The two parental cultivars showed different responses to the flooding treatment: 'Tamahomare' showed 0.0% GR and 0.0% SR 7 days after sowing, while 'Peking' showed 93.8% GR and 99.7% SR (Fig. 5.1a and b). Thus 'Peking' was a strong tolerant cultivar as expected, while 'Tamahomare' was a susceptible cultivar.

The frequency of the lines with 0% GR was significantly higher in the $F_{2:3}$ population from 'Peking' $\times$ 'Tamahomare' than in the $F_{2:3}$ population from its reciprocal cross (Fig. 5.1a). In the former population, about half of the lines showed 0% GR, and the population was divided into two distinct groups for both GR and SR.
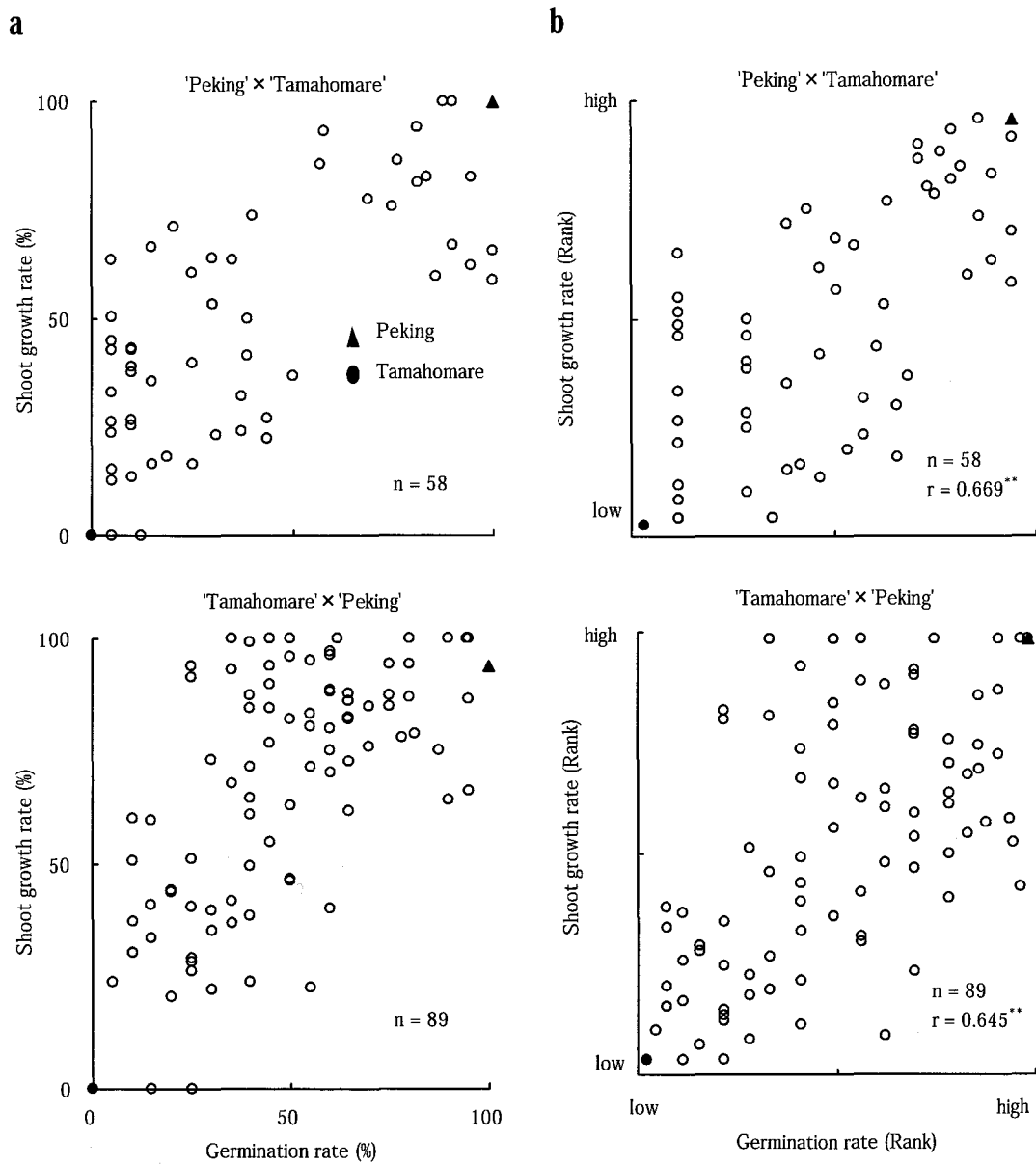
**Fig. 5.1** Distributions of germination rate (GR) and shoot growth rate (SR) in $F_{2:3}$ populations derived from reciprocal crosses between 'Tamahomare' and 'Peking'. Double asterisks attached to correlation coefficients indicate the significance at the 1% level.

On the contrary, in the latter population, GR and SR showed an approximately continuous distribution, but not a normal distribution, ranging from 0 to 100%, although there were no lines with about 10% SR.

**Shoot growth rate as a new index of pregermination flooding tolerance**

The frequency distribution curbs for GR and SR were not normal; therefore, the data of these two traits were ranked and subjected to a non-parametrical statistical analysis (Fig. 5.1b), where the average rank was given to tie values. There was a significant positive correlation between GR and SR in both populations. However, not all the lines with higher GR exhibited higher SR. For instance, two $F_{2:3}$ lines, $PTF_{2:3}$-24 and $PTF_{2:3}$-44, derived from 'Peking' $\times$ 'Tamahomare' showed about 90% GR, but their SR were about 60% almost equivalent to the average value of all the lines (Fig. 5.2).

**Relationships with seed size, seed shape and seed weight**

Great differences between the two parental cultivars were found in seed size, seed shape and seed weight. The $F_1$ plants ($F_{1:2}$) took values almost equal to the mid-parent values for all the three characters (Table 5.1). In addition, these characters showed a continuous frequency distribution without any transgressive segregants in both the $F_{2:3}$ populations, suggesting that these characters might be governed by polygenes. It was also found that in most cases not only GR but also SR was negatively correlated with seed characters (Table 5.2).

**Correlations between flooding tolerance and seed coat color**

The PCA revealed that 98.7% of the total variation of seed coat coloration could be explained by the first principal component ($Z_1$). $Z_1$ represented the isometric
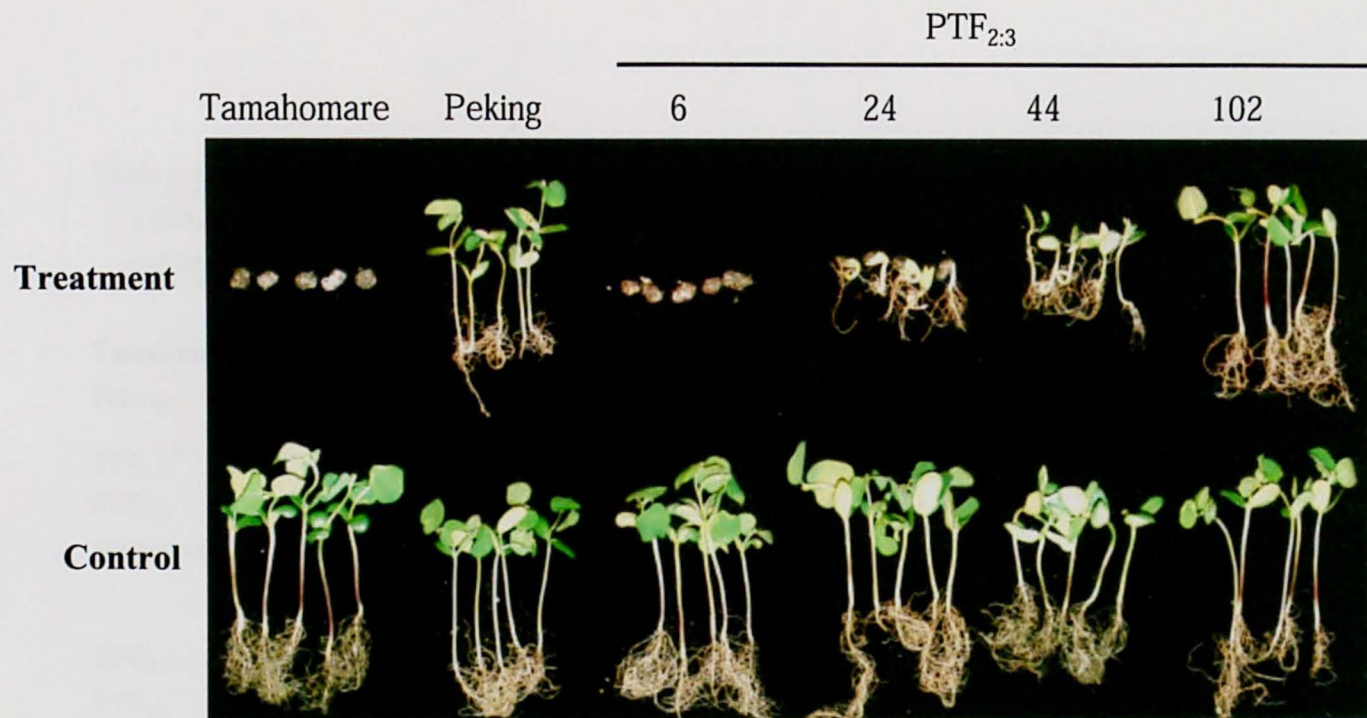
**Fig. 5.2** Seven days old seedlings of four $F_{2:3}$ lines grown in a greenhouse after pregermination flooding treatment. $PTF_{2:3}$ means the $F_{2:3}$ lines derived from the cross 'Peking' × 'Tamahomare'.

