Studies on Genetic Diversity, Al Tolerance Selection Method and Effectiveness of Al Tolerance Breeding Program in Sorghum (*Sorghum bicolor* (L.) Moench)

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A N A S

Studies on Genetic Diversity, Al Tolerance Selection Method and Effectiveness of Al Tolerance Breeding Program in Sorghum (*Sorghum bicolor* (L.) Moench)

by

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1. Anas and Yoshida, T. 2000. Screening of Al–tolerant sorghum by hematoxylin staining and growth response. Plant Prod. Sci. 3:246 – 253.

2. Anas and Yoshida, T. 2002. Genotypic difference of *Sorghum bicolor* in the callus formation and callus growth on aluminum-containing medium. Plant Prod. Sci. 5:242 – 247.

3. Anas and Yoshida, T. 2004. Genetic Diversity among Japanese Cultivated Sorghum Assessed with Simple Sequence Repeats Markers. Plant Prod. Sci. Vol.7. No.2. *In press.*

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Contents

General Summary

Breeding of sorghum tolerance to aluminum toxicity is necessary to improve sorghum production in the arid and semi-arid tropical area. Therefore, evaluation of sorghum germplasm, developments of Al tolerance screening technique and development of sorghum genotypes with Al tolerance were carried out in this study.

SSR markers could discriminate the sorghum germplasm. Sorghum materials used in this study revealed a wide genetic background. The field experiment also exhibited a wide phenotypic and genotypic variation and a significant correlation was found with genetic diversity assessed by SSR markers.

Hematoxylin staining method was proven to be an accurate indicator for Al tolerance and showed a significant correlation with the method by Al-added soil in pots. Some candidate parental lines were selected for using in the breeding program.

Crossings between Al-tolerant and high yield genotypes were conducted. The moderately low heritability of Al tolerance and small genetic gain were observed. Dry weight and plant height were most closely associated with Al tolerance based on path analysis, indicating that selection for high dry weight and selection of Al tolerance in early generations was more appropriate to maximize the Al tolerance.

Tissue culture was proven to be a useful tool for selecting Al-tolerant plants at the callus level. The differences in callus growth and the percentage of callus formation among genotypes were in agreement with those in the hematoxylin staining screening method. Direct gene transfer into florets-derived sorghum callus using particle bombardment gave GUS gene (gene encoding β-glucuronidase) activities in the callus.

Summary

Results of experiments that were carried out in order to develop high yield sorghum genotypes with Al tolerance are shown as follows:

1. All nine simple sequence repeats (SSR) markers showed polymorphic. Diversity indices of each locus marker ranged from 0.70 (Sb6-34) to 0.94 (Sb1-10). Japanese cultivated sorghum showed a wide genetic background, however genotypes from the same place of collection tended to have a close genetic background. The breeding materials in this study that included genotypes from ICRISAT, Japan and USA showed a narrower genetic background than Japanese cultivated sorghum and were distinctly separated from them.
2. Agronomic traits as 1000-grain weight, harvest index, length of head, plant height, stalk diameter and dry weight showed highly significant differences and the variation of heritabilities was observed. Principal component analysis could explain 71% of the total variation. The Spearman rank dissimilarity matrix obtained using phenotypic data was significantly correlated with that obtained with SSR markers. All combinations of phenotypic data were highly correlated with SSR marker data.
3. Hematoxylin staining screening method, which was conducted by staining seedling roots, could be used to screen sorghum tolerance to Al toxicity. Variation of Al tolerance was observed among sorghum genotypes and could be classified into three groups: (i) tolerant genotypes (G4, Real 60, SPA2, SPAD, H11/C8, H11/H2); (ii) susceptible genotypes (G2, G8, G9-1, C9/D12, C9/H11, C9/H13, C9/H2, H13/D12); (iii) intermediate genotypes (G3, G6, G7, H11/H13, H11/D12, C8/D12, TX403/H13).

4. By the growth-response method, which was conducted in Al-added soil in pots, eight genotypes (G3, G4, G7, H11/C8, Real 60, SPA2, SPAD and PI533869) showed tolerance to Al toxicity. Five genotypes (G4, H11/C8, Real 60, SPA2, SPAD) showed tolerance to Al toxicity by both screening methods. C9/H13 showed susceptibility to Al by both methods. These facts show that a similar evaluation for Al tolerance can be obtained by these two methods. For evaluation of large number of genotypes, the hematoxylin staining method is more convenient than the growth-response method.
5. Heritability of Al tolerance in sorghum was moderately low (H = 0.35 and H = 0.43) in two crosses. Consequently, low genetic gain of Al tolerance was also observed. High allocation of resources in early generations for Al tolerance and high selection intensity of Al tolerance must be applied if higher gain for Al tolerance is to be obtained. Low genetic correlations in some agronomic traits and inconsistent relationship with the phenotypic correlations were observed.
6. The correlations among agronomic traits for two crosses showed that dry weight, length of head, grain weight plant^{-1} and plant height were significantly positively correlated to each other. The same directions of correlations were shown by agronomic traits and Al tolerance in both crosses. However, for one cross dry weight and grain weight plant^{-1} were highly significantly correlated with Al tolerance in F_3, whereas dry weight, length of head, grain weight plant^{-1}, days to flowering, plant height and Al tolerance in F_2 population showed a strong correlation with Al tolerance in F_3 for another cross.
7. The multiple linear regression model of two populations for six-independent

agronomic variables explained the 50%~60% variation of Al tolerance. Dry weight and plant height were important components in relationships with Al tolerance. However, based on the overall correlation data and path analysis data, the increasing of dry weight and high selection intensity of Al tolerance was more appropriate to maximize the Al tolerance in sorghum.

8. The percentage of callus formation and the callus growth on Al-containing medium varied with the genotypes, indicating that the tissue culture could be used to screen Al-tolerant genotypes. The differences in callus growth and the percentage of callus formation among genotypes were in agreement with those in the hematoxylin staining.
9. Activities of GUS genes were observed in floret-derived calli that were bombarded with three DNA plasmids (pAct1-D, pWI-GUS and pEX7113). GUS activity was observed in all of 2~3 weeks old floret-derived calli, which was higher than that in 6~7 weeks old floret-derived callus. The pWI-GUS construct carrying double CaMV 35S promoter gave the highest gene transformation in sorghum callus.
10. In conclusion, genetic diversity of sorghum germplasm was assessed using DNA markers, which also showed association with the phenotypic variation in field experiment. Precise and reliable Al screening technique was established and effectiveness of selection could be improved by available information of genetic gain and direct or indirect effect of agronomic traits on Al tolerance. Tissue culture could be used to assist in identification of Al-tolerant genotype. Direct gene transfer using particle bombardment was possible. All of these findings could contribute to the Al tolerance breeding in sorghum.

I. Introduction

Originally, sorghum (*Sorghum bicolor* (L.) Moench) is found in Africa about 5000 years ago. Ethiopia including adjacent areas of northeast of Africa is known as a source of genetic diversity in native sorghum in the world (Poehlman and Sleper, 1995). The world's production of sorghum is about 54.5 million metric ton in 2002, making it the world's fifth leading cereal grain, after maize, rice, wheat, and barley (FAO, 2003).

Sorghum is extensively grown in the semi-arid tropics of Africa and Asia, and was occupied more than 81% of the total area harvest in the world (FAO, 2003). Sorghum also has the distinct advantage compared to maize of being drought-resistant and is grown on a subsistence level by farmers in these regions as a staple food crop for home consumption (Murty and Kumar, 1995). However, sorghum yields hectare^{-1} in Africa and Asia are 0.86 and 0.98 ton, respectively, which are much lower than sorghum yield in Europe (3.36 ton), Australia (2.55 ton) and USA (3.76 ton).

Although sorghum is well adapted in the semi-arid tropical area, the yield is limited by chemical constraints such as high levels of phytotoxic minerals in the soil. The most of the soil in this area are acidic in nature (Fig.1) and aluminum (Al) has been recognized as a major problem in most of acidic soils (Baligar et al., 1989). Approximately 68% of America's tropical land, 38% of Asia's tropical land and 27% of Africa's tropical land are classified as acid soil (Martinez and Estrella, 1999; Delhaize and Ryan, 1995).

Sorghum is generally not tolerant to high Al saturation in the acid soil. Liming is commonly recommended to raise the pH and change the Al exchangeable in order to improve plant growth. However, lime is costly, difficult to obtain in many countries and highly impractical for wide areas. There has been, therefore, a consideration to

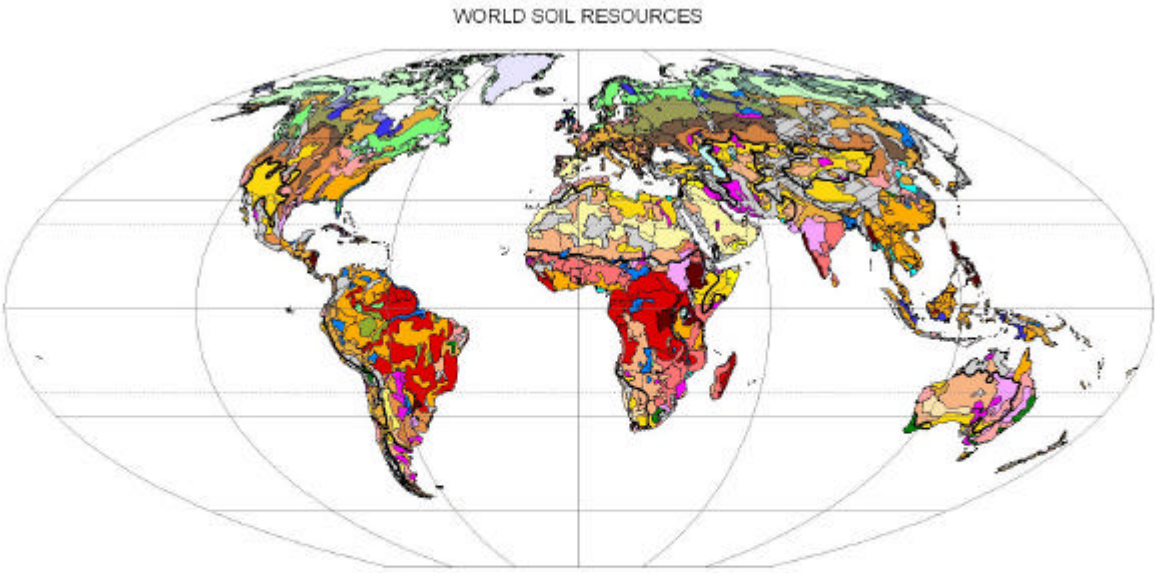


Fig. 1. Map of world soil resources based on WRB and the FAO/UNESCO soil map of the world. Three colors (, ,) show acid soil and soil with high Al contents (FAO/AGL-WRB Map of Soil Resource, 1990).

make efforts in breeding sorghum tolerant to Al.

Three of the important decisions in selection of parents for population development include (i) identifying sources of parental germplasm, (ii) identifying the characters to be improved, (iii) understanding how the characters are inherited (Fehr, 1987). Generally, evaluation of germplasm is the first step in the utilization of germplasm. Evaluation of germplasm involves characterization of the plant performance, observation of the genetic diversity and estimation of the genetic relationship among plants. Genetic distance or genetic diversity among plants in germplasm will provide an opportunity in selection of superior plants that can be used as a parent in a cultivar development program.

Simple sequence repeats (SSR) technique is powerful for the studying genetic diversity in some crops because it utilizes hypervariable regions of the genome comprised of tandemly repeated simple sequences (Karp et al., 1996). There were some utilization

of SSR markers in study of the genetic diversity and genetic redundancy in sorghum germplasm (Dje et al., 2000; Ghebru et al., 2002; Dean et al., 1999). However, information of the genetic diversity among Japanese cultivated sorghum using high-throughput SSR technology has not established yet. Moreover, information of the relationship between Japanese cultivated sorghum and the breeding materials that have some superior characters is very useful for an efficient sorghum-breeding program.

Performance of a plant in field will determine whether it would be chosen in a breeding program. Phenotypic variances that are basically resulted from interaction between environment variance and genotypic variance should have correlations with genetic diversity in the molecular level. If genetic basis of plant variation is known, effectiveness of plant breeding will also increase (Fehr, 1987). Therefore, phenotypic variance that closely related to the gene diversity would increase the effectiveness of plant breeding. Reif et al. (2003) reported that there was relationship between SSR markers and the heterosis grouping in a tropical maize population. However, few papers about correlations between phenotypic performance and SSR data markers in sorghum were reported.

The effectiveness of Al tolerance breeding program also depends on the efficiency of the screening method that can visually identify the most desirable character of a plant. Development of Al-screening method that has high accuracy in screening of Al-tolerant plants is very helpful in a plant breeding program. Almost all the Al-screening methods have used seedling or root development as parameters of Al tolerance. Konzak et al. (1976) suggested that the screening systems not dependent on the rate of seedling or root development would greatly improve the success of the Al-screening procedure.

Hematoxylin staining screening method that could combine the accuracy and simplicity would be more useful than other screening methods. However, there have been

no reports on the use of hematoxylin staining method in the screening of Al-tolerant sorghum.

It is hard to find the homogeneous acid soil in a field and environmental factors interacting with soil Al may sometimes mask the expression of Al tolerance (Campbell and Carter, 1990). Therefore, screening by using the growth response to Al added to the soil in pots in a greenhouse may be superior in this respect. The hematoxylin staining screening methods will be more powerful if it also shows a correlation with Al-screening in the pots.

Understanding of the inherited pattern of Al tolerance will determine the efficiency of Al-screening methods (Fehr 1987). Genotypic variance of Al tolerance in sorghum has been reported (Flores et al., 1988) and variation in genetic controls of Al tolerance was also reported (Martinez et al., 1999). Boye-Goni and Marcarian (1985) estimated the high narrow-sense and broad-sense heritability of Al tolerance in sorghum using nutrient solution culture method. On the other hand, Gourley et al. (1990) reported that Al tolerance was inherited as a dominant character with low narrow sense heritability for shoot and root dry matter production based on green house acid soil study. Therefore, estimation of Al tolerance heritability using hematoxylin staining screening method is necessary in this respect.