**Table 5.1** Seed characters of 'Tamahomare', 'Peking' and their reciprocally crossed progeny lines.

| Cultivar and lines | Seed size (mm) | | | Seed shape | | 100-seed weight (g) |
|---|---|---|---|---|---|---|
| | Length (L) | Width (W) | Thickness (T) | W/L | T/W | |
| | | | Mean | | | |
| Tamahomare | 8.1 a¶ | 7.5 a | 6.6 a | 0.93 a | 0.88 a | 27.0 a |
| Peking | 8.4 a | 5.5 b | 3.6 b | 0.66 b | 0.65 b | 9.1 b |
| $TPF_{1:2}$[†] | 8.0 a | 6.8 c | 5.1 c | 0.85 c | 0.76 c | 18.1 c |
| $PTF_{1:2}$ | 7.8 a | 6.6 c | 5.0 c | 0.86 c | 0.76 c | 17.9 c |
| Midparent | 8.3 a | 6.5 c | 5.1 c | 0.80 c | 0.77 c | 18.1 c |
| | | | Range | | | |
| $TPF_{2:3}$ | 7.2-9.6 | 5.5-7.1 | 4.0-6.1 | 0.71-0.87 | 0.65-0.91 | 11.6-22.3 |
| $PTF_{2:3}$ | 7.0-9.6 | 5.5-7.7 | 4.3-6.2 | 0.66-0.89 | 0.68-0.87 | 11.8-26.9 |

[†] TP and PT indicate the lines derived from crosses 'Tamahomare'×'Peking' and 'Peking'×'Tamahomare', respectively.

¶ Values within a colum followed by the same letter do not differ significantly at the 5% level.

**Table 5.2** Correlation coefficients of germination rate rank and shoot growth rate rank with seed characters
in two $F_{2:3}$ populations from reciprocal crosses between 'Peking' and 'Tamahomare'

| Flooding tolerance | Seed size | | | Seed shape | | Seed weight |
|---|---|---|---|---|---|---|
| | Length | Width | Thickness | W/L | T/W | |
| Tamahomare × Peking (n = 89) | | | | | | |
| Germination rate | -0.085 ns | -0.235 * | -0.304 ** | -0.147 ns | -0.206 ns | -0.306 ** |
| Shoot growth rate | -0.066 ns | -0.191 ns | -0.316 ** | -0.253 * | -0.269 ** | -0.274 ** |
| Peking × Tamahomare (n = 58) | | | | | | |
| Germination rate | 0.259 * | -0.293 ** | -0.368 ** | -0.407 ** | -0.305 ** | -0.216 * |
| Shoot growth rate | 0.235 * | -0.252 * | -0.340 ** | -0.415 ** | -0.305 ** | -0.208 * |

\* and \*\* Significant at the 5 and 1% levels, respectively; ns = nonsignificant.

phase of variation of the intensity of red and brightness: the lines with a larger $Z_1$ score had a deep red and bright seed coat, which was visually recognized as yellow, while the lines with a smaller $Z_1$ score had a dark seed coat (without a red color), which was visually recognized as black. According to the $Z_1$ score, each $F_{2:3}$ population was divided into four groups, yellow, tan, brown, and black groups, although the yellow group showed a great variation and involved dark, light, and greenish yellow lines.

The relationships between GR and SR in the four groups from 'Tamahomare' $\times$ 'Peking' are shown in Fig. 5.3. There are great differences in GR among the groups: the black group showed significantly higher GR than brown, tan and yellow groups. For SR, all the groups took similar average values when the lines with 0% GR were excluded. Both GR and SR were not correlated with $Z_1$ score in all the groups (data not shown).

**Relationship of flooding tolerance with seed coat ratio**

The seed coat ratios of 'Tamahomare' and 'Peking' were 7.5 and 13.8%, respectively. In both the $F_{2:3}$ populations, the black and brown groups showed a significantly higher mean seed coat ratio than those of the tan and yellow groups. There were seven lines with the seed coat ratio over than 12%: out of them, four belonged to the black group; two belonged to the brown group; and one belonged to the yellow group. The four lines in the black group all showed a high GR, but the three lines in other groups showed a low to middle GR (Fig. 5.4). The three lines in the brown and yellow groups had crackness on seed coat. SR was not correlated with seed coat ratio.
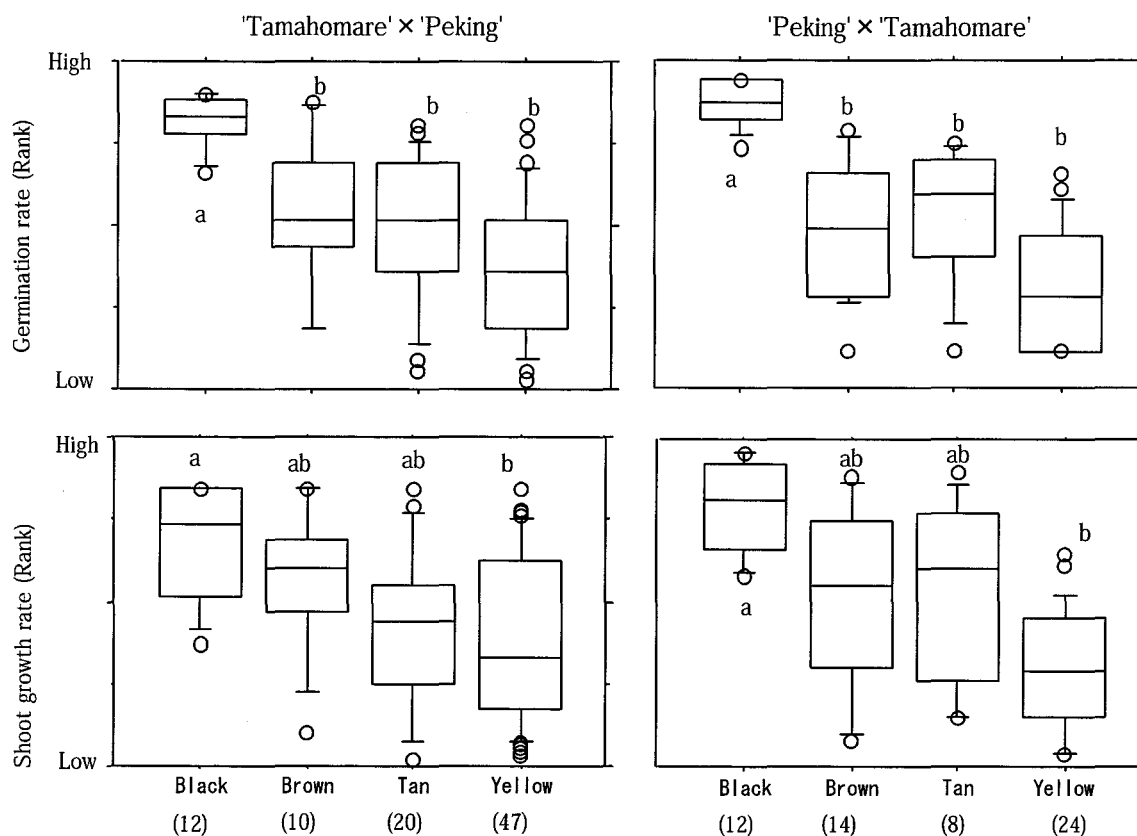
**Fig. 5.3** Distributions of lines for germination rate (GR) rank and shoot growth rate (SR) rank in four seed coat coloration groups in the $F_{2:3}$ populations derived from reciprocal crosses between 'Tamahomare' and 'Peking'. For each group, 50% of lines are included in a box, and 80% of them are included within the range from top to bottom bar. The number in parenthesis indicates the number of lines in each group. The four seed coat color groups followed by the same letter do not differ significantly at the 1% level by the Scheffe's test.
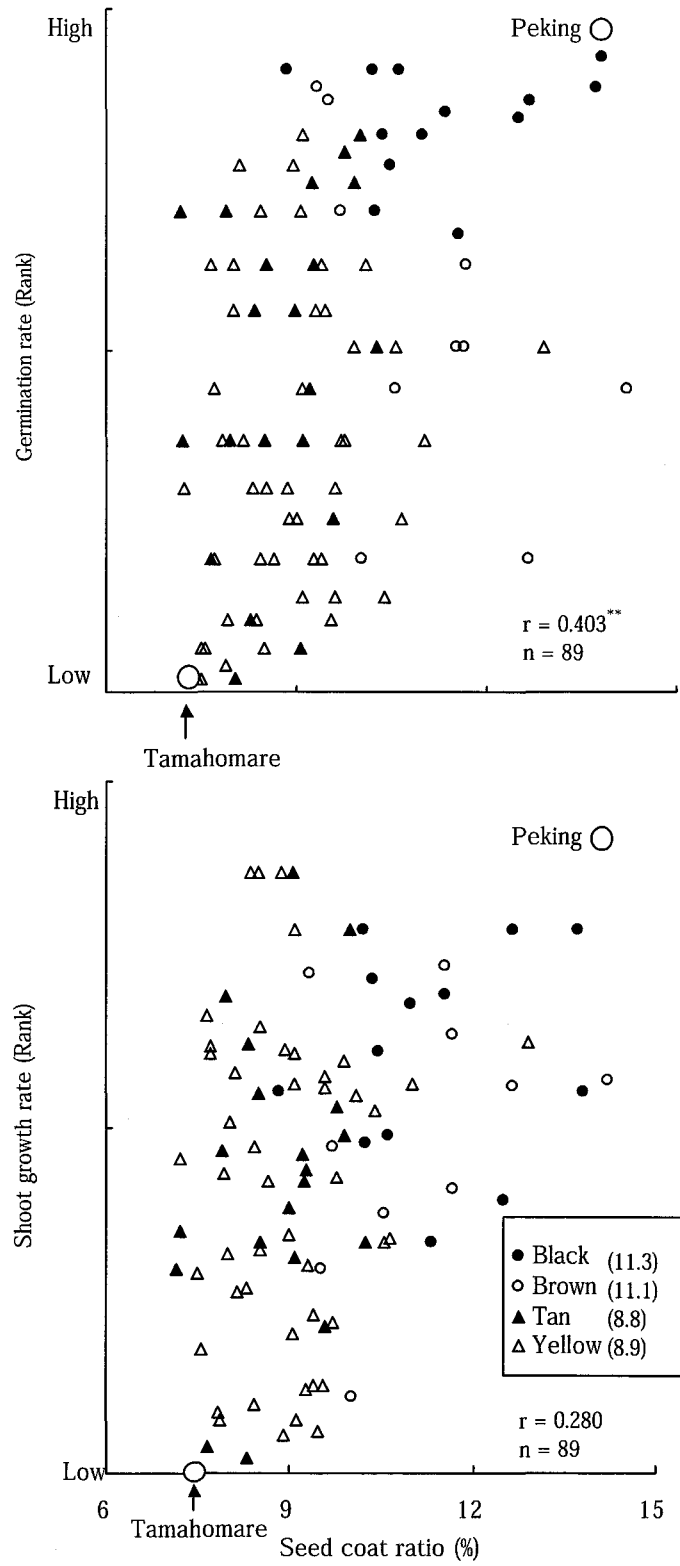
**Fig 5.4** Relationships of germination rate (GR) and shoot growth rate (SR) with seed coat ratio (seed coat weight/ whole seed weight) in the $F_{2:3}$ populations derived from the cross 'Tamahomare' × 'Peking'. The number in parenthesis indicates the mean seed coat ratio (%) in each group.

## 5.4 Discussion

It is known that once soybean seeds encountered the anoxia stress caused by excessive water or heavy rainfall during the period of germination, the growth of the plants are retarded markedly, resulting in noticeable reduction of grain yield (Arihara and Kanno 1998). Furthermore, the present study showed that SR was genetically independent of GR. Therefore, SR should be taken into account when evaluating flooding tolerance of soybean seeds.

The two $F_{2:3}$ populations derived from reciprocal crosses showed different distributions for both GR and SR: the frequency of the lines with 0% GR was significantly higher in the $F_{2:3}$ population from 'Peking' × 'Tamahomare' than in the $F_{2:3}$ population from its reciprocal cross. This indicates that cytoplasmic and/or maternal factors, as well as nucleic factors, are concerned with pregermination flooding tolerance. Such effects have not been reported in higher plants at yet.

Experimental results confirmed that 'Peking' with a small seed size had strong pregermination flooding tolerance. It is known that strong pregermination flooding tolerant cultivars mostly have a small seed size and black seed coat coloration (Hou and Thseng 1991). However, GR and SR were not correlated with seed size and seed shape in the $F_{2:3}$ populations. This indicates that the gene(s) conferring strong tolerance to 'Peking' is independent of those for these two characters, and can be introduced into commercial cultivars without changing these two characters. In contrast, GR was strongly correlated with seed coat coloration (Table 5.2): the seeds with black or brown seed coat color tended to show high GR. Previous studies

indicated that the cultivars with black and brown seed coat had higher contents of phenolic compounds than those with tan and yellow seed coat, and that phenolic compounds would repress the water uptake rate of seed (Kannenberg and Allard 1964, Marbach and Mayer 1974, Tully et al. 1981, Kuo 1989). In this study, however, 'Peking' and 'Tamahomare' did not differ in the increasing rate of water content during seed soaking (data not shown). Thseng et al. (1996) reported that pregermination flooding tolerance was not related with the water uptake rate of seed under excessive water conditions. Therefore, low water uptake rate is not considered as a factor conferring strong pregermination flooding tolerance to the cultivars with black or brown seed coat coloration.

Based on $Z_1$ score extracted from the PCA for seed coat coloration, each $F_{2:3}$ population was divided into four groups. The black and brown groups were clearly distinguished from other groups, but the difference between the tan and yellow groups were not clear. The tan and yellow groups each involved the lines with a green, dark yellow, or light yellow seed coat, which was probably due to that some of the loci responsible for seed coat coloration were heterozygous. In tan and yellow groups, the amount of pigmentation in seed coat, which was roughly estimated from $Z_1$ score, was related with neither GR nor SR. Furthermore, some of the lines with a black seed coat showed decreased viability by the flooding treatment. Thus the gene(s) responsible for seed coat pigmentation is not a unique factor for pregermination flooding tolerance, contrary to Hou and Thseng (1991).

In this study, the lines of black or brown seed coat group have a significantly higher seed coat ratio than those of tan or yellow group. Since seed coat ratio is

probably related to seed coat thickness, the lines with a black and brown seed coat showed strong tolerance to flooding treatment by preventing seeds from collapsing under excessive water conditions. However, it was considered that there were a few factors, other than seed coat ratio, causing a large variation of pregermination flooding tolerance.

In conclusion, not only GR but also SR should be considered in evaluating pregermination flooding tolerance because experimental results showed the possibility that the former was related to seed coat structure, while the latter was related to respiration ability of cotyledonous and/or embryo cell. To ascertain the second point, the comparison of SR between flooding and anoxia treatments will be needed. It is also concluded that breeding of cultivars with strong pregermination flooding tolerance will be possible without changing seven seed characters examined here.

# Chapter 6. Exploitation of New Rating System of Pregermination Flooding Tolerance

## 6.1 Introduction

Hou et al. (1992) reported that *in vitro* germination rate after flooding treatment of soybean seeds is closely related with *in vivo* germination rate. This indicates that pregermination flooding tolerance of soybean could be evaluated *in vitro*.