Since the Al tolerance must be identified as an important agricultural trait, correlations between Al tolerance with primary agronomic traits will be most important. The studies on correlated characters are important to know how the improvement of one character will cause simultaneous changes in other characters (Falconer and Mackay, 1996). Therefore, studies on genetic correlations between Al tolerance and several agronomic characters will lead to the efficiency in Al-breeding methods.

Consideration of correlated responses suggests that indirect selection might be

possible to achieve more rapid progress than direct selection for the desired character itself (Falconer and Mackay, 1996). Significant correlations might be observed between Al tolerance and several agronomic characters and path analysis is necessary to partition the relative contribution of components via standardized partial-regression coefficient and measures the direct influence of one variable upon another (Dewey and Lu, 1959).

Genetic gain of Al tolerance is very important information for allocation of resources in each generation of selection. Selection intensity and effectiveness of direct selection can be determined appropriately if the genetic gain information is also available. Moreover, information of genetic gain would guide the breeders toward optimum allocation of resources in one stage of selection (Martin and Futi, 2000). However, few published researches about genetic gain of Al tolerance in sorghum are available.

The use of tissue culture to select Al tolerance genotypes at the cellular level has been reported in a number of crop species (Ojima and Ohira, 1983; Smith et al., 1983; Duncan et al., 1995; Conner and Meredith, 1985a; Barnabas et al., 2000). Unfortunately, almost all of the previous selection of tolerant callus was dependent on the variation of Al tolerance appeared during the culture process, but was not in the original explants. Parrot and Bouton (1990) reported that alfalfa plants from the Al-tolerant germplasm rapidly expressed Al tolerance at the callus stage and consequently the selection by tissue culture can be applied to identify Al-tolerant plants. However, this approach has not been applied to sorghum.

The biolistic system, particle bombardment, is widely used for delivery of genetic materials directly into intact cells and tissues. Liu et al. (2003) reported that the promoter played a crucial role in successful gene transformation studies. The different activities of some promoters were observed in sorghum (Emani et al., 2002), sugarcane (Liu et al., 2003) and tall fescue (Takamizo, 1996). The usefulness of promoters (DNA plasmids) in

genetic engineering depends on the number of genes expressed in target cells or tissues after bombardment. Therefore, preliminary evaluation of usefulness of DNA plasmid must be constructed on the transient GUS expression in anther and floret-derived callus of sorghum.

Some series of experiments were carried out in this study in order to resolve the problems in breeding of Al-tolerant sorghum, and look for the efficient selection and breeding method of Al tolerance in sorghum. Generally, all experiments in this study can be summarized into four main topics as follow: (1) germplasm evaluation; (2) identification of character to be improved; (3) understanding how the character is inherited, (4) practical aspect of a development program. Illustration of the entire experiments, which show the systematical method employed in this study, is shown in Fig. 2. In this study, a series of experiments are included as follows:

1. Genetic diversity among Japanese cultivated sorghum assessed with molecular analysis using simple sequence repeats (SSR) markers.
2. Correlation between molecular analysis using simple sequence repeats (SSR) markers and phenotypic performance data in sorghum.
3. Screening of Al-tolerant sorghum by hematoxylin staining method.
4. Screening of Al-tolerant sorghum by growth-response method.
5. Genetic correlation, heritabiity and genetic gain of Al tolerance.
6. Path coefficient analysis of Al tolerance and several agronomic characters of sorghum.
7. Genotypic difference of sorghum in the callus formation and callus growth on Al-containing medium.
8. Direct gene transfer to anther and floret-derived callus of sorghum by particle bombardment.

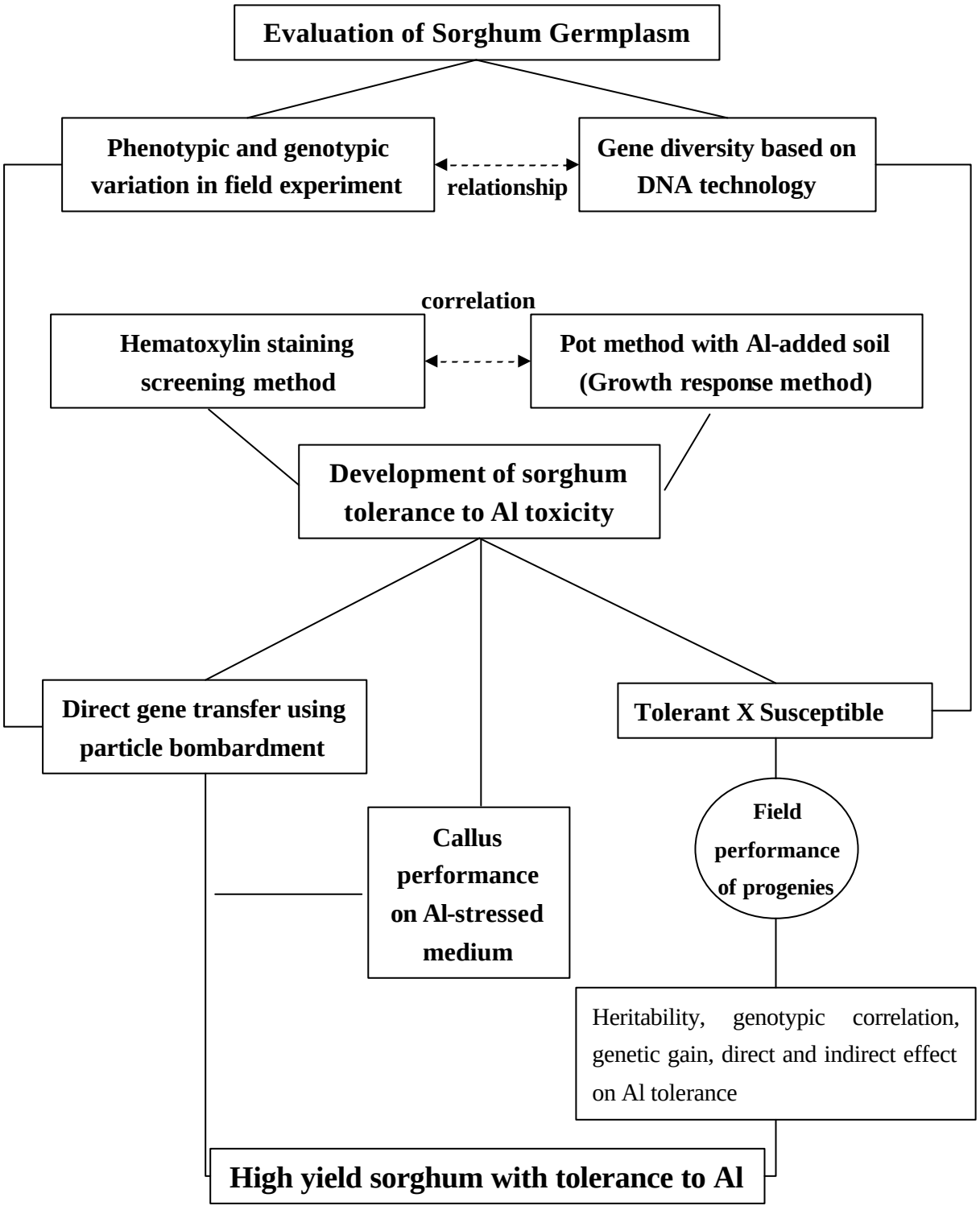


Fig. 2. A flow chart of the systematical method of Al tolerance breeding employed in this study.

II. Evaluation of Sorghum Germplasm

2.1. Genetic Diversity among Japanese Cultivated Sorghum Assessed with Simple Sequence Repeats (SSR) Markers

Simple sequence repeats (SSR) or microsatellite is a short specific sequence of DNA bases or nucleotides, which can contains mono, di, tri, or tetra tandem repeats. Microsatellites are highly variable between individuals and distribute widely throughout the genome. SSR markers are highly discriminative and are often used to distinguish varieties, or even individuals, and reveal parentage and identity (Karp et al., 1996).

SSR markers are useful indices for the studying the genetic diversity of the world sorghum germplasm, and were used for the study of Eritrean sorghum landraces, the elite sorghum inbred lines and the sorghum from Southern Africa (Djè et al., 2000; Ghebru et al., 2002; Smith, et al., 2000; Uptmoor et al., 2003); genetic redundancy in the 'Orange' sorghum (Dean et al., 1999); and mapping of sorghum genome (Bowers et al., 2003; Menz et al., 2002). SSR fingerprints revealed parentage and identity (Karp et al., 1996) and grouping the maize germplasm based on heterotic groups (Reif et al., 2003). The other advantages of SSR markers include the need of only small quantities of a DNA sample, and technical simplicity.

Sorghum is believed to have been brought to Japan about 700~1100 years ago, probably from China through Korea (Matano, 2000). Recently, sorghum is generally used as feed and green fodder for livestock in Japan. In recent years, sorghum has been introduced to Japan from USA and some sorghum cultivars in Japan are probably derived from them. For effective conservation, selection and use of crop genetic resources in plant breeding programs, a population should be characterized by the amount and type of genetic variability it contains (Fehr, 1987). However, the detail of the pedigree history information of Japanese cultivated sorghum was not known and information of genetic

background and genetic diversity of Japanese cultivated sorghum was not reported yet at least at the DNA level.

The author and co-workers had bred inbred sorghum lines with superior characters, such as early maturity, short plant height, high yield and tolerance to Al toxicity (Anas and Yoshida, 2000; 2002; Can and Yoshida, 1999a; 1999b; Can et al., 1997). Almost all of the breeding materials were from ICRISAT-India, Kansas-USA and Chugoku Agriculture Experimental Station-Japan. However, information about genetic background of these materials using DNA-based techniques and relationship with Japanese cultivated sorghum was not observed yet.

The objectives of this study were (i) to determine the genetic diversity of Japanese cultivated sorghum, (ii) to know the diversity of the breeding germplasm and their relationship with Japanese cultivated sorghum, using high throughput SSR technology.

Materials and Methods

Plant materials

The sorghum genotypes evaluated in this study, their origins and genotype grouping are listed in Table 1. Dr. H. Nakagawa (National Institute of Livestock and Grassland Science-Japan) kindly provided the seeds of Japanese cultivated sorghum, which are the commercial hybrid sorghum cultivated widely in Japan (entry no. 13~31). Some of Japanese cultivated sorghums were introduced from USA and some of them were collected from Nagano and Hiroshima prefecture Japan. They include grass sorghum and sweet sorghum.

Seed sources of the breeding germplasm (entry no. 1~12) that were derived from the Institute for the Semi-Arid Tropics (ICRISAT), USA and Chugoku Agriculture Experimental Station – Japan were obtained as described by Can and Yoshida (1999a).

Table 1. List of genotypes used in this study.

Entry No.	Name	Origin/donated	Line grouping	Agronomic groups and morphological note
1	C9/H11	Utsunomiya University	Japan-U.S.A. (JU)	grain sorghum, early maturity, Al - susceptible
2	C9/H13	Utsunomiya University	Japan-U.S.A. (JU)	grain sorghum, tall, late maturity, Al - susceptible
3	H11	Kansas University	U.S.A. (U)	grain sorghum
4	H13	Kansas University	U.S.A. (U)	grain sorghum
5	C9	Chugoku Natl. Agric. Exp. Stn.	Japan (J)	grain sorghum, early maturity
6	C8	Chugoku Natl. Agric. Exp. Stn.	Japan (J)	grain sorghum
7	ICR3	ICRISAT-India	ICRISAT (I)	grain sorghum, dwarf, late maturity, Al tolerance
8	SPA2	ICRISAT-India	ICRISAT (I)	grain sorghum, Al tolerance
9	Real 60	ICRISAT-India	ICRISAT (I)	Al tolerance
10	D12	ICRISAT-India	ICRISAT (I)	grain sorghum
11	SPAD	ICRISAT-India	ICRISAT (I)	grain sorghum, Al tolerance
12	TX403	Kansas	U.S.A. (U)	
13	Haysudan	U.S.A.	U.S.A. (U)	sudangrass
14	Green A	Hiroshima Pref. Agric. Res. Cent.	Japan (J)	sudangrass
15	Tentaka	Nagano Animal Exp. Stn.	Japan (J)	very late
16	High Sugar Sorgo	U.S.A.	U.S.A. (U)	sorgo
17	Piper	U.S.A.	U.S.A. (U)	sudangrass
18	Suzuho	Nagano Animal Exp. Stn.	Japan (J)	sorgo, silage, fodder
19	Touzan Kou 20	Nagano Animal Exp. Stn.	Japan (J)	
20	Sendachi	Hiroshima Pref. Agric. Res. Cent.	Japan (J)	sudangrass
21	Dry Sudan II	U.S.A.	U.S.A. (U)	sudangrass
22	Green Hope	Hiroshima Pref. Agric. Res. Cent.	Japan (J)	sudangrass, early green
23	Ryuujin-wase	Nagano Animal Exp. Stn.	Japan (J)	sorgo, early green, silage, fodder
24	Koutobun	Snow Brand Seed Co. Ltd.	U.S.A. (U)	sorgo, high sugar
25	Hybrid Sorgo NK326	Takii & Co. Ltd.	U.S.A. (U)	
26	Green A (010134)	U.S.A.	U.S.A. (U)	
27	Cyrup Sorgo No.2	U.S.A.	U.S.A. (U)	sorgo
28	MTCI-Sunrise	U.S.A.	U.S.A. (U)	sudangrass
29	Kazetachi	Nagano Animal Exp. Stn.	Japan (J)	very late, dwarf
30	Sugar Grace	U.S.A.	U.S.A. (U)	sorgo, high sugar
31	Natsuibuki	Nagano Animal Exp. Stn.	Japan (J)	sorgo, silage, fodder

Note: Entry No.1 to 12 are sorghum breeding germplasm and No.13 to 31 is Japanese cultivated sorghum (sorghum lines cultivated in Japan).

Entry no. 1~12 had been selected by the pedigree selection method (early maturity, short plant height, high yield and Al tolerance) in the same place at the Crop Science Laboratory – Utsunomiya University.