It is known that once soybean seeds encountered the anoxia stress caused by excessive water or heavy rainfall during the period of germination, the growth of the plants are retarded markedly, resulting in noticeable reduction of grain yield (Arihara and Kanno 1998). Therefore, the growth of established seedling, as well as germination rate, should be taken into account when evaluating flooding tolerance of soybean seeds.

In this chapter, the author attempted to evaluate of pregermination flooding tolerance using the *in vitro* screening method and to exploit new characters, other than germination rate, available for rating the tolerance. Based on the results, a new rating system of pregermination flooding tolerance *in vitro* was proposed.

## 6.2 Materials and Methods

### Plant materials

Two soybean cultivars, 'Peking' and 'Tamahomare' were used to evaluate the *in vitro* screening method of pregermination flooding tolerance. When using conventional screening method, 'Peking' is regarded as a strong pregermination

73

flooding tolerant cultivar with a black seed coat and very small seed size, while 'Tamahomare' is regarded as a weak pregermination flooding tolerant cultivar with a yellow seed coat and medium seed size. To investigate the relationships between pregermination flooding tolerance and seed characters using the *in vitro* screening method, nine cultivars in addition to the above two cultivars were used: 'Tanbaguro' and 'Gankui' with a black seed coat and very large seed size; 'PI408251' with a black seed coat and very small seed size; 'Tenshin-ao-daizu' with a green seed coat and medium seed size; 'Aburamame', 'Clark' and 'Tamamusume' with a yellow seed coat and small medium seed size; 'Enrei' with a yellow seed coat and medium seed size; and 'Mizukuguri' with a green seed coat and small large seed size. 'Tamahomare', 'Peking', 'Tenshin-ao-daizu' and 'Gankui' were kindly provided by Chushin Agricultural Experimental Station, Nagano. 'PI408251', 'Aburamame', 'Tamamusume', 'Clark', 'Tanbaguro' and 'Enrei' were provided by National Institute of Agrobiological Sciences. 'Mizukuguri' was provided by Laboratory of Crop Science, Kyoto University.

**Germination test**

0.7% agar gel was used as a germination medium, which was developed by Hyogo Prefectural Agricultural Institute. In this method, however, damaged seeds by flooding treatments were easily infected with fungi. Therefore, the effects of anti-fungus reagent (25, 50, 100 and 200 ppm of antiformin) in the agar medium on the growth of fungus and germination of soybean seeds were first investigated. Subsequently, using the 0.7% agar medium with antiformin, the germination test of seeds was conducted according the completely randomized design with three

replications. The flooding treatment was conducted by soaking seeds in distilled water for 1 or 2 days. For each plot, ten seeds were used.

**Pregermination flooding tolerance assay**

The method of Hou and Thseng (1992) was used for the flooding treatment with several modifications: seeds were sterilized with 99% ethanol, and were soaked with distilled water at 25°C for 1 to 8 d. After air-dried on filter paper for about 6 h, seeds were sown on 0.7% agar media with 50 ppm antiformin in an incubator at 25°C. Seeds with more than 1cm root were counted as germinated seeds. Immediately after germination, they were transplanted to a small pot with sterilized soil, which comprised vermiculite, coarse sand and gravel in the ratio 2:1:2 by volume, respectively, and grown for 4 d in an incubator at 25°C. The lengths of shoot and root of 4 d old seedlings were measured and their root tips were visually rated. Flooding tolerance assay was conducted with two replications.

# 6.3 Results

**The effect of antiformin concentration**

In both 'Peking' and 'Tamahomare', any antiformin concentration in the agar media used here did not affect the germination rate of soybean seeds (Table 6.1). The multiplication of fungi was significantly repressed by adding more than 50 ppm of antiformin to the agar media. It is therefore considered that germination tests can be smoothly conducted on the 0.7% agar media, including at least 50ppm of antiformin.

**Germination rate and seedling growth rate after flooding treatment**

The relationship between germination rate and soaking time was investigated

**Table 6.1** Effects of antiformin concentration on germination rate of soybean seeds after flooding treatment

| Cultivar | Source | D.F. | MS | F |
|---|---|---|---|---|
| Peking | Flooding treatment[†] | 2 | 30.42 | 7.61 [**] |
| | Antiformin concentration[¶] | 4 | 4.26 | 1.06 |
| | Error | 30 | 4.00 | |
| Tamahomare | Flooding treatment | 2 | 224.47 | 53.44 [**] |
| | Antiformin concentration | 4 | 1.70 | 0.40 |
| | Error | 30 | 4.20 | |

[†]Seeds were soaked in distilled water for 0, 1 and 2 days before germination, respectively.
[¶]Antiformin concentrations in germination agar medium were 0, 25, 50, 100 and 200 ppm, respectively.

76

when using the 0.7% agar media including 50ppm of antiformin. The germination rate of 'Peking' was almost constant at around 80% without regard to flooding time, while that of 'Tamahomare' was reduced to about 30% even by 1 d flooding treatment and no seeds germinated by more than 3 d treatments due to seed coat collapse (Fig. 6.1). Some seeds of 'Peking' germinated during flooding treatment.

'Peking' and 'Tamahomare' showed significantly reduced shoot and root lengths when subjected to the flooding treatment (Fig. 6.2). 'Peking' did not show any difference in both shoot and root length among treatments, while the shoot and root length of 'Tamahomare' tended to decrease with the increase of flooding treatment time. Root length was damaged more markedly than shoot length by the flooding treatment. The root tips of most of the seedlings with serious damage were dead.

**Genetic variation of pregermination flooding tolerance**

Eleven soybean cultivars different in seed appearance from each other were investigated for pregermination flooding tolerance using the *in vitro* screening method. By 3 d flooding treatment, a great intervarietal variation in pregermination flooding tolerance was found: the cultivars were clearly classified into three groups (Fig. 6.3). In 'Aburamame', 'Tamamusume' and 'Tamahomare', almost all the seeds were disrupted due to seed coat collapse, while in 'Enrei', 'Gankui', 'Clark' and 'Mizukuguri', more than half of the seeds were not disrupted although they did not germinate. 'PI408251', 'Peking', 'Tenshin-ao-daizu' and 'Tanbaguro' showed strong pregermination flooding tolerance without disruption. However, established seedlings of all the cultivars except 'Tamahomare' and 'Tamamusume' showed remarkable growth inhibition.
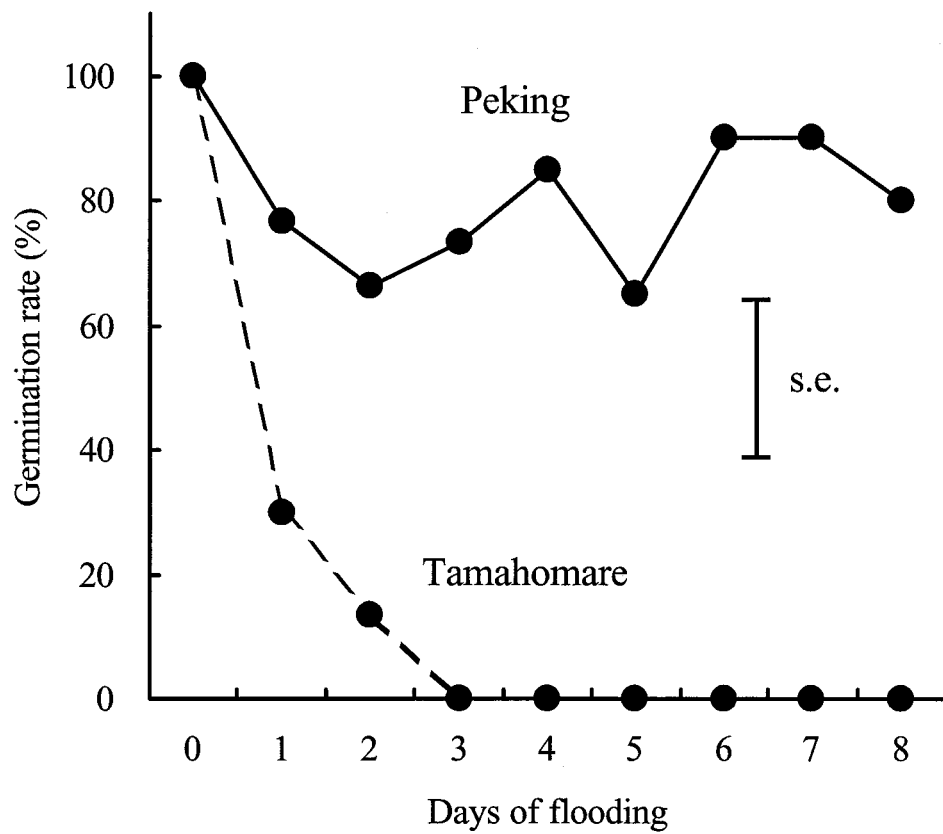
**Fig. 6.1** Effect of flooding treatment time on the germination rates of soybean cultivars 'Peking' and 'Tamahomare'.
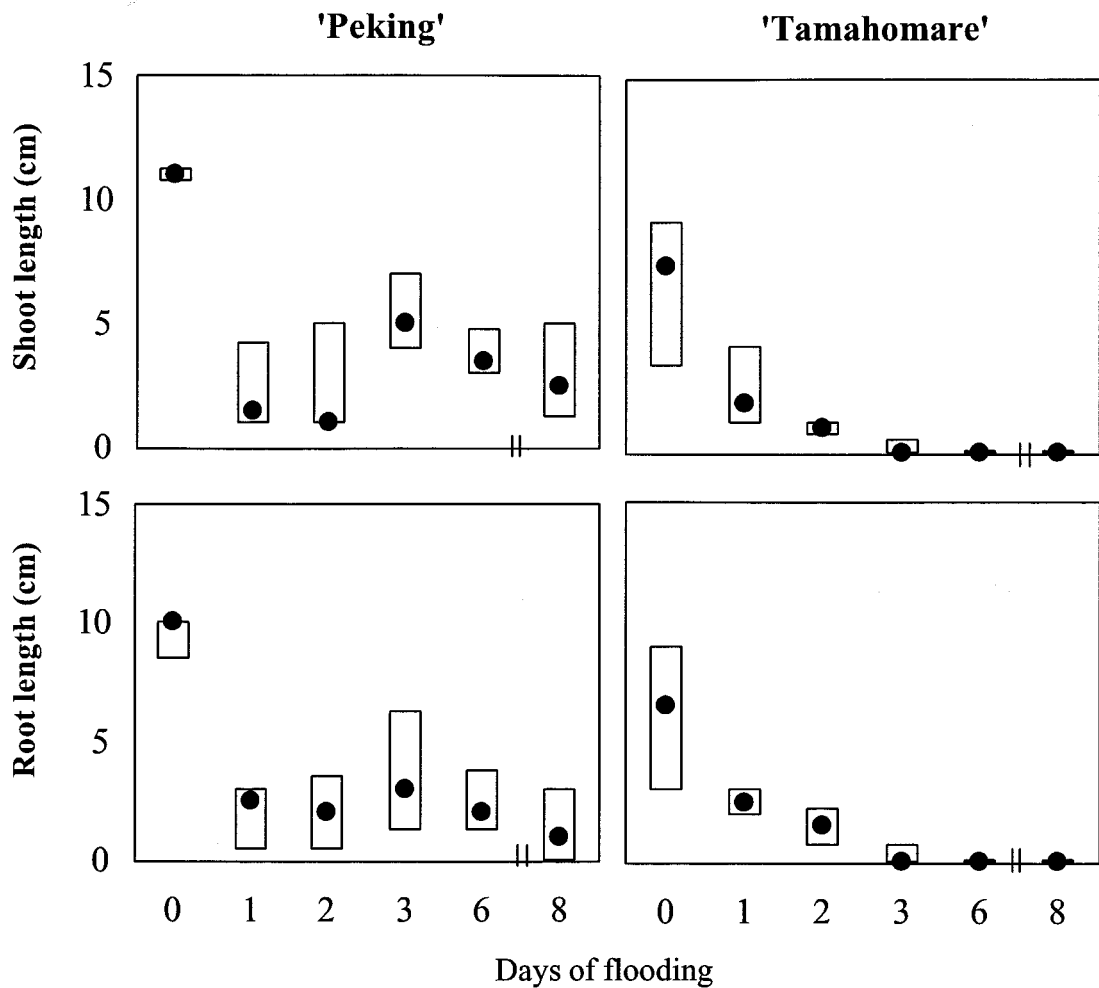
**Fig. 6.2** Effects of flooding treatment time on shoot and root length in 4 days old seedlings of soybean cultivars 'Peking' and 'Tamahomare'. Box indicates the range of the shoot or root length.

Cultivar

| | SR | RR |
|---|---|---|
| PI408251 | 63 | 91 |
| Peking | 57 | 57 |
| Tenshin-ao-daizu | 66 | 39 |
| Tanbaguro | 68 | 62 |
| Enrei | 49 | 42 |
| Gankui | 66 | 30 |
| C l a r k | 65 | 47 |
| Mizukuguri | 53 | 32 |
| Aburamame | 25 | 12 |
| Tamamusume | | |
| Tamahomare | | |

0%                    50%                    100%

□ Seed coat collapse   ▨ Non-germinate   ▥ Unvital root tip   ■ Established seedling
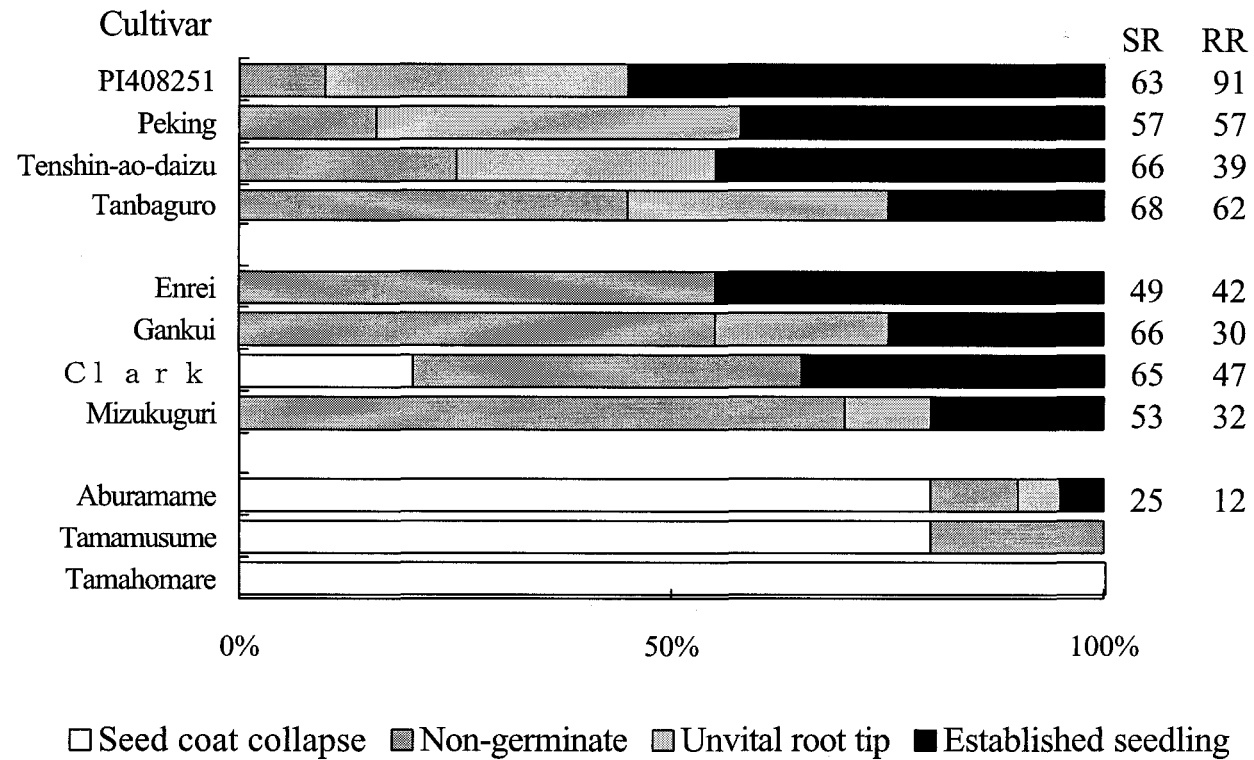
**Fig. 6.3** Percentages of seed coat collapse, non-germination, unvital root tip, and established seedling in 4 days old seedlings after 3 days flooding treatment in 11 soybean culitvars. SR and RR mean shoot growth rate (%) and root growth rate (%) of established seedlings, respectively.