DNA preparation

Seeds were grown in a growth chamber at 27°C with 12 hours daylight. Genomic sorghum DNA was isolated from leaves collected from 10- to 15-day-old seedlings using Nucleon Phytopure (Amersham). Total DNA was extracted from 0.1g fresh weight of leaves following supplied instructions that yielded about 20 to 100ng total DNA. A 100 dilution of these total DNA extracts was consecutively used for PCR reactions.

SSR primers

In all, nine primer pairs were used for genotyping (Table 2). These oligonucleotide sequences were derived from SSR-containing clones isolated from a size-fractionated genomic DNA library of sorghum cultivar RTx430 (Brown et al., 1996). The SSR markers used are distributed widely across the sorghum linkage groups (A-I), thus giving the comprehensive coverage of the sorghum genome.

PCR amplification

PCR amplification was performed in a volume of 20µL containing approximately 20ng of templete DNA solution (1µL), 1.23 µg to 4.55 µg of each primer, 1 U Qiagen PCR buffer, 200 µM of each dNTP, 15 mM $MgCl_2$ and 2.5 U *Taq* DNA Polymerase (Core Kit-Qiagen). For maximum yield and specificity, annealing temperature (T_m) and cyling times were optimized for each primer pair. Annealing temperature was generally set 2°C below the highest T_m of each primer pair.

Reactions were run in a Takara PCR Thermocycler MP TP3000 (Takara, Biomedicals) with an initial denaturation step for 4 minutes at 94°C; followed by 30 cycles of 94°C for 1 minute, 60°C to 70°C (depend on primers, Table 2) for 2 minutes and

72°C for 1 minute; followed by a final extension at 72°C for 10 minutes. Samples were not diluted prior to electrophoresis.

Electrophoresis and detection

In order to obtain a precise estimate of fragment size and to identify small size differences between fragments, polymorphic PCR products were electrophoresed on 8% non-denaturing polyacrylamide gels (10 cm length) running vertically. After ethidium bromide staining, amplified fragments were visualized using LAS-1000UV mini (Fuji Photo Film Co, LTD) connected with Image Reader Software Version 1.01 (Fuji Photo Film Co, LTD) and size of DNA fragments was determined using Science Lab. 2001 – Image Gauge Ver.4.0 software (Fuji Photo Film Co, LTD), which could automatically recognize the lane and band (the peaks region) and measure the fragment size. Only allele size of loci that were recognized in some literatures as an amplification product of SSR was used for genotyping (Table 2). The size of the DNA fragments was determined relative to the size standard from the 1-kb size marker GIBCO/BRL.

Statistical analyses

Genotypes of individual plants were represented by the allele size at all SSR loci. Genetic distance was estimated by the pairwise difference method and was calculated for each haplotype pair using the parameters F_{ST} in Arlequin ver. 2.000 software program (Schneider et al., 2000). Distances (d_{xy}) were estimated by simply counting the number of different alleles between two haplotypes:

$$\hat{d}_{xy} = \sum_{i=1}^{L} d_{xy}(i)$$

where $d_{xy}(i)$ is the Kronecker function, equal to 1 if the alleles of the i^{th} locus are identical for both haplotypes and equal to 0 otherwise.

The resulting dissimilarity matrix was employed to construct dendograms by the

neighbor-joining method of Saitou and Nei (1987) and the UPGMA method of clustering using 'Neighbor' program in the Phylip package programs (Felsenstein, 1993). Performance of tree was optimized by 'Treeview' application software (Page, 1996).

The genetic variation of each locus which shows the utility and general information of the informativeness of each SSR as a tool was measured in terms of the number of observed alleles and diversity index (Saghai-Maroof et al., 1994) as DI = 1 - $\sum p_i^2$, where p_i is the frequency of the i^{th} SSR allele.

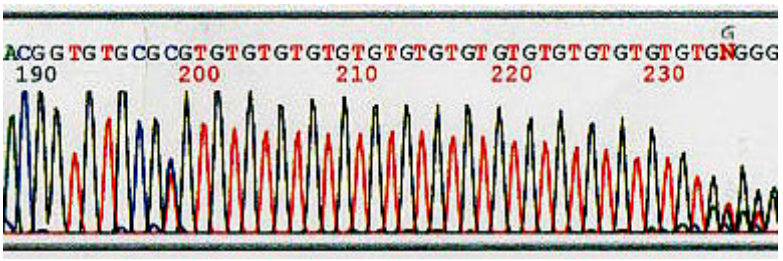
Results

Amplification product and estimation of genetic diversity

In this study, null alleles were consistently observed for Sb6-342 in entry no. 4 and its progeny (entry no. 2) that was developed from crossing of the parent. The higher molecular weight (heteroduplex and faint minor band) than SSR allele band was also observed in some amplification product. However, nine SSR markers produced all the expected SSR allele size as were reported in literatures. To confirm the nature of these SSR alleles, an example of polymorphic SSR alleles of 'Tentaka' (entry no. 15) and 'Touzan Kou 20' (entry no. 19) was sequenced. Different repeats numbers in SSR alleles of these genotypes that were amplified using Sb6-342 primer were observed (Fig. 3).

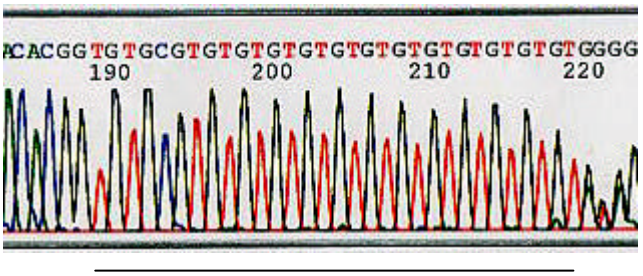
In total, 70 SSR alleles (different fragment size) were observed in this study (Table 2). All nine loci were polymorphic. Fig. 4 shows the example of the polymorphic PCR product from 31 sorghum genotypes amplified with SSR-specific sorghum primer pair Sb6-342. Total number of observed alleles was five. A summary of the number of alleles and diversity indices for each locus is presented in Table 2. The number of alleles per locus ranged from four (Sb5-256 and Sb6-34) to 20 (Sb1-10), and the average number of alleles per locus was 7.2. Diversity indices of each locus ranged from 0.70 (Sb6-34) to 0.94 (Sb1-10).

(A)



repeat region

(B)



repeat region

Fig. 3. Sequence analysis of the SSR alleles amplified with reverse primer Sb6-342 in association with $(AC)_n$ repeat number. (A) 'Tentaka' $(AC)_{17}$ (entry no. 15); (B) 'Touzan Kou 20' $(AC)_{13}$ (entry no. 19).

Genetic relationship among genotypes

All of the sorghum genotypes included in this study could be uniquely identified by nine SSR loci. The clustering of the sorghum genotypes is shown in Fig. 5. All genotypes were distinctly placed in this dendogram, and showed clustering into six groups (A – F).

All genotypes from ICRISAT (I genotype) and their progenies (entry no. 7, 8, 9, 10, 11) were clustered into a cluster group B, which were closely neighboring. Genotypes from Japan (J genotype) were widely scattered into five cluster groups (A, C, D, E and F) and genotypes from USA (U genotype) scattered into five cluster groups (A, B, C, D, and E).

Table 2. Characteristic of the nine SSR loci analyzed.

SSR locus	Flanking sequences	RM[1)]	LG[2)]	FS[3)]	A_T[4)]	DI[5)]
Sb5-236	F: TGC TTG TGA GAG TGC CTC CCT	$(AG)_{20}$	G	165-229	10	0.82
	R: GTG AAC CTG CTG CTT TAG TCG ATG					
Sb6-342	F: TGC TTG TGA GAG TGC CTC CCT	$(AC)_{25}$	A	265-304	5	0.75
	R: GTG AAC CTG CTG CTT TAG TCG ATG					
Sb1-1	F: TCC TGT TTG ACA AGC GCT TAT A	$(AG)_{16}$	H	227-295	9	0.82
	R: AAA CAT CAT ACG AGC TCA TCA ATG					
Sb1-10	F: GTG CCG CTT TGC TCG CA	$(AG)_{27}$	D	225-492	20	0.94
	R: TGC TAT GTT GTT TGC TTC TCC CTT CTC					
Sb5-256	F: AAT TTG CTT TTT GGT CCG TTT	$(AG)_8$	C	154-188	4	0.87
	R: TAG GAA AGA CAG TAC TAG AGG TCA					
Sb6-84	F: CGC TCT CGG GAT GAA TGA	$(AG)_{14}$	F	164-218	6	0.80
	R: TAA CGG ACC ACT AAC AAA TGA TT					
Sb4-72	F: TGC CAC CAC TCT GGA AAA GGC TA	$(AG)_{16}$	B	181-209	7	0.74
	R: CTG AGG ACT GCC CCA AAT GTA GG					
Sb5-206	F: ATT CAT CAT CCT CAT CCT CGT AGA A	$(AC)_{13}$	E	101-149	5	0.76
	R: AAA AAC CAA CCC GAC CCA CTC	$/(AG)_{20}$				
Sb6-34	F: AAC AGC AGT AAT GCC ACA C	(AC)	I	180-202	4	0.70
	R: TGA CTT GGT AGA GAA CTT GTC TTC	$/(AG)_{15}$				

[1)] RM=repeat motif original publications (Brown et al.,1996); [2)] LG=linkage group (Dean et al.,1999); [3)] FS=fragment size (bp); [4)] A_T=Total No. of observe alleles; [5)] DI=diversity index (Saghai-Maroof et al., 1994).

All genotypes from Nagano or Hiroshima prefecture or Chugoku National Agriculture Experimental Station tended to be placed in the same cluster group. Genotypes from Nagano were placed in cluster groups E and F, while genotypes from Hiroshima were clustered within groups C and D. Genotypes from Chugoku National Agriculture Experimental Station was clustered within a group A.

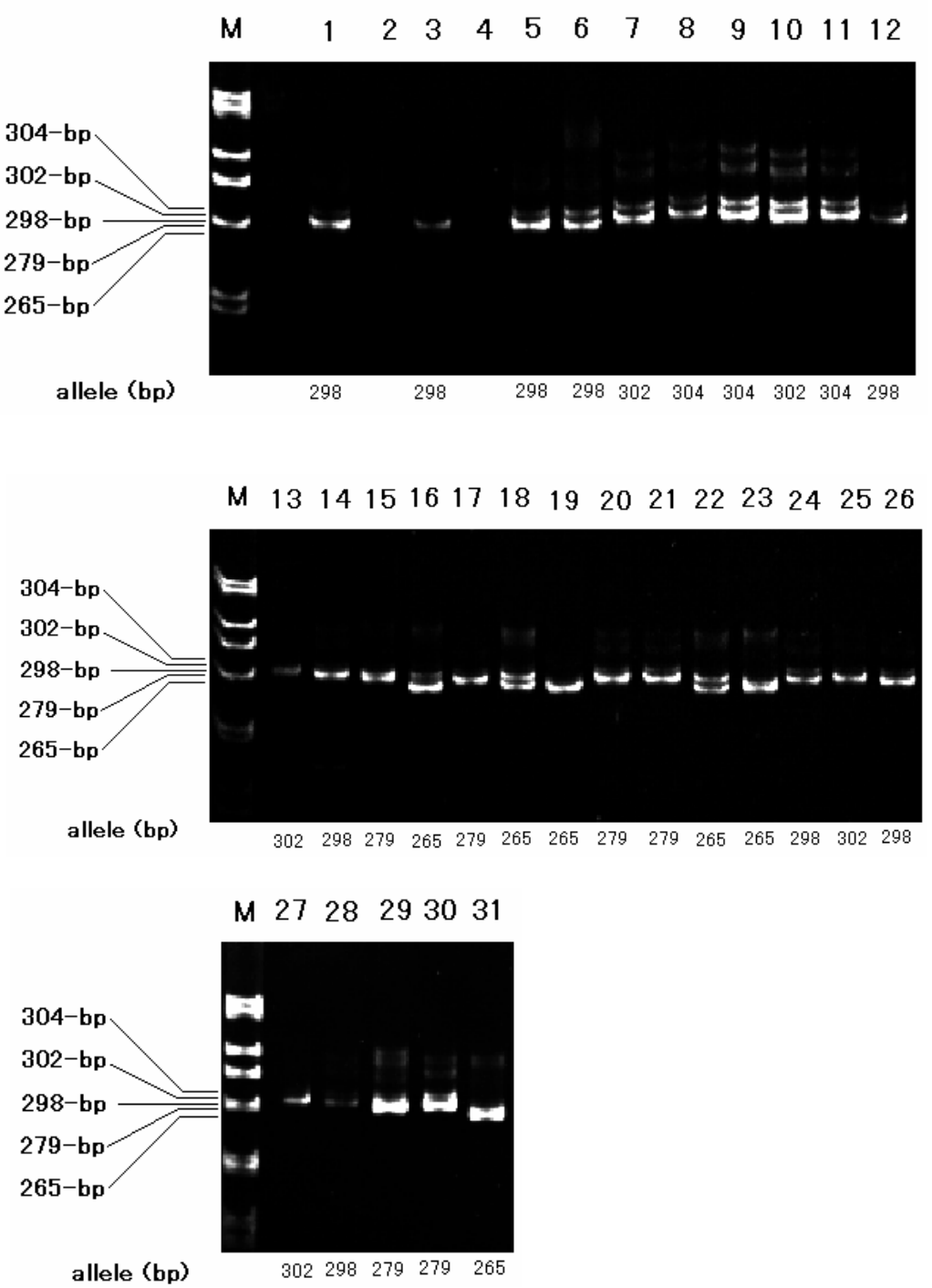


Fig. 4. Photograph of EtBr-stained polyacrylamide gel of polymorphic PCR products from 31 sorghum genotypes amplified with SSR-specific sorghum primer pair Sb6-342. Total number of alleles (different fragment size) was five. Lanes no. = entry no. is shown in Table 1. M=1-kb size marker from GIBCO/BRL. Fragments 220-bp~344-bp were recognized as SSR alleles. Fragments out of the range were not considered in genotyping.