## 6.4 Discussion

Excessive water before germination negatively affects physical and physiological aspects of soybean seeds. In this study, the physical disruption of seeds was found to be the main cause of the reduced germination rate after flooding treatment. Therefore, reducing the seed coat collapse after soaking would be a major objective of breeding strong pregermination-flooding tolerant cultivar. Even though seeds were not disrupted by flooding, many of them did not germinate. This suggests that seeds were damaged also by some physiological factors. These factors probably reduce or inhibit the activity of cell division in both seed and seedling.

In contrast to *in vivo* screening, *in vitro* screening of pregermination flooding tolerance, performed in this experiment, could identify the causes of seed deterioration after flooding, such as seed coat collapse, non-germination of seed without seed coat collapse, and growth inhibition of seedling. Therefore, *in vitro* screening could detect the genetic variation in pregermination flooding tolerance more precisely than *in vivo* screening.

Based on these results, a new rating system of pregermination flooding tolerance *in vitro* based on the following four characters could be proposed: 1) presence of seed coat collapse, 2) germination ability, 3) viability of root tip, and 4) growth rate of seedling. If using this system, physical and physiological factors related to pregermination flooding tolerance could be separately estimated.

# Summary

Soybean is one of the most susceptible crops to environmental conditions, breeding of cultivars that achieve stable and high production of high quality seeds has been required in various counties. For the fatty acid composition of soybean seeds, there is a great genetic variation. Using these genetic resources, breeders have successfully enhanced the oxidative stability of soybean seed oil. However, the stable production of desirable fatty acid composition of seeds is still difficult, indicating that the significance of investigating genetic mechanism and environmental effects on fatty acid composition. In soybean cultivation, excessive water before germination causes severe seed deterioration, resulting in decreased grain yield at maturity. Therefore, breeding of pregermination flooding tolerant cultivars has been desired in various countries with much rainfall during sowing time. The complex nature of seed damages due to excessive water makes it difficult to elucidate the physiological and genetic mechanisms of pregermination flooding tolerance. Therefore, further investigations on pregermination flooding tolerance should be done considering various physical and physiological factors. In this study, the author deals with two important traits, fatty acid composition (Part I) and pregermination flooding tolerance (Part II), aiming at stable and high production of cultivars with superior seed oil composition.

I. Genetic Analysis of Fatty Acid Composition of Soybean Seeds

In this part, (1) genetic variation in fatty acid composition of mature seeds, (2)

fatty acid accumulation pattern in developing seeds, (3) expression of fatty acid desaturase genes, and (4) fatty acid flux in developing seeds were investigated.

1. Genetic Variation of Fatty Acid Composition in Mature Seeds

Sixty soybean cultivars from Japan and USA formed five maturity groups (IIb-Vc) based on number of days from sowing to flowering and number of days from flowering to maturity. Highly significant intervarietal difference in fatty acid composition was found in all the maturity groups, especially in IIc. Stearic and oleic acids showed a larger variation than the palmitic, linoleic and $\alpha$-linolenic acid. The principal component analysis suggested that the total variation of fatty acid composition depended mainly on the desaturation levels from oleic to linoleic acid.

2. Fatty Acid Accumulation Pattern in Developing Seeds

Three cultivars exhibiting unique fatty acid composition, together with a standard cultivar, were examined for the contents of the five fatty acids as well as crude oil at eight seed filling stage. For all the four cultivars, it was found that crude oil content increased sigmoidally with the advance of filling stage, and that the accumulation patterns of palmitic, linoleic, and $\alpha$-linolenic acids were similar to that of crude oil. But the accumulation pattern of stearic acid was different from that of crude oil and divided the cultivars into two distinct groups. For oleic acid, only the cultivar 'Aburamame' showed a rapid increase in proportion with the advance of filling stage, although not differing markedly in accumulation content from other cultivars. These results indicate that analyzing the accumulation patterns of fatty

acids could explain the latent genetic variation of fatty acid composition in soybean seeds.

3. Expression of Fatty Acid Desaturase Genes

Three cultivars exhibiting unique fatty acid composition, together with a standard cultivar, were examined for the transcription levels of genes encoding fatty acid desaturase at three seed filling stages. For all the four cultivars, it was found that *SACPD*, *FAD2-1* and *FAD3* mRNAs increased with the advance of the filling stage, and decreased in the mature seeds. *FAD2-2* expressed at all the seed filling stages and even in mature seeds. The transcription level of *FAD2-1* in 'Cutler71' and that of *FAD2-2* in 'Aburamame' were significantly lower than those of three other cultivars. There were no intervarietal differences in base sequence of open reading frame in *FAD2-1*. From the analysis of genomic region of *FAD2-1*, however, the restriction fragment length polymorphism was found between two cultivar groups (Group 1: 'Cutler71' and 'Clark', Group 2: 'Aburamame' and 'Tamamusume'). This indicates that base substitution has occurred around the *FAD2-1* genomic region.

4. Algorithmic Analysis of Fatty Acid Flux in Developing Seeds

Environmental and genetic effects on fatty acid composition in soybean seeds were investigated using four cultivars. The contents of all the five fatty acids were highest when plants were grown in a proper season. For the percentage of each fatty acid content to the total fatty acid content, effects of genotype (all the five fatty acid), year (palmitic and stearic acids), and cropping season (oleic, linoleic and $\alpha$-linolenic

acids) were statistically significant, and genotype × year interactions were also detected for percentages of oleic, linoleic and $\alpha$-linolenic acids. The percentages of palmitic and oleic acids were positively correlated with mean temperature during the post-flowering developmental phase, while those of stearic and linoleic acids were negatively correlated. As a result of algorithmic analyses, fatty acid fluxes at the first three biosynthetic steps were synchronized with each other and had two active phases, while that at the last step (linoleic to $\alpha$-linolenic acid) was lower in amount and had only one active phase. For the flux at each step, there were great genotypic and environmental variations. Based on the results, the effects of environments on biochemical mechanisms of fatty acid syntheses were discussed.

II. Genetic Analysis of Pregermination Flooding Tolerance of Soybean Seeds

In this part, inheritance of pregermination flooding tolerance and its relationship with seed characters were investigated. Subsequently, a new rating system of pregermination flooding tolerance was exploited.

5. Inheritance of Pregermination Flooding Tolerance and its Relationship with Seed Characters

The inheritance of pregermination flooding tolerance in soybean was analyzed using seeds of $F_2$ plants ($F_{2:3}$ lines) derived from reciprocal crosses between tolerant and susceptible cultivars. Experimental results showed that flooding tolerance should be evaluated by two indices, germination rate and shoot growth rate (shoot length of treatment/ that of control), which were controlled by different genetic factors. These

two indices were not associated with seed size, seed weight, and seed shape in the two $F_{2:3}$ populations, indicating that strong flooding tolerance can be introduced into commercial cultivars without changing these characters. Each $F_{2:3}$ population was divided into four seed coat coloration groups, yellow, tan, brown and black groups. The average germination rate of the black seed coat group was higher than those of three other groups, while the average shoot growth rate did not vary among the groups. The black group showed a higher seed coat ratio (seed coat weight/ whole seed weight) than three other groups, and seed coat ratio was positively correlated with germination rate. However, several of the $F_{2:3}$ lines with a tan or yellow seed coat exhibited a higher germination rate. This indicates that the strength of flooding tolerance is determined not only by seed coat coloration, which has been considered as a key factor, but also by other factors.

6. Exploitation of New Rating System of Pregermination Flooding Tolerance

Excessive water before germination of soybean causes the deterioration of seeds, resulting in decreased field emergence, especially in the cultivation with direct seeding. To establish a new screening method for pregermination flooding tolerance, new characters available for rating the tolerance were exploited. Experimental results showed that the pregermination flooding tolerance could be rated by the following four characters: presence of seed coat collapse, germination rate, viability of root tip, and growth rate of seedling.

The present study showed that there are great intervarietal genetic and

86

environmental variations in fatty acid composition of soybean seeds, suggesting the possibility of developing the cultivars with stable and specific fatty acid composition. Genetic analyses indicated that pregermination flooding tolerance could be easily introduced into commercial cultivars without changing seed appearance. These finding will help develop various kinds of excellent cultivars with specific fatty acid composition and strong flooding tolerance.