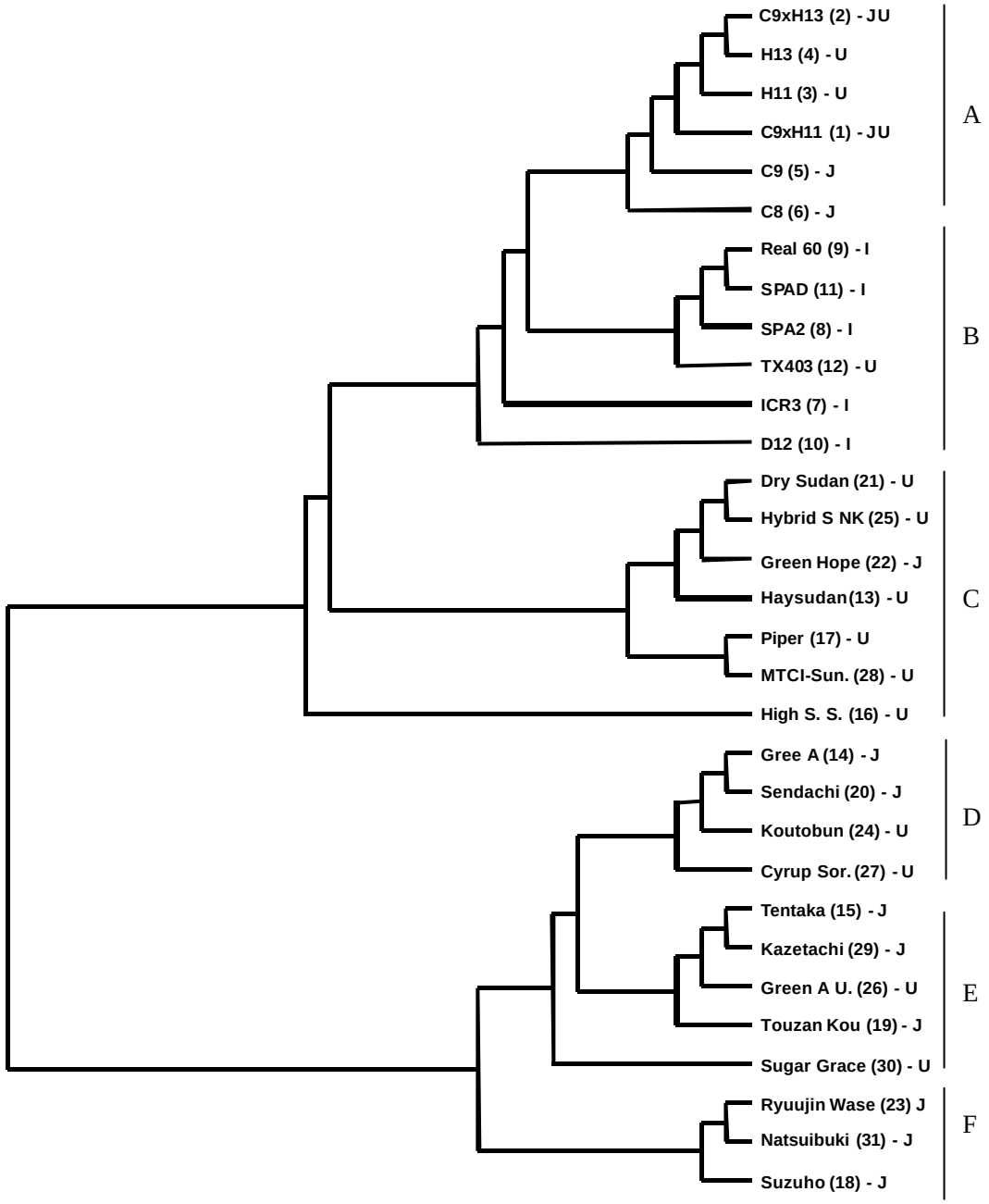


Fig. 5. Dendogram showing genetic relationship among Japanese cultivated sorghum and the breeding germplasm. The numbers in parentheses indicate the entry number shown in Table 1. Letters on the right of genotype's name indicate genotype grouping shown in Table 1 (J=Japan; U=USA; JU=Japan-USA; I=ICRISAT). Letters on the right indicate cluster grouping. Note that branch lengths are not proportional to genetic distance.

A cluster group A had two genotypes from USA and a cluster group C included five genotypes from USA. While two and one genotype from USA were clustered within groups D and E, respectively.

The breeding germplasm collections that included genotypes from Japan, USA, ICRISAT and Japan-USA were clustered into cluster groups A and B, and they were distinctly separated from Japanese cultivated sorghum (cluster groups C, D, E and F). Within the breeding germplasm, all genotypes from ICRISAT were distinctly placed in a different cluster group from the genotypes from Japan and USA.

Discussion

SSR diversity and genotyping

Nine of the SSR markers that were applied for the genetic diversity in this study showed polymorphisms with a relatively high diversity index (Table 2). Brown, et al. (1996) also observed polymorphisms with a high diversity index. The diversity index of each SSR locus ranged from 0.70~0.94, which allowed to discriminate each of 31 sorghum genotypes. This suggests that the utility of each SSR maker as a tool in categorizing sorghum germplasm is high. The diversity index value for comparison with other types of molecular marker can also be used (Brown, et al., 1996). Nine SSR markers listed in this study almost covered all different sorghum genomes (Dean et al., 1999). Thus, these markers should represent a genetic diversity among these sorghum genotypes.

Null alleles were observed only in one inbred line (parent) and its progeny. There seemed to be a close relationship between the parents and the progeny in the capability of SSR amplification. Dean et al. (1999) also observed null alleles for one SSR marker in all five individual samples of sorghum 'Orange'. Studies on null alleles in microsatellite amplification in humans and other mammals have revealed that null alleles are generally

as a result of mutation in the priming region (Callen et al., 1993; Paetkau and Strobeck, 1995).

The minor bands (heteroduplex, faint and shadow bands) have no affect on occurrence of the SSR allele bands; in fact, they can be useful during gel scoring for genotype verification because the minor bands are generally consistent (Wang et al., 2003; Rodriguez et al., 2001). Fait bands were believed to be PCR artifact in homozygous individuals and heteroduplex was caused by the reannealing of non-complementary strands during the later rounds of PCR and only occur in heterozygous individuals (Rodriguez et al., 2001). However in this study, heteroduplex and faint bands were not considered in scoring and genotyping.

Genetic diversity of sorghum germplasm

Japanese cultivated sorghum that included genotypes from Japan and USA (genotype grouping J and U) showed a wide genetic background (scattered into groups C, D, E and F). However, the greater degrees of gene diversity among Japanese cultivated sorghum were found to be associated with the genotypes from Japan (genotype grouping J), which were widely scattered in these cluster groups.

Japanese cultivated sorghum that had been collected from various origins in Japan including research centers and private companies affected apparently a wide genetic diversity in their background. However, genotypes from the same place of collection tended have a close genetic background. For example, three pairs of genotypes from Japan that were placed in the same branch in this study were from the same origin: (1) entry no.14 and 20; (2) entry no.15 and 29; and (3) entry no.23 and 31.

Yang et al. (1996) using DNA markers (RAPDs and ISSRs) also reported that half of the Chinese sorghum from the same area (Liaoning) belonged to one group. This suggests that sorghum genotypes introduced or collected from the same place or country

tended to have a close genetic background. The same or limited genetic source of one's country or research center might have caused low genetic diversity among breeding lines.

Except for entry no. 19 (no maturity information), all genotypes from Nagano within a cluster group E were late or very late maturing (data not shown). This might be why they were distinctly separated from cluster group F. Ghebru et al. (2002) using Eritrean sorghum landraces reported that SSR data reflected their morphological characteristic.

The breeding germplasm (cluster groups A and B) that included genotypes from ICRISAT, Japan, USA and Japan-USA showed a narrower genetic background than Japanese cultivated sorghum. Entry no. 4 and 5 were parents of no. 2 and they were placed in the same branch, while no. 1 is the progeny of no. 3 and no. 5 and they were also placed in the same cluster. Except for no. 9, most of the genotypes from ICRISAT were selected for resistance to leaf diseases, early vigor, stay-greenness, high yield and Al tolerance. Smith et al. (2000) reported that SSR data was in agreement with the pedigree information or performance information.

Conclusion

To my knowledge, this is the first report on genetic relationship among Japanese cultivated sorghum genotypes based on microsatellite markers. Japanese cultivated sorghum that included genotypes from Japan and USA showed a wide genetic background, however genotypes from the same place of collection tended have a close genetic background. The breeding germplasm that included genotypes from ICRISAT, Japan, USA and Japan-USA showed a narrower genetic background than Japanese cultivated sorghum and were distinctly separated from them. The efficiency of using germplasm as a genetic resource and its use can be improved if genetic diversity information is available. For

example, further crossings between the breeding materials and genotypes located in cluster groups D~F might give a wide range of ancestors, which could include promising lines with high yield.

2.2. Correlation between Molecular Analysis Using Simple Sequence Repeats (SSR) Markers and Phenotypic Performance Data in Sorghum

Measurement of phenotypic and genotypic variance in field trials is a common and traditional approach to resolve the genetic differences among genotypes. A field experiment is simpler and cheaper than DNA-based technology. However, environmental variation in the field trials that cannot be removed may mask the actual genetic potency of a genotype. On the other hand, the SSR markers have been proved as a powerful technique for studying the genetic diversity in sorghum (Dje et al., 2000; Ghebru et al., 2002; Smith, et al., 2000; Yang et al., 1996).

Reif et al. (2003) reported a relationship between genetic distance based on SSR markers and heterosis in tropical maize population. This suggested the relationship between phenotypic performance and SSR molecular data. However, there are few reports about the relationship between molecular data and the phenotypic performance in sorghum.

The objectives of this study are: (i) to elucidate the relationship between phenotypic performance and genetic diversity determined with SSR markers; (ii) to study the possibility of grouping inbred lines based on phenotypic performances; and (iii) to estimate the genotypic and phenotypic variation of yield, yield components and primary agronomic traits among inbred lines.

Materials and methods

Field experiment

Twenty-two inbred lines were grown in the field (Table 3). Most of the

Table 3. List of inbred lines and their origin for the study of field and SSR analysis.

Inbred line	Note
H11*, H13*, H2, TX403*	Parental inbred lines, Kansas, USA
C9*, C8*	Parental inbred lines, Chugoku Natl. Agric. Exp. Station, Japan.
SPA2*, SPAD*, D12*	Parental inbred lines, ICRISAT, India
C9/D12, C9/H11*, C9/H13*, C9/H2, C9/TX403, H11/D12, C8/D12, C8/H2, TX403/H11, TX403/H2, TX403/D12, H13/D12, H13/H2	Developed inbred lines. Crop Sci. Lab., Utsunomiya Univ., selected from the cross mentioned in the name.

* = Lines were used both in field experiment and SSR analysis marker; SPA2 = SPA2 94039B; SPAD = SPAD 940006B.

parental inbred lines were derived from ICRISAT - India, Kansas - USA and Chugoku Agriculture Experimental Station - Japan. The experimental design was a randomized complete block design with two replications. The plot size was 1.5 x 2.0 cm^2 with 25 cm spacing between plants and 75 cm spacing between rows. The nominal plant density was 10 plants m^{-2}. Fertilizer was applied at the rate of 2 g m^{-2} of N, P_2O_5 and K_2O as a basal dressing. Hand weeding and watering were practiced as necessary.

Seed yield (grain weight per plot) was recorded for every plot. The yield components consisted of the length of head (from the base of head to tip) and harvest index (ratio of grain weight to total above-ground weight). The primary agronomy traits involved plant height at maturity (from the ground to the tip of panicle of the main stalk), days to flowering (50% of plants in the plot was heading), dry weight, stalk diameter (three cm above the ground) and 1000-grain weight.

Plant height, stalk diameter and days to flowering were measured before harvesting. Grain weight plot^{-1}, length of head, dry weight, 1000-grain weight and harvest index were recorded after harvesting. Stalks (including leaves) were dried at 80^{o}C for four days and weighed to determine total plant dry matter. Heads were dried at 40^{o}C for three days, weighed and threshed to determine grain yield, head yield and 1000-grain weight. The yield components and primary agronomy traits were examined for six plants in the middle of each plot.

SSR amplification

Ten inbred lines with an asterisk were evaluated for SSR analysis (Table 3). Nine primer pairs used for genotyping, DNA sample preparation, PCR amplification and electrophoresis were same with materials and methods in section 2.1.

Statistical analysis

The components of variances were obtainable from the analysis of variance (ANOVA) (Halloran, 1979). Phenotypic variance (σ^2_{ph}) and genotypic variance (σ^2_g) among population of inbred lines were estimated from expected means square (EMS). All phenotypic observation variables were subjected as phenotypic data in genetic population analysis. Associations among the populations were revealed by principal component analysis (PCA) based on Pearson's correlation coefficients.

The Spearman rank dissimilarity (SDM) analysis was performed to estimate the similarity among inbred lines (Steel and Torrie, 1980) for both SSR marker data (base pair size) and phenotypic marker data (phenotypic performance). The Mantel test using Pearson's correlation coefficient was performed to study the correlation between two matrixes and to evaluate the significance of the matrixes.

Results

Field experiment

Six traits of the 21 sorghum lines, 1000-grain weight, harvest index, length of head, plant height, stalk diameter and dry weight, showed highly significant ($p<0.01$) differences in analysis of variance (ANOVA), but for grain weight per plot and days to flowering did not (Table 4). Generally, coefficients of phenotypic variance were slightly higher than coefficients of genotypic variance in the six traits. The highest coefficient of genotypic variance was observed in 1000-grain weight (4.91) and the lowest in days to flowering (1.89). Phenotypic variance of grain weight per plot and days to flowering showed a great difference from their genotypic variance.

The six traits showed variation of heritability. The highest heritability was observed in plant height (0.95) and the lowest in grain weight per plot (0.08) (Table 4). The days to flowering also showed low heritability in comparison with other traits. The days to flowering ranged from 40 days to 62 days. SPA2 flowered very late and produced only a few seeds.

Table 5 shows the phenotypic performance and ranking position of inbred lines. The ranking position of inbred lines for each trait was intended for principal component analysis (PCA). D12 showed the highest 1000-grain weight and TX403 the lowest 1000-grain weight (Table 5). D12 also showed a high harvest index (48.34), whereas, H13, TX403, SPAD and SPA2 showed a low harvest index. These inbred lines also showed a poor vegetative growth. **I**n the principal component analysis using Pearson's correlation coefficient for six phenotypes in 22 lines, the first and second principal component (PC 1 and PC 2) explained 48% and 24% of the total variation, respectively

Table 4. ANOVA of performance of eight traits in 21 inbred lines.

	Range	Means squared	σ^2_g	σ^2_{ph}	σ^2_e	H
Grain weight / plot (g)	62.99 - 226.20	235.82 ^{ns}	9.590 (2.07)	117.91 (1.27)	9.85	0.08
Harvest index (%)	20.00 - 60.24	8.56**	3.120 (4.27)	4.278 (5.00)	3.68	0.73
1000 grain weight (g)	7.09 - 31.19	2.27**	0.808 (4.91)	1.133 (5.81)	4.40	0.71
Length of head (cm)	13.76 - 29.70	1.34**	0.614 (4.18)	0.672 (4.37)	1.80	0.91
Plant height (cm)	75.83 - 179.83	83.45**	39.558 (4.69)	41.727 (4.82)	1.55	0.95
Stalk diameter (cm)	1.07 - 1.93	0.004**	0.002 (2.90)	0.002 (3.31)	2.26	0.77
Dry weight (g)	29.27 - 51.51	3.61**	1.439 (2.98)	1.806 (3.34)	2.13	0.79
Days to flowering (d)	40.50 - 59.0	2.13 ^{ns}	0.426 (1.89)	1.066 (2.99)	3.27	0.40

SPA2 was excluded from the analysis of variance; Number in parentheses are coefficient of variances; σ^2_g = genotypic variance; σ^2_{ph} = phenotypic variance; σ^2_e = environment (error) variance; H (σ^2_g / σ^2_{ph}) = heritability; ** = significant at the 1% level; ns = not significant.

Table 5. Phenotypic performance and ranking position of ten inbred lines in the field experiment.

Inbred lines	100-grain weight (g)	r	Dry weight (g)	r	Length of head (cm)	r	Plant height (cm)	r	Stalk diameter (cm)	r	Harvest index (%)	r
D12	31.19	1	30.55	10	24.82	1	137.08	4	1.20	9	48.34	2
H11	16.93	6	39.81	8	18.93	5	124.75	6	1.19	10	46.27	3
C8	17.23	5	44.11	4	15.41	8	131.67	5	1.31	6	34.34	5
C9	21.16	2	46.87	2	19.43	3	144.67	3	1.25	7	30.33	6
C9/H11	18.19	4	51.51	1	20.03	2	162.33	2	1.24	8	40.86	4
H13	15.07	7	42.38	6	13.76	10	119.08	7	1.41	4	28.62	7
C9/H13	18.30	3	41.63	7	17.11	6	172.17	1	1.43	3	52.00	1
TX403	7.09	10	38.05	9	19.35	4	75.83	10	1.93	1	22.84	8
SPAD	12.12	8	44.57	3	15.76	7	101.58	8	1.37	5	28.27	9
SPA2	10.55	9	43.01	5	14.00	9	100	9	1.58	2	20.00	10

r = ranking position.

(Fig. 6). These principal components could explain 71% of the total variation. Sixteen lines were pooled in one group and six lines were clearly separated from the group (D12, H2, TX403, SPA2, SPAD and H13).

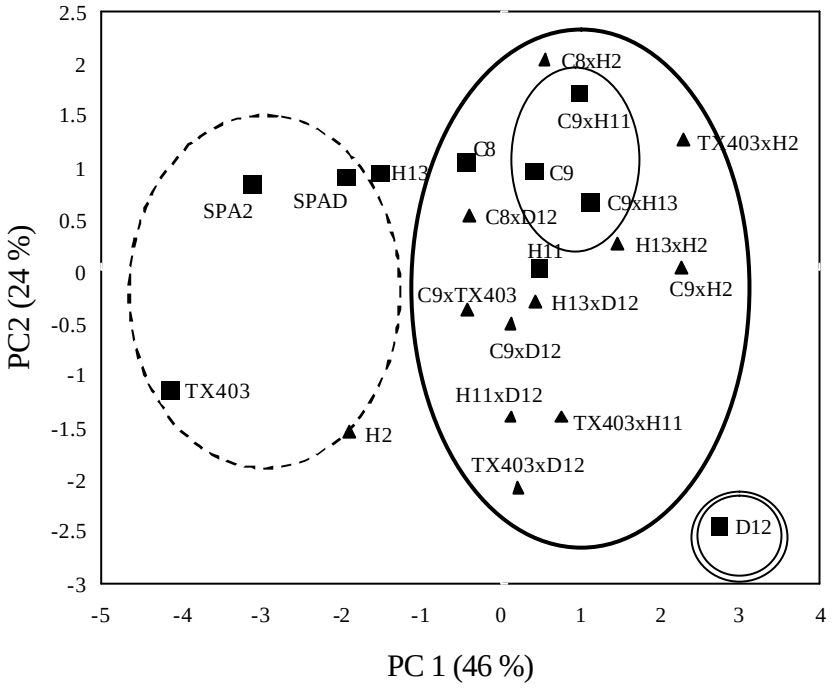


Fig. 6. Principal component analysis of 22 inbred lines using data for six phenotypic data. PC1 and PC2 are the first and second principal component, respectively. ¦ = inbred lines used in both field experiment and SSR analysis marker; ? = inbred lines used only in field experiments; Italic letters are parental inbred lines; Plain letters are developed inbred lines.

Relationship between phenotypic and SSR marker data

Ten inbred lines were analyzed with both field experiment and SSR markers. The dissimilarity matrix of molecular data was plotted against the dissimilarity matrix of single phenotypic to a combination of six phenotypic data in a mantel test and was analyzed using Pearson's correlation (Table 6). Generally, the Spearman rank

Table 6. Correlations of dissimilarity distance matrix between SSR markers and all possible combinations of phenotypic data based on Pearson's correlation.

Correlation between SSR data and single phenotypic data.					
	DW	LE	PH	SD	HI
SSR	**ns**	**ns**	**ns**	**ns**	**ns**
Correlation between SSR data and a combination of two phenotypic data.					
	DW,LE	DW,PH	DW,SD	DW,HI	
SSR	**ns**	**ns**	**ns**	**ns**	
	LE,PH	LE,SD	LE,HI		
SSR	0.325*	0.574**	**ns**		
	PH,SD	PH,HI			
SSR	0.320*	0.306*			
	SD,HI				
SSR	0.446*				
Correlation between SSR data and a combination of three phenotypic data.					
	DW,LE,PH	DW,PH,SD	DW,SD,HI		
SSR	**ns**	0.297*	0.396*		
	LE,PH,SD	LE,SD,HI			
SSR	0.606**	0.589**			
	PH,SD,HI				
SSR	0.460**				
Correlation between SSR data and a combination of four phenotypic data.					
	DW,LE,PH,SD	DW,PH,SD,HI	LE,PH,SD,HI		
SSR	0.549**	0.409*	0.630**		
Correlation between SSR data and a combination of five and six phenotypic data.					
	DW,LE,PH,SD,HI	GW,DW,LE,PH,SD,HI			
SSR	0.568**	0.444**			

Correlations between matrixes were analyzed by the Mantel test; GW was included only in a combination of six phenotypic data; DW=dry weight; LE=length of head; PH=plant height; SD=stalk diameter; HI=harvest index; GW=1000-grain weight; *, ** = significant at the 5% and the 1% level, respectively; ns = not significant.

dissimilarity obtained using of SSR markers was significantly correlated with that obtained with phenotypic data (Fig. 7).

None of the dissimilarity distance matrixes based on single phenotypic data showed significant correlation with the dissimilarity distance matrix of SSR markers (Table 6). Five matrixes obtained for the combination of two phenotypic data significantly correlated with the SSR marker data. All combinations of four, five and six phenotypic data gave results highly correlated with SSR marker data.

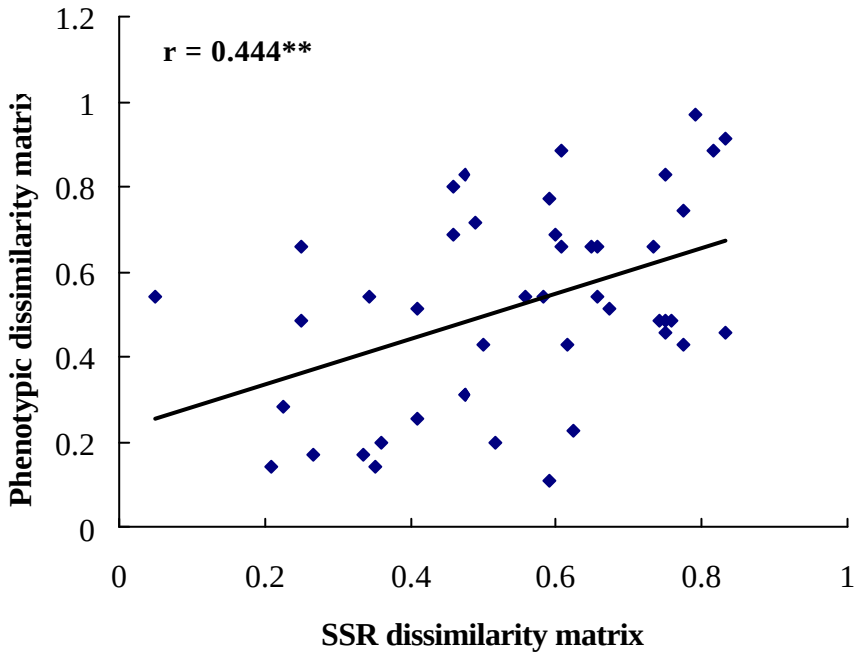


Fig. 7. Correlation between dissimilarity matrix obtained using SSR markers and dissimilarity matrix obtained using a combination of six phenotypic data.

Discussion

Variation of phenotypic traits

Six traits showed high heritability values in this study. Genetic factors greatly affected the 1000-grain weight, dry weight, length of head, plant height, stalk diameter and harvest index. On the other hand, environmental factors influenced performance of grain yield per plot. Grain weight per plot showed a very low habitability. Including the grain yield in principal component analysis and Spearman rank dissimilarity analysis significantly reduced the correlations of their dissimilarity matrix (data not shown). Therefore, grain yield per plot was not included as a phenotypic marker in correlation analysis, hereafter.

There was no significant difference in days to flowering among inbred lines. All sorghum inbred lines used in this study, except SPA2 and SPAD, were routinely planted in the same area. Craufurd et al. (1999) reported that adaptation of sorghum to the diverse environments was largely determined by photoperiod sensitivity and the minimum time to flower. Consequently, a very small genetic variation will be observed in days to flowering (Table 4), and therefore, days to flowering might not be a useful phenotypic marker in this study.

Grouping of inbred lines based on principal component analysis

Principal component analysis using phenotypic data clearly separated the developed inbred lines from the parental inbred lines. On the basis of their performance data, developed inbred lines were included in one group (bold circle in Fig. 6) because the members of this group had a relatively similar phenotypic performance (Table 5). All inbred lines in this group were previously selected for short plant statue, early

maturity and high yield. In addition, they were grown at the same place for many years.

Two inbred lines from ICRISAT (SPA2 and SPAD) showed a poor performance in the field experiment. SPA2, SPAD and TX403 showed a similar in 1000-grain weight, plant height and harvest index (Table 5). According to the principal component analysis, TX403, SPA2 and SPAD were clearly separated from the above-mentioned group and positioned relatively close to each other (broken circle in Fig. 6).

D12 showed a great difference from other inbred lines in 1000-grain weight, length of head and harvest index. D12 was greatly separated from SPA2, SPAD and TX403 in PCA analysis (double circle in Fig. 6). Two inbred lines (C9/H11 and C9/H13) were generated from the same parent and have a genetic background closely related to C9.

Correlations between phenotypic performance and molecular data

Reif et al. (2003) reported that there were significant correlations between SSR molecular data and panmictic midparent heterosis for grain yield, days to silking and plant height in tropical maize population. Their study is theoretically slightly different from this study, but it suggested that there was a relationship between SSR molecular data and phenotypic performance data.

The significant correlation between dissimilarity matrix of molecular data and phenotypic data (Fig. 7) suggested that there was a close association between distribution of the SSR markers in sorghum genome and distribution of genes which controlled the six phenotypic variables in sorghum genome. The correlation values increased in parallel with the increase in the number of phenotypic analyzed in

combination (Table 6). The Spearman rank dissimilarity for less than four phenotypic data in combination showed a lower value with the molecular marker data. Single phenotypic data did not show any significant correlation with the molecular data marker (Table 6). This suggested that using more phenotypic markers should provide a good description of the genotype.

All combinations of phenotypic data including length of head and stalk diameter always gave a high correlation with the nine SSR data markers. Even in the combination of two phenotypic data, these two phenotypes showed a highly significant correlation with SSR markers (Table 6). This suggested that combination performance of length of head and stalk diameter were portrayed by nine SSR markers.

Conclusion

The results of analysis using a combination of more than four phenotypic data fairly correlated with SSR marker data. The SSR markers highly correlated with the combination of phenotypic data that included the length of head and stalk diameter. Generally, grouping of inbred lines based on their phenotypic performance data (combination of the six phenotypic data) was similar to that based on SSR markers. However, SSR markers gave more accurate and detailed grouping of inbred lines. On the basis of the phenotypic performance, D12, H11, C9/H13 and C9/H11 were promising parents that could be used in future plant breeding programs.

III. Development of Al tolerance Screening Method

3.1. Screening of Al-tolerant Sorghums by Hematoxylin Staining Method

Aluminum (Al^{3+}) toxicity is considered as a major constraint for the production of maize, sorghum, and rice in acid soil. Al damages the root apex (root cap) and inhibits root hair growth, resulting in nutrient deficiency and leaf disorder (Delhaize and Ryan, 1995; David et al., 1997; Chang et al., 1998; Godbold and Jentschke, 1998).