# References

Arihara, J. and A. Kanno (1998) Effects of oxygen concentration on the growth and yield of soybean (*Glycine max* (L.) Merr.). Jpn. J. Crop Sci. 67 extra issue 1:44-45.

Arondel, V., B. Lemieux, I. Hwang, S. Gibsonm, H.M. Goodman and C.R. Somerville (1992) Map-based cloning of a gene controlling $\omega$ -3 fatty acid desaturase in *Arabidopsis*. Science 258:1353-1354.

Bernard, R.L., G.A. Juvick, E.E. Hartwig and C.J. Edwards Jr. (1988) Origins and pedigrees of public soybean varieties in the United States and Canada. USDA Technical Bulletin No. 1746.

Brim, C.A. and J.P. Ross (1966) Registration of Pickett soybeans. Crop Sci. 6:305.

Brim, C.A. and J.W. Burton (1979) Recurrent selection in soybeans. II. Selection for increased percent protein in seeds. Crop Sci. 19:494-498.

Browse, J. and C. Somerville (1991). Glycerolipid synthesis: biochemistry and regulation. Annal. Rev. Plant Physiol. Plant Mol. Biol. 42:467-506.

Burton, J.W. and C.A. Brim (1981) Recurrent selection in soybeans. III. Selection for increased percent oil in seeds. Crop Sci. 21:31-34.

Burton, J.W., R.F. Wilson and C.A. Brim (1983) Recurrent selection in soybeans. IV. Selection for increased oleic acid percentage in seed oil. Crop Sci. 23:744-747

Carver, B.F., J.W. Burton, T.E. Carter Jr. and R.F. Wilson (1986) Response to environmental variation of soybean lines selected for altered unsaturated fatty acid composition. Crop Sci. 26:1176-1181

Cheesbrough T.M. (1989) Changes in the enzymes for fatty acid synthesis and desaturation during acclimation of developing seeds to altered growth temperature. Plant Physiol. 90:760-764.

Cheesbrough T.M. (1990) Decreased growth temperature increases soybean stearoylacyl carrier protein desaturase activity. Plant Physiol. 93:555-559.

Chen L., Y. Moon, J. Shanklin, B. Nikolau and A.G. Atherly (1995) Cloning and sequence of a cDNA encoding stearoyl-acyl carrier protein desaturase from Glycine max (PGR95-105). Plant Physiol. 109:1498.

Cherry, J.H., L. Bishop, N. Leopold, C. Pikaard and P.M. Hasegawa (1984) Patterns of fatty acid deposition during development of soybean seed. Phytochemistry 23:2183-2186

Cole, G., S. Coughlan, N. Frey, J. Hazebroek and C. Jennings (1998) New sunflower and soybean cultivars for novel vegetable oil types. Fett/Lipid 100:177-181.

Cramer, M.M. and W.D. Beversdorf (1984) Effect of genotype $\times$ environment interactions on selection for low linolenic acid soybeans. Crop Sci. 24:327-330.

Dornbos, D.L.Jr. and M.B. McDonald Jr. (1986) Mass and composition of developing soybean seeds at five reproductive growth stages. Crop Sci. 26:624-630.

Egley, G.H., R.N. Paul, K.C. Vaughan and S.O. Duke (1983) Role of peroxidase in the development of water-impermeable seed coats in Sida spinosa L. Planta 157:224-232.

Endres, J.G. (1992) Niche marketing for new oilseeds: An industrial perspective. In: S.L. MacKenzie and D.C. Taylor (eds) Seed Oils for the Future, pp. 1-8. Am. Oil Chem. Soc. Press, Champaign, IL.

Fagerstedt, K.V. and R.M.M. Crawford (1987) Is anoxia tolerance related to flooding tolerance? Functional Ecology 1:49-55.

Fausey, N.R., T.T. VanToai and M.B. McDonald Jr. (1985) Response of ten corn cultivars to flooding. Trans. ASAE 28:1794-1797.

Fehr, W.R., C.E. Caviness, D.T. Burmood and J.S. Pennington (1971) Stage of development descriptions for soybeans. Crop Sci. 11:929-931.

Fukui, J. and M. Arai (1951) Ecological studies on Japanese soy-bean varieties. I. Classification of soy-bean varieties on the basis of the days from germination to blooming and from blooming to ripening with special reference to their geographical differentiation. Jpn. J. Breed. 1:27-39.

Gbikpi, P.J. and R.K. Crookston (1981) Effect of flowering date on accumulation of dry matter and protein in soybean seeds. Crop Sci. 21:652-655.

Graef, G.L., W.R. Fehr and E.G. Hammond (1985a) Inheritance of three stearic acid mutants of soybean. Crop Sci. 25:1076-1079.

Graef, G.L., L.A. Miller, W.R. Fehr and E.G. Hammond (1985b) Fatty acid development in a soybean mutant with high stearic acid. J. Am. Oil Chem. Soc.

62:773-775.

Hammond, E.G. and W.R. Fehr (1983) Registration of A6 germ plasm line of soybean. Crop Sci. 23:192-193.

Hanson, W.D. (1992) Modified seed maturation rates and seed yield potentials in soybean. Crop Sci. 32:972-976.

Harris, P. and A.T. James (1969) Effect of low temperature on fatty acid biosynthesis in seeds. Biochim. Biophys. Acta 187:13-18.

Harwood, J.L. (1996) Recent advances in the biosynthesis of plant fatty acids. Biochim. Biophys. Acta 1301:7-56.

Hawkins S.E., W.R. Fehr, E.G. Hammond and S. Rodriguez de Cianzio (1983) Use of tropical environments in breeding for oil composition of soybean genotypes adapted to temperate climates. Crop Sci. 23:897-899.

Heppard E.P., A.J. Kinney, K.L. Stecca and G.H. Miao (1996) Development and growth temperature regulation of two different microsomal $\omega$-6 desaturase genes in soybeans. Plant Physiol. 110:311-319.

Hou, F.F. and F.S. Thseng (1991) Studies on the flooding tolerance of soybean seed: varietal differences. Euphytica 57:169-173.

Hou, F.F. and F.S. Thseng (1992) Studies on the screening technique for pre-germination flooding tolerance in soybean. Jpn. J. Crop Sci. 61:447-453.

Hou, F.F., F.S. Thseng, S.T. Wu and K. Takeda (1995) Varietal differences and diallel analysis of pre-germination flooding tolerance in soybean seed. Bull. Res. Inst. Bioresour. Okayama Univ. 3:35-41.

Ishikawa, G., H. Hasegawa, Y. Takagi and T. Tanisaka (2001) The accumulation pattern in developing seeds and its relation to fatty acid variation in soybean. Plant Breed. 120:417-423.

Kane, M.K., C.C. Steele, L.J. Grabau, C.T. MacKown and D.F. Hildebrand (1997) Early-maturing soybean cropping system: III. Protein and oil contents and oil composition. Agron. J. 89:464-469.

Kannenberg, L.W. and R.W. Allard (1964) An association between pigment and lignin formation in the seed coat of the limabean. Crop Sci. 4:621-622.

Kinney, A.J. (1997) Genetic engineering of oilseeds for desired traits. In: J.K. Setlow

(ed.) Genetic Engineering, Principles and Methods, Vol. 19, pp. 149-166, Plenum Publishing, Wiesbaden, Germany.

Kinney A.J. (1998) Plant as industrial chemical factories – new oils from genetically engineered soybeans. Fett/Lipid 100:173-176.

Kinney A.J. and S. Knowlton (1998) Designer oils: the high oleic acid soybean. In: S. Roller and S. Harlander (eds) Genetic Modification in the Food Industry, pp. 193-213. Blackie, London.

Kuo, W.H.J. (1989) Delayed permeability of soybean seed: Characteristics and screening methodology. Seed Sci. Technol. 17:134-142.

Kwanyuen, P. and R.F. Wilson (1986) Isolation and purification of diacylglycerol acyltransferase from germinating soybean cotyledons. Biochim. Biophys. Acta. 877:238-245.

List, G.R., T.L. Mounts, F. Orthoefer and W.E. Neff (1996) Potential margarine oils from genetically modified soybeans. J. Am. Oil Chem. Soc. 73:729-732.

Liu, K.S. (1999) Soy oil modification: products, applications. INFORM 10:868-878.

Luedders, V.D. (1977) Genetic improvement in yield of soybeans. Crop Sci. 17:268-271.

Lühs, W. and W. Friedt (1994) Major oil crops. In: D.J. Murphy (ed.), Designer Oil Crops, pp. 5-71. VCH Verlagsges, Weinheim.