In general, the Al-screening technique can be classified into laboratory screening and field screening. Laboratory screening methods include screening of plants with solution-soaked paper and solution culture (Konzak, et al., 1976), soil-petri dish (Hill et al., 1989), and screening in pots in a greenhouse (Baligar et al., 1989). For sorghum, screenings in the field (Duncan, 1988; Flores et al., 1988; Miller et al., 1992), and in pots or nutrient solution (Furlani and Clark, 1981; Boye-Goni and Marcarian, 1985; Gourley et al., 1990; Miller et al., 1992) are commonly used for selection of Al-tolerant genotypes.

A rapid screening method is needed to select a large number of new genotypes or new inbred lines in plant breeding, such as solution-soaked paper, solution culture and soil-petri dish methods used to evaluate Al-tolerant sorghum. All of these rapid screening techniques use the response to Al of the rate of seedling germination and root development. However, the method using such growth responses would curtail the accuracy of screening. Detection systems not dependent on the rate of seedling or root development would greatly improve the success of screening procedure (Konzak et al., 1976).

Screening by using hematoxylin staining of seedling roots (hematoxylin staining method), which requires less time and simpler pH management than the other methods, is very useful for selection or screening a relatively large population in a breeding program. Measurement of Al tolerance is based on the staining pattern of the root. The hematoxylin staining method is a very common technique for the evaluation of Al tolerance in wheat (Polle et al., 1978; Takagi et al., 1981; Wallace et al., 1982) and barley (Minella and Sorrells, 1992), but there have been no reports on the screening of Al-tolerant sorghum.

Materials and Methods

Genotype

Twenty-two sorghum genotypes were evaluated, which consisted of parental lines, inbred lines and Al-tolerant lines. Parental lines and inbred lines were obtained from the germplasm collection of Crop Science Laboratory, Utsunomiya University and Chuugoku National Agricultural Experiment Station, Japan. ICRISAT (India) and USDA (USA) kindly provided tolerant lines. Real 60, SPA2 and SPAD (tolerant lines) have been screened under 60%-80% Al saturation at Carimagua, Matazul and Chilichau-Columbia by ICRISAT (Reddy, personal communication). Table 7 lists the genotypes and their origin.

Hematoxylin staining method

A hematoxylin staining method based on the technique of Polle et al. (1978) with some modifications was used. Levels of Al concentration of 0, 17.87, 35.73, 53.60, and 71.46 ppm Al (0, 74, 148, 222, and 296 µM) were designed. Al was added to the

Table 7. List of genotypes and their origin for hematoxylin screening.

Genotypes	Name	Origin
Parental lines	G2, G3, G4, G8, G9-1	Collection of Crop Sci. Lab., Utsunomiya Univ.
	G6, G7	Chugoku Natl. Agric. Exp. Station, Japan.
Inbred lines	C9/D12, C9/H11 (high yield), C9/H13, C9/H2, H11/C8 (high yield), H11/H2, H11/H13, H11/D12, C8/D12, TX403/H13, H13/D12	Breeding lines, Crop Sci. Lab., Utsunomiya Univ., selected from the cross-mentioned in the name.
Tolerant lines	Real 60, SPA2, SPAD	ICRISAT (INDIA)
	PI 533869	USDA (USA)

SPA2 = SPA2 94039B; SPAD = SPAD 940006B.

distilled water from 0.1 M $AlCl_3 \cdot 6H_2O$ stock solution. The choice of these Al concentrations was based on the sorghum screening for Al tolerance in nutrient solution (Furlani and Clark, 1981).

Seeds were germinated directly on the planting tray in the plastic container filled with distilled water and kept in an incubator in darkness at 25°C for 29 hours. The tray was covered with thin clear plastic plate. Planting trays (19.3cm x 25.3cm) had 560 holes (0.7cm x 0.7cm) to place the seeds. A nylon fabric screen was glued onto the tray. After the radicle had completely emerged, water in the plastic container was replaced with nutrient solution (4.0 mM $CaCl_2 \cdot H2O$, 6.5 mM KNO_3, 2.5 mM $MgCl_2 \cdot 6H_2O$, 0.1 mM $(NH_4)_2SO_4$, 0.4 mM NH_4NO_3) that was adjusted to pH 4.0 with 0.25 M HCl. Seedlings were grown for 31 hours on the above nutrient solution in the growth chamber under the light at 25°C. The surface of the planting tray with the seedlings was covered with a thin clear plastic plate. The seedlings were then grown for 17 hours on

the nutrient solution with 0 to 296 µM Al without plastic cover and kept in the growth chamber in the light at 25°C. The planting tray was then placed on distilled water for 30 minutes and placed in a container with 0.2% hematoxylin solution for 15 minutes. Prior to evaluation of staining, seedlings were washed with distilled water for 30 minutes. Stained root tips of six seedlings per genotype were then evaluated for scoring based on the staining of roots as shown in Table 8.

Table 8. Scoring of hematoxylin staining at various Al concentrations and the relative root length (RRL) in acid soil containing 447 ppm in relation to Al tolerance.

Staining pattern of root tips				RRL (%)		
Al concentration (ppm)					Score	Remark
17.87	35.73	53.60	71.46	447 ppm Al		
NS	NS	NS	NS	79.2 ≤ 91.9	1	Very tolerant
NS	NS	NS	S	66.5 ≤ 79.2	2	Tolerant
NS	NS	S	S	53.8 ≤ 66.5	3	Intermediate
NS	S	S	S	41.1 ≤ 53.8	4	Susceptible
S	S	S	S	0 ≤ 41.1	5	Very susceptible

NS = not stained; S = stained; RRL = relative root length, which will be described in the 3.2 section.

Results and Discussion

Six seedlings per genotype with well-developed roots 1.5 cm or longer were chosen for scoring in the hematoxylin staining test. Although the length of seedling roots varied either in the solution with or without Al, Polle et al. (1978) found no relationship between the staining response of genotype and the length of seedling roots in the hematoxylin staining test. In addition, the seedlings were treated with Al for a

short time.

The score ranged from 1 to 5 (Table 9). Eight genotypes showed the score for 4 or 5 (susceptible); seven genotypes showed score 3 (intermediately tolerant); and six genotypes showed score 1 or 2 (tolerant). Variation in the hematoxylin score from complete staining to no staining was also observed in wheat, barley and pearl millet (Takagi et al., 1981; Minella and Sorrells, 1992; Yoshida and Shigemune, 1999). Minella and Sorrells (1992) observed difference in staining pattern of root to select 37 barley genotypes, and determined the loci number and allelic relationships among barley genotypes of diverse origins.

Roots of G4 and SPA2 were not stained at any Al level, indicating that they were very tolerant (Table 9 and Fig. 8). Polle et al. (1978) and Takagi et al. (1981) reported that the roots of a tolerant wheat genotype were not stained with hematoxylin. This may be mainly due to the high pH of the cell wall in a tolerant plant. The high pH immobilizes Al and thus protects the plants from Al-toxicity (Ownby, 1993; Andrade et al., 1997).

All genotypes from ICRISAT (Real 60, SPA2, SPAD) showed tolerant conformity with the score of hematoxylin staining. These genotypes were classified by ICRISAT as tolerant genotypes, and their roots were not stained in a solution even with 53.60 ppm Al (Table 8). In contrast, roots of G2, G8 and G9-1 exhibited complete staining at all Al levels and were classified as very susceptible genotypes.

G3, G6, G7, H11/H13, H11/D12, C8/D12 and TX403/H13 were classified as genotypes with intermediate tolerance. Their roots exhibited staining either at 53.60 or

Table 9. Aluminum tolerance of sorghum genotypes evaluated by hematoxylin staining and growth response method.

No	Geno-type	Score of hematoxylin staining[1)]	Score of RRL[2)]	No	Geno-type	Score of hematoxylin staining	Score of RRL
1	G2	5	-	12	H11/C8	2	2
2	G3	3	1	13	H11/H2	2	-
3	G4	1	1	14	H11/H13	3	-
4	G6	3	4	15	H11/D12	3	-
5	G7	3	1	16	C8/D12	3	3
6	G8	5	3	17	TX403/H13	3	-
7	G9-1	5	-	18	H13/D12	4	-
8	C9/D12	4	-	19	Real 60	2	1
9	C9/H11	4	3	20	SPA2	1	1
10	C9/H13	4	5	21	SPAD	2	1
11	C9/H2	4	-	22	PI 533869	-	2

SPA2 = SPA294039B; SPAD = SPAD940006B; [1)] Score of hematoxylin staining was based on staining pattern of root; [2)] Score of growth-response was based on RRL at 447 ppm Al, which will be described in the next section.

71.46 ppm Al. Although the segregation pattern was not studied here, crossing between the parents of intermediate tolerance (G7 and G3) resulted in a susceptible progeny (C9/H13). Boye-Goni and Marcarian (1985) reported that predominantly additive genetic effects with some degree of dominance controlled the Al tolerance trait in sorghum, while Gourley et al. (1990) reported that tolerance to Al-toxicity in sorghum

was inherited as a dominant character. C9/H13 had high yields in the field (Can, personal communication) but was shown to be susceptible to Al toxicity.

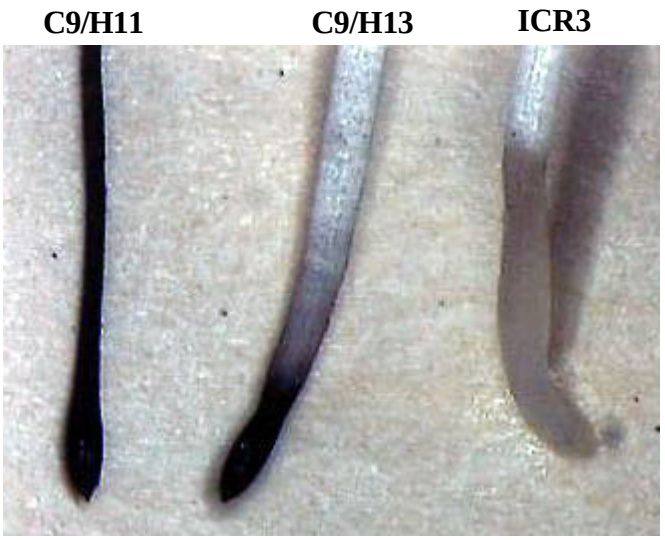


Fig. 8. Staining of a root tip of a susceptible and a tolerant genotype by the hematoxylin staining method.

Conclusion

Hematoxylin staining screening method could be used to screen sorghum tolerance to Al toxicity. Variation of Al tolerance was observed among sorghum genotypes and could be classified into three groups: (i) tolerant genotypes (G4, Real 60, SPA2, SPAD H11/C8, H11/H2); (ii) susceptible genotypes (G2, G8, G9-1, C9/D12, C9/H11, C9/H13, C9/H2, H13/D12); (iii) intermediate genotypes (G3, G6, G7, H11/H13, H11/D12, C8/D12, TX403/H13).

3.2. Screening of Al-tolerant Sorghums by Growth-response Method

For selection of Al-tolerant genotypes, a precise screening technique to evaluate sensitivity of plants to Al is necessary. Therefore, the results of the hematoxylin staining method should be compared with other screening methods. It is important to compare the laboratory screening methods with the field screening methods.

Field screening for Al tolerance would be the best approximate for selecting Al-tolerant plants. In practice, however, reliable ranking of tolerance in the field screening is difficult because the Al concentration in soil may not be uniform and because environmental factors interact with soil Al to mask the expression of Al tolerance (Campbell and Carter, 1990). Screening by using the growth response to Al added to the soil in pots in a greenhouse (referred to as growth-response method hereafter) may be superior in this respect.

There was a correlation between the performance of sorghum in the greenhouse study and grain yield in the field (Baligar et al., 1989). The plants that showed severe reduction of shoot or root weight in a greenhouse showed also low grain yield in the field. There was also a similar genotype response to Al-induced stress in nutrient solution and to acid-soil stress in the field (Duncan et al., 1983).

The objectives of this research are to compare the results of Al tolerance assessed by hematoxylin staining screening method with those obtained by the growth-response method mentioned above.

Materials and Methods

Genotype

Twelve genotypes selected by the hematoxylin staining method were evaluated again for acid soil tolerance in a pot: G3, G4, G6, G7, G8, H11/C8, C9/H11, C8/D12, C9/H13, Real 60, SPA2, and SPAD. These genotypes were chosen for a wide range of Al tolerance. Based on the data obtained by hematoxylin staining method, SPA2 and C9/H11 were selected as representative tolerant and susceptible genotypes, respectively. Although C9/H11 genotype was not the most susceptible genotype with the hematoxylin staining method, it was chosen because it had also early maturity character in the field (Can and Yoshida, 1999c, d). Genotype PI 533869 was evaluated only by the culture on acid soil, because the number of seeds was very limited. In addition, the growth of PI 533869 genotype was very poor during the preliminary test by the hematoxylin staining method.

Growth-response method

The homogenous soil *"Kumiai Kokuryuubaido"* (*Zennou*, Japan) was used for pot experiment. The *"Kumiai Kokuryuubaido"* contains micronutrients, heat dried humus and fertilizer (0.28 N Kg^{-1}, 0.28 P Kg^{-1}, 0.28 K Kg^{-1}). About 350 g soil in a pot, 9.5 cm in diameter and 9 cm in depth, was mixed with 100 mL $AlCl_3 \cdot 6H_2O$ solution at various concentrations (8.32, 16.5 and 24.9 mM) to prepare the soil with different acidity.

The Al solution was adjusted to around pH 3 to 3.5 by HCl. The soil mixed with Al solution was kept for 24 hours and then the seeds were sown in a greenhouse.

The greenhouse temperature during the experiment ranged from 25°C to 28°C in the daytime and 23°C to 25°C at night. Five seeds were sown per pot and thinned to four plants after seedling emergence. Three days after sowing, 40 mL of distilled water was added to keep the soil moist. Seven days after planting, 40 mL of $AlCl_3 \cdot 6H_2O$ solution was added to the soil.

In a preliminary experiment, Al at various concentrations (11.18 to 671.00 ppm Al) was added to the soil to determine the Al concentration that gives the widest range of toxic symptoms in the root. The soils containing 224 ppm, 447 ppm and 671 ppm Al (8.32, 16.5 and 24.9 mM) gave significant differences in the root symptoms (data not shown), and used for screening in this experiment. Baligar et al. (1993) used dark red latosol soil with 2, 41 and 64 % Al saturation to observe the growth and uptake parameter of sorghum. Flores et al. (1988) used 40% and 60% Al saturation of acid ultisol soil to determine the effects of Al saturation on the growth and yield of sorghum. De Sousa (1998) used acid soil (pH 4.2 to 4.9) that contained 25 to 42.7 mM Al for classification of Al tolerance of wheat cultivars. On the other hand, the Al at the concentration of 0 to 296 µM was usually added to the complete nutrient solution for the selection of tolerant sorghum or physiological investigation (Gourley et al., 1990; Tan and Keltjens, 1995; Furlani and Clark, 1981).

Plants were harvested 15 days after sowing, roots were rinsed with stream flow of water tap, and data for the longest root length (from the base of the stem to root tip) and shoot length (from the base of stem to the longest tip of the longest leaf) was measured. The relative root length (RRL) ([the longest root length in soil with Al / the

longest root length in soil without Al] x 100) was scored as shown in Table 8.

To achieve a greater degree of precision for genotype comparison than for Al concentration response, the split plot experimental design was used with genotype assigned to the sub plot and Al concentration to the main plot.

Results and Discussion

The solubility of the Al compound and severity of their toxic effect on the plant are influenced by many chemicals and physical factors such as pH and organic matter (Dong et al., 1999; Shuman et al., 1990; Kapland and Estes, 1985). The soil pH was still under 4.7 in all pots at the end of experiment, indicating that Al^{3+} in the soil was available to the plant throughout the experiment, whereas high concentration of Al (8.32, 16.5 and 24.9 mM) was needed to obtain significant effect on root growth among genotypes.

RRL of all genotypes decreased in response to the increase in Al concentration (Fig. 9), and roots tended to become swollen with a stubby appearance in response to Al. Tan and Keltjens (1995) reported that Al toxicity was expressed by the direct damage of roots (stubby and discolored root) with a concomitant reduction in specific root length. Negative values of correlation coefficients between RRL and Al concentration were observed for all genotypes (Table 10). Inhibition of root elongation is widely recognized as Al stress symptoms in plants (Delhaize and Ryan, 1995; Gallego and Benito, 1997; Martinez and Estrella, 1999).

Utilizing shoot length as a criterion of selection for Al toxicity was not reliable,

because relative shoot length (RSL) of G6 did not show a significant correlation (r = -0.523) with Al concentration, and some genotypes showed a weak correlation with Al concentration (Table 10). Shoot growth measurements often produced contradictory results compared with total root length measurements (Bushamuka and Zobel, 1998).

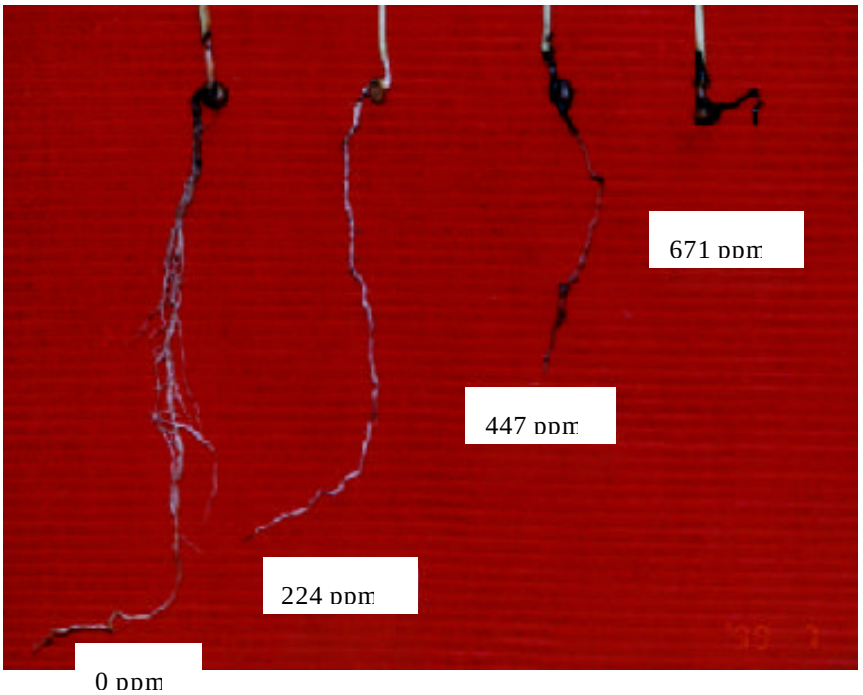


Fig. 9. Decrease of relative root length (RRL) in response to increased Al concentrations determined by the growth-response method, which will be described in the 3.2 section.

Analysis of variance showed that the effect of Al concentration was not significant in this experiment (Table 11). With split plot design, the precision for the measurement of the effects of the main-plot factor (Al concentration) was sacrificed to improve that of the subplot factor (genotype) (Gomez and Gomez, 1984). Interaction between the genotype and Al concentration (G x C) was significant, indicating the different response of genotypes with the Al concentration.

Table 10. Correlation coefficients between root length (RRL) or relative shoot length (RSL) and Al concentration of 13 genotypes determined by the growth-response method.

Genotype	Root length	Shoot length
G3	- 0.843 **	- 0.617 *
G4	- 0.873 **	- 0.859 **
G6	- 0.984 **	- 0.523
G7	- 0.819 **	- 0.995 **
G8	- 0.936**	- 0.936 **
H11/C8	- 0.924 **	- 0.958 **
C9/H11	- 0.969 **	- 0.934 **
C8/D12	- 0.968 **	- 0.993 **
C9/H13	- 0.969 **	- 0.854 **
Real 60	- 0.867 **	- 0.972 **
SPA2	- 0.950 **	- 0.605 *
SPAD	- 0.834 **	- 0.877 **
PI533869	- 0.931 **	- 0.878 **

*, ** = significant at the 5% and 1% level, respectively.

Table 11. Analysis of variance for relative root length (RRL) and relative shoot length (RSL) determined by the growth-response method.

Source	df	Means square	
		RRL	RSL
Replication	1	164.934	135.387
Al level (C)	3	35529.999	16882.172
Error a	3	135.864	268.862
Genotype (G)	12	503.238**	947.498**
G x C	36	275.501**	523.770**
Error b	48	128.119	220.754

** = significant at the 1% level.

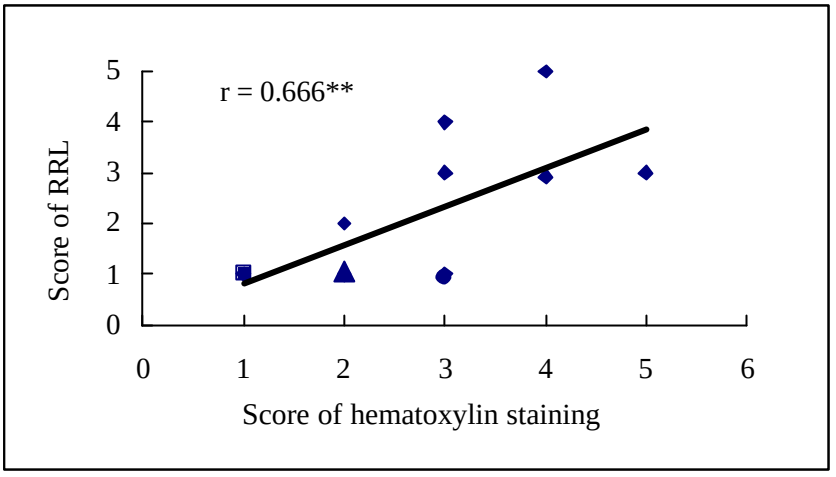


Fig 10. Correlation between the score of hematoxylin staining and that of relative root length (RRL). **Significant at the 1% level. ? G6, G8, C9/H11, C9/H13, H11/C8, C8/D12; ¦ ¦ G4 and SPA2; ? ? Real 60 and SPAD; ?? G3 and G7.

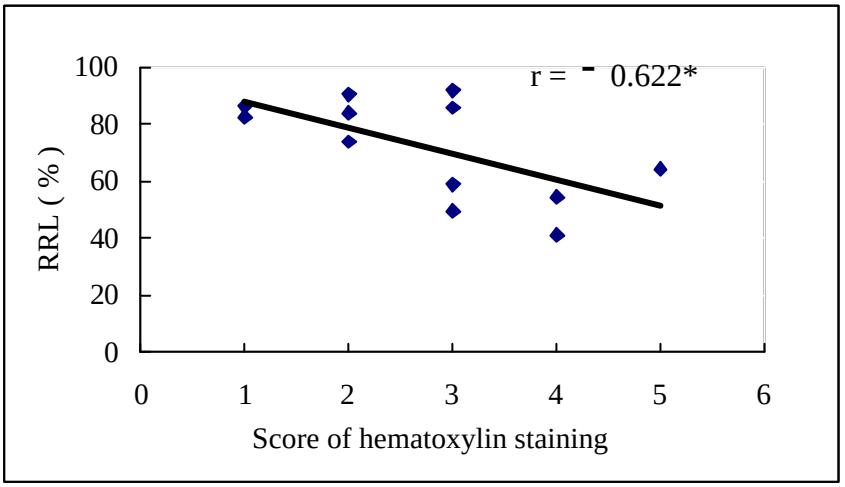


Fig 11. Correlation between the score of hematoxylin staining and relative root length (RRL) in the soil containing 447 ppm Al. * Significant at the 5% level.

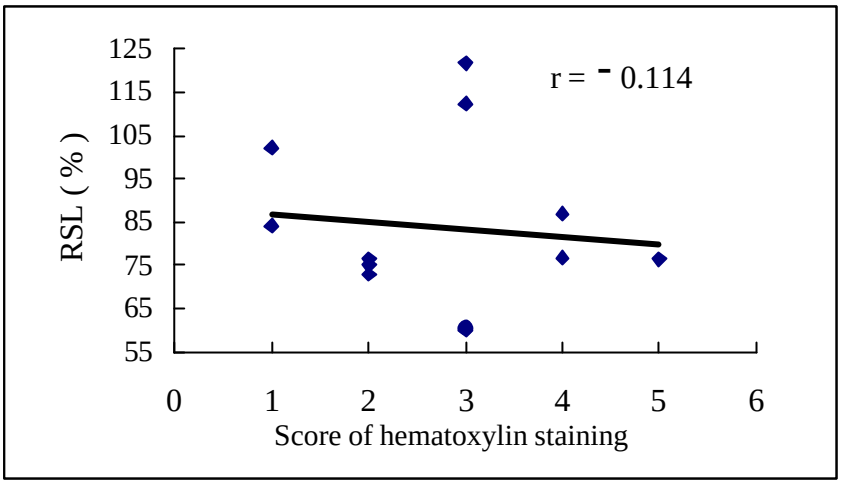


Fig. 12. Correlation between the score of hematoxylin staining and relative shoot length (RSL) determined in the soil containing 447 ppm Al. ?? G7 and C8xD12, ? G3, G4, G6, G7, C9/H11, C9/H13, H11/C8, Real 60, SPA2, and SPAD.

Tan and Keltjesns (1995) reported that root damage well reflected genetic differences in response to Al at a high acidity.

The mean RRL at different Al concentrations showed that the 447 ppm Al gave the widest range of toxic response among genotypes. Eight genotypes were tolerant, three intermediately tolerant and two susceptible (Table 9). G3, G4, G7, Real 60, SPA2 and SPAD were significantly more tolerant than G6, G8, C9/H13 and C9/H11 (Table 12). RRL of plants grown with 224 ppm Al and 671 ppm Al showed Al toxicity symptoms but differences among genotypes were difficult to distinguish (Table 12).

Correlation between hematoxylin staining method and growth-response method

The score of hematoxylin staining showed a significant correlation with the score of RRL (r = 0.666**) (Fig.10) and RRL (r = - 0.622*) (Fig.11). Genotypes that were not stained (tolerance) by hematoxylin staining method also tended to have long roots (tolerance) in the soil with the growth-response method (Table 9). Polle et al. (1978) reported that the results of the hematoxylin test were in good agreement with

those of the acid soil-screening method in wheat. Takagi et al. (1981) also reported that scoring of Al tolerance of wheat by the hematoxylin staining method strongly coincided with the scoring of Al tolerance in nutrient solution with Al.

Table 12. Means of relative root length (RRL) and relative shoot length (RSL) of 13 genotypes determined by the growth-response method.

Genotype	RRL (%)				RSL (%)			
	Al concentration (ppm)							
	0	224	447	671	0	224	447	671
G3	100 a	100.910 a	85.830 ab	4.505 b	100 a	110.805 ab	121.670 a	32.120 b
G4	100 a	80.865 ab	82.500 abc	13.160 b	100 a	73.405 cd	84.120 abc	32.860 b
G6	100 a	62.135 b	49.585 de	18.430 b	100 a	57.635 d	112.220 ab	30.910 b
G7	100 a	99.500 a	91.900 a	8.470 b	100 a	82.200 a-d	60.190 d	31.855 b
G8	100 a	99.380 a	64.215 b-e	17.145 b	100 a	95.780 abc	76.495 cd	37.590 b
H11/C8	100 a	84.155 ab	73.880 a-d	15.330 b	100 a	94.060 abc	72.835 cd	39.990 b
C9/H11	100 a	87.430 a	54.530 de	7.115 b	100 a	90.895 a-d	76.765 cd	32.090 b
C8/D12	100 a	87.270 a	58.910 cde	12.710 b	100 a	78.050 bcd	60.400 d	28.945 b
C9/H13	100 a	92.075 a	41.135 e	5.660 b	100 a	78.340 bcd	86.880 bcd	32.910 b
Real 60	100 a	89.295 a	83.805 abc	11.260 b	100 a	86.375 a-d	75.040 cd	46.785 b
SPA2	100 a	97.360 a	86.185 ab	67.600 a	100 a	114.615 a	102.235 abc	118.385 a
SPAD	100 a	96.360 a	90.345 ab	17.365 b	100 a	111.805 ab	76.525 cd	47.285 b
PI 533869	100 a	100.920 a	68.635 a-d	26.675 b	100 a	114.310 a	58.715 d	38.520 b

Means followed by a common letter in the column are not significantly different at the 5% level by DMRT.