Marbach, I. and A.M. Mayer (1974) Permeability of seed coats to water as related to drying conditions and metabolism of phenolics. Plant Physiol. 54:817-820.

Miquel, M. and J. Browse (1994) Lipid biosynthesis in developing seeds. In: G. Galili, J. Kigel and M. Negli (eds) Seed Development and Germination, pp. 169-193, New York, Marcel Dekker.

Murphy, D.J. (1995) The use of conventional and molecular genetics to produce new diversity in seed oil composition for the use of plant breeders: Progress, problems and future prospects. Euphytica 85:433-440.

Murphy D.J. and P. Piffanelli (1998) Fatty acid desaturase: structure, mechanism and regulation. In: J.L. Harwood (ed.) Plant lipid biosynthesis: fundamentals and agricultural applications, pp. 95-130, Cambridge University Press, UK.

Murray, M.G. and W.F. Thompson (1980). Rapid isolation of high molecular weight

plant DNA. Nucl. Acids Res. 8:4321-4325.

Nielsen N.C. (1996) Soybean seed composition In: D.P.S. Verma and R.C. Shoemaker (eds) Soybean – Genetics, Molecular Biology and Biotechnology, pp. 127-163, CAB International, Wallingford, UK

Ohlrogge, J.B. (1994) Design of new plant products: Engineering of fatty acid metabolism. Plant Physiol. 104:821-826.

Ohlrogge, J.B. and J.G. Jaworski (1997) Regulation of fatty acid synthesis. Annu. Rev. Plant Physiol. Plant Mol. Biol. 48:109-136.

Ohnishi, J. and M. Yamada (1980) Glycerolipid synthesis in *Avena sativa* cultivar Victory I leaves during greening of etiolated seedlings: 2. α-Linolenic acid synthesis. Plant Cell Physiol. 21:1607-1618.

Okuley, J., J. Lightner, K. Feldmann, N. Yadav, E. Lark and J. Browse (1994) *Arabidopsis FAD2* gene encodes the enzyme that is essential for polyunsaturated lipid synthesis. Plant Cell 6:147-158.

Palmer, R.G., T. Hymowitz and R.L. Nelson (1996) Germplasm diversity within soybean. In: D.P.S. Verma and R.C. Shoemaker (eds) Soybean – Genetics, Molecular Biology and Biotechnology, pp. 1-35, CAB International, Wallingford, UK.

Pantalone, V.R., G.J. Rebetzke, J.W. Burton and R.F. Wilson (1997) Genetic regulation of linolenic acid concentration in wild soybean *Glycine soja* accessions. J. Am. Oil Chem. Soc. 74:159-163.

Pereira, L.A.G. and C.H. Andrews (1985) Comparison of non-wrinkled and wrinkled soybean seed coats by scanning electron microscopy. Seed Sci. Technol. 13:853-860.

Powell, A.A. and S. Matthews (1978) The damaging effect of water on dry pea embryos during imbibition. J. Exp. Bot. 29:1215-1229.

Rahman, S.M., Y. Takagi and T. Kinoshita (1996) Genetic control of high oleic acid content in the seed oil of two soybean mutants. Crop Sci. 36:1125-1128.

Rahman, S.M., Y. Takagi and T. Kinoshita (1997) Genetic control of high stearic acid content in seed oil of two soybean mutants. Theor. Appl. Genet. 95:772-776.

Rebetzke, G.J., V.R. Pantalone, J.W. Burton, B.F. Carver and R.F. Wilson (1997) Genotypic variation for fatty acid content in selected *Glycine max* × *Glycine*

*soja* populations. Crop Sci. 37:1636-1640.

Roehm, J.N. and O.S. Privett (1970) Changes in the structure of soybean triglycerides during maturation. Lipids 5:353-358

Salados-Navarron, L.R., K. Hinson and T.R. Sinclair (1985) Nitrogen partitioning and dry matter allocation in soybeans (*Glycine max*) with different seed protein concentration. Crop Sci. 25:451-455.

Sangwan, N.K., K. Gupta and K.S. Dhindsa (1986) Fatty acid composition of developing soybeans. J. Agric. Food Chem. 34:415-417.

Shanklin, J. and C. Somerville (1991) Stearoylacyl-carrier-protein desaturase from higher plants is structurally unrelated to the animal and fungal homologs. Proc. Natl. Acad. Sci. USA 88:2510-2514.

Shen, N., W. Fehr, L. Johnson and P. White (1997) Oxidative stabilities of soybean oils with elevated palmitate and reduced linolenate contents. J. Am. Oil Chem. Soc. 74:299-302.

Smith, R.R. and C.R. Weber (1968) Mass selection by specific gravity for protein and oil in soybean population. Crop Sci. 8:373-377.

Specht, J.E. and G.L. Graef (1996) Limitations and potential of genetic manipulation of soybean. In: D.P.S. Verma and R.C. Shoemaker (eds) Soybean – Genetics, Molecular Biology and Biotechnology, pp. 91-106, CAB International, Wallingford, UK.

Stobart, A.K. and S. Stymne (1985) The regulation of the fatty-acid composition of the triacylglycerols in microsomal preparations from avocado mesocarp and the developing cotyledons of safflower. Planta 163:119-125.

Takagi, Y., T. Matsuo and K. Ikeda (1979) On the varietal differences and heritabilities for fatty acid composition of soybean seeds in Japan and Korea. Bull. Fac. Agr. Saga Univ. 47:53-64.

Takagi, Y., A.B.M.M. Hossain, T. Yanagita and S. Kusaba (1989) High linolenic acid mutant in soybean induced by X-ray irradiation. Jpn. J. Breed. 39:403-409.

Takeda K. and T. Fukuyama (1987) Tolerance to pre-germination flooding in the world collection of barley varieties. Proc. of the 5th Int. Barley Genetics 5:735-740.

Thseng, F.S., F.F. Hou and S.T. Wu (1996a) Varietal differences in germination rate,

storability, and flooding tolerance of soybean seeds produced from different cultural seasons and location. Jpn. J Crop Sci. 65:525-531.

Thseng F.S., F.F. Hou and K. Takeda (1996b) Studies on the flooding tolerance and water uptake of seed and elongation force of seedling in soybeans. Jpn. J Crop Sci. 65:103-107.

Tully, R.E., M.E. Muzgrave and A.C. Leopold (1981) The seed coat as a control of imbibition chilling injury. Crop Sci. 21:312-317.

Ueno, K. and H. Takahashi (1997) Varietal variation and physiological basis for inhibition of wheat seed germination after excessive water treatment. Euphytica 94:169-173.

Wilson, R.F. and R.W. Rinne (1978) Lipid molecular species composition in developing soybean cotyledons. Plant Physiol. 61:830-833.

Wilson, R.F., J.W. Burton and C.A. Brim (1981) Progress in the selection for altered fatty acid composition in soybean. Crop Sci. 21:788-791.

Wilson, R.F. (1987) Seed metabolism. In: Soybeans: Improvement, Production, and Uses, 2nd edition, pp. 643-686. Agronomy Monograph no. 16. ASA-CSSA-SSSA, Madison, USA

Yadav, N.S., A. Wierzbicki, M. Aegerter, C.S. Caster, L. Perez-Grau, A.J. Kinney, W.D. Hitz, J.R. Booth Jr., B. Schweiger, K.L. Stecca, S.M. Allen, M. Blackwell, R.S. Reiter, T.J. Carlson, S.H. Russell, K.A. Feldmann, J. Pierce and J. Browse (1993) Cloning of higher plant omega-3 fatty acid desaturases. Plant Physiol. 103:467-476.

Yadav, N.S. (1996) Genetic modification of soybean oil quality. In: D.P.S. Verma and R.C. Shoemaker (Eds) Soybean – Genetics, Molecular Biology and Biotechnology, pp. 165-188, CAB International, Wallingford, UK.

Zanakis, G.N., R.H. Ellis and R.J. Summerfield (1994) Seed quality in relation to seed development and maturation in three genotype of soybean (Glycine max). Experimental Agriculture 30:139-215.

Zou, J., Y. Wei, C. Jako, A. Kumar, G. Selvaraj and D.C. Taylor (1999) The Arabidopsis thaliana TAG1 mutants has a mutation in a diacylglycerol acyltransferase gene. Plant J. 19:645-653.

# Acknowledgements