The relative shoot length (RSL) showed no significant correlation with the score of hematoxylin staining (Fig. 12). This is in agreement with the fact that shoot length was not correlated with Al concentration in the growth-response method.

Conclusion

Eight genotypes (G3, G4, G7, H11/C8, Real 60, SPA2, SPAD and PI533869) showed tolerance to Al toxicity by the growth-response method. Five genotypes (G4, H11/C8, Real 60, SPA2, SPAD) showed tolerance to Al toxicity by both screening methods. C9/H13 showed susceptibility to Al by both methods. These facts show that a similar evaluation for Al tolerance can be obtained by these two methods.

For evaluation of large number of genotypes, the hematoxylin staining method is more convenient than the growth-response method. The hematoxylin staining procedure is simple and requires little space and labor. However, screening Al-tolerant sorghum to Al toxicity in the field (natural acid soil) is still necessary, especially for comprehensive information about yield and growth of the tolerant plants. The growth-response method can be used as a preliminary test for the field test.

IV. Development of Sorghum Tolerance to Al toxicity

4.1. Genetic Correlation, Heritability and Genetic Gain of Al tolerance

The different genetic controls of Al tolerance were reported among crop species. Boye-Goni and Marcarian (1985) estimated the high narrow-sense and broad sense heritability among sorghum genotypes by a nutrient solution screening method. While, Gourley et al. (1990) reported that Al tolerance was inherited as a dominant character with low narrow sense heritability for shoot and root dry matter production based on green house acid soil study.

Genetic gain of Al tolerance is very important information for allocation of resources in each generation of selection. Testing of genotypes in a large number of plants is very costly and handling of selection will be more difficult if the number of plants and genotypes to be tested increase. Discarding inferior genotypes as early as possible needs genetic gain information.

Furthermore, application of selection intensity in each generation can be more appropriate if genetic gain information is available. Information of genetic gain would guide breeders toward optimum allocation of resources in one stage of selection (Martin and Futi, 2000). However, there are few reports about genetic gain of Al tolerance and several agronomic characters in sorghum.

In this section, sorghum genotypes from various origins were used as parents. Crossings were done between Al-tolerant and susceptible genotypes, and two cycles of selection for Al tolerance using hematoxilyn staining screening method were conducted for genetic correlation, heritability and genetic gain study.

The objectives of this study are: (i) to estimate the realized heritability of Al tolerance and genetic correlation between Al tolerance and several agronomic traits and yield components (easily visible characters), (ii) to estimate genetic gain of Al tolerance and several agronomic traits of sorghum.

Materials and Methods

Plant materials

The materials used were two series of crosses, C9/H11//ICR3 and C9/H13//ICR3. C9/H11 originated from a cross between C9 (introduced from Chugoku Exp. Sta.-Japan, Can et al., 1997) and H11 (introduced from USA). H13 was introduced from USA and ICR3 was introduced from ICRISAT. ICR3 was used as an Al-tolerant parent in this experiment. General characters of genotypes are shown in Table 13.

Table 13. General characters of the parents for genetic correlation and path coefficient analysis study.

	C9/H11	C9/H13	ICR3
Al tolerance	susceptible	susceptible	tolerance
Day to flowering	early (± 47 d)	late (± 68 d)	medium (± 54 d)
Plant height	medium (± 104 cm)	tall (± 241 cm)	short (± 66 cm)
Yield	high	high	medium

Two cycles of selection of Al tolerance and agronomic traits were conducted in F_2 and F_3 population. Two hundred F_2 plants and sixty F_3 plants were grown in field

for first and second year, respectively. For genetic gain study, F_3 population was separated into three categories (population) based on information obtained from field and laboratory. They were an Al-tolerant population, an early maturity population and a short plant height population. The term population refers to the character that selection was applied directly to it. Genetic gain was estimated from each population.

The field experiment and data collection was conducted as described in the previous chapters.

Hematoxylin-staining screening method at 148 µM and 222 µM aluminum was applied for evaluating Al tolerance for six seedlings per Al concentration with three replications. The group with no roots stained was scored 1 (very tolerant) and the group in which the roots of five seedlings stained was scored 5 (very susceptible).

Calculation

The realized heritability was calculated as follows (Fehr, 1987);

$$\text{Realized heritability} = (M_{\text{high F3}} - M_{\text{low F3}}) / (M_{\text{high F2}} - M_{\text{low F2}})$$

where, $M_{\text{high F3}}$ is a mean performance of F_3 progenies of F_2 plants selected in the high group; $M_{\text{low F3}}$ is a mean performance of F_3 progenies of F_2 plants selected in the low group; $M_{\text{high F2}}$ is a mean performance of F_2 plants in the high group; and $M_{\text{low F2}}$ is a mean performance of F_2 plants in the low group.

Genetic correlation (r_A) was calculated as follows (Fehr, 1987);

$$r_A = CR_x\, i_x\, h_x / R_x\, i_y\, h_y$$

where, CR_x is amount of improvement of primary character obtained by indirect selection for secondary character; R_x is amount of improvement obtained by direct selection for primary character; i_x and i_y are selection intensity for primary and

secondary character, respectively; h_x and h_y are square root of realized heritability, instead of narrow sense heritability, of primary and secondary character, respectively.

The realized genetic gain is calculated as follows (Martin and Futi, 2000);

Genetic gain = (Xs – Xps) – (X – Xpo)

where, Xs is a mean of selected population in successor test, and Xps is a mean of parents in successor test; X is a mean of original population; Xpo is a mean of parents in original population.

The changing of the selection intensity for each character at any population was estimated by calculating the selection differential. Selection differential is calculated as follows;

Selection differential = $(Xs – X) / \sigma_p$

where, Xs is a mean of selected plants; and X is a overall mean of the original population and σ_p is a standard deviation of original population.

Results and Discussion

Genetic correlation and heritability of Al tolerance and agronomic traits

Genotypic correlations of length of head, early days to flowering and short plant height with Al tolerance were similar in both C9/H11//ICR3 and C9/H13//ICR3 crosses (Table 14). The relatively high positive genotypic correlations with Al tolerance were observed for high performance of dry weight and grain weight plant^{-1} in the C9/H11//ICR3 cross. On the other hand, early days to flowering and harvest index showed relatively high genotypic correlations with Al tolerance in the C9/H13//ICR3 cross.

Table 14. Heritability and genotypic correlation between six agronomic traits and Al tolerance in sorghum.

	Dry weight	Length of head	Grain weight plant^{-1}	Days to flowering	Plant height	Harvest index	H[1)]
Al tolerance (C9/H11//ICR3)	0.497	0.249	0.706	-0.391	0.060	-0.485	0.35
Al tolerance (C9/H13//ICR3)	0.119	0.344	0.025	-0.468	-0.158	0.728	0.43

[1)] H=heritability; High performances of dry weight, grain weight per plant and harvest index, short plant height and early days to flowering were used in genotypic correlation analysis.

Table 15. The phenotypic correlations between Al tolerance and six agronomic traits of sorghum.

	Dry weight	Length of head	Grain weight plant^{-1}	Days to flowering	Plant height	Harvest index	Al tol. in F_2
Al tolerance in F_2	-0.119	-0.174	-0.201	-0.232	0.053	-0.201	
	-0.265*	-0.258	-0.209	-0.382**	-0.376**	-0.183	
Al tolerance in F_3	-0.505**	-0.267*	-0.488**	-0.207	0.115	-0.350**	0.268*
	-0.387**	-0.408**	-0.370**	-0.385**	-0.661**	-0.254	0.597**

Upper value is C9/H11//ICR3 crossing and low value is C9/H13//ICR3 crossing.

Some agronomic traits showed a small genotypic correlation with Al tolerance. A small genotypic correlation and inconsistent relationship with the phenotypic correlation suggested that environmental factors extremely affected phenotypic performance in field experiment.

Genotypic correlations between days to flowering and Al tolerance were negative as in the phenotypic correlations in both series of crossing (Table 15). This not only showed negative phenotypic correlations between days to flowering and Al

tolerance but also suggested that early maturity and low Al tolerance were linked genetically or they might be pleiotropically controlled.

The genotypic correlation of harvest index with Al tolerance was different between C9/H11//ICR3 and C9/H13//ICR3 crosses. This result is somewhat disturbing, but perhaps these might result from the use of different parents for the cross. There are some reports showing that different genes control Al tolerance in the same plant species. It was reported that several genes in some of wheat cultivars controlled Al tolerance but simple dominant genes controlled Al tolerance in other wheat cultivars (Martinez et al., 1999).

The realized heritability of Al tolerance was moderately low (H = 0.35 and H = 0.43) in C9/H11//ICR3 and C9/H13//ICR3 crossing (Table 14). Using a nutrient solution culture, Boye-Goni and Marcarian (1985) reported that narrow-sense and broad-sense heritabilities were 0.78 and 0.99, respectively. Gourley et al. (1990) reported that the narrow-sense heritability assessed by the green house acid soil study method was different from that assessed by the solution culture method even when the same materials were being examined. The narrow-sense heritabilities assessed by the green house acid soil method and solution culture method were between 0.05~0.31 and 0.65~0.72, respectively.

Heritability was moderately low in this study, confirming that several genes controlled Al tolerance. Al tolerance in wheat cv. Atlas 66 was controlled by two or more major genes (Berzonsky, 1992); dominant or semidominant genes in *Arabidopsis thaliana* (Larsen et al., 1996).

Al tolerance in sorghum was inherited as a dominant character and

predominantly additive genetic effect with some degree of dominance controlled it (Boye-Goni and Marcarian, 1985). Gourley et al. (1990) using solution culture screening method also reported that the additive variance of Al tolerance in sorghum was much greater than the variance in the degree of dominance. However, direct selection of Al tolerance might difficult for improving Al tolerance character judging from the data obtained in this study. Consequently, indirect selections, selections with a more accurate evaluation method for Al tolerance, use of wider germplasm with high Al tolerance and a larger number of progeny plants are necessary for increasing Al tolerance.

Genetic gain

Overall mean genetic gain of Al tolerance for all populations was 0.86, despite selection differential of –94.2% for Al tolerance (Table 16). This suggested that multiple genes rather than a single gene might control Al tolerance character in sorghum. These results also confirmed the reports that two or more dominant genes controlled Al tolerance character in a lot of plant. Therefore, for improvement of Al tolerance characters, several cycles of selections might be needed to make this character more stable.

The overall genetic gain for plant height and dry weight were relatively high –1.12 and –0.35, respectively (Table 16 and 17). This showed that there were significant reductions of plant height and dry weight in successor population. Significant reduction in plant height and dry weight may also cause a small improvement of genetic gain in Al tolerance. The author observed that dry weight and plant height were important component factors in Al tolerance path analysis, in which these characters gave the

Table 16. Standardized genetic gain (GG), unstandardized genetic gain (RV) and selection differential (SD) of Al tolerance, dry weight, length of head and grain weight plant^{-1} conducted in the early stage of sorghum development.

Population	Al tolerance			Dry weight			Length of head			Grain weight plant^{-1}		
	GG[1)]	RV	SD (%)	GG	RV (g)	SD (%)	GG	RV (cm)	SD (%)	GG	RV (g)	SD (%)
Al-tolerant population	0.50	0.56	-94.2	-0.12	-1.61	18.7	4.93	7.40	28.9	0.43	6.03	119.4
Early maturity population	0.99	1.11	-3.6	-0.58	-7.64	-39.7	1.99	7.71	10.6	-0.52	-6.37	23.5
Short plant height population	0.81	0.98	0.9	-0.76	-10.23	-63.5	1.87	7.34	-14.7	0.29	6.95	-21.6
Overall[2)]	0.86			-0.35			1.95			0.26		

Genetic gains of particular populations are within a row and genetic gains of particular character are within a column. [1)] GG = standardized genetic gain of average from two series of crossing C9/H11//ICR3 and C9/H13//ICR3; [2)] Overall genetic gain of particular characters over three populations sorghum.

Table 17. Standardized genetic gain (GG), unstandardized genetic gain (RV) and selection differential (SD) of harvest index, plant height and days to flowering conducted in the early stage of sorghum development.

Population	Harvest index			Plant height			Days to flowering		
	GG[1)]	RV	SD (%)	GG	RV (cm)	SD (%)	GG	RV (d)	SD (%)
Al-tolerant population	0.02	0.60	71.5	-0.91	-40.31	1.0	0.04	0.51	-6.1
Early maturity population	-0.16	-3.97	56.0	-1.21	-54.23	-31.8	-0.15	-1.71	-83.0
Short plant height population	0.32	7.30	-22.0	-1.39	-62.46	-98.4	0.22	2.74	-32.4
Overall[2)]	0.11			-1.12			0.21		

Genetic gains of particular populations are within a row and genetic gains of particular character are within a column; [1)] GG = standardized genetic gain from two series of crossing C9/H11//ICR3 and C9/H13//ICR3; [2)] Overall genetic gain of particula r characters over three populations sorghum.

great influence both directly and indirectly on Al tolerance. More detailed explanation about Al path analysis can be seen in section 4.2.

Relatively high genetic gain of 0.50 for Al tolerance in the population that was specially selected for Al tolerance (Al tolerance population) was because of the greatest selection differential (selection intensity) for Al tolerance in this population, compared to the other populations, early maturity and short plant height population (Table 16). This suggests that high allocation of resources in early generations for Al tolerance and high selection intensity must be applied if relatively high genetic gain of Al tolerance is to be obtained.

Selection pressure for early maturity gave negative gains for grain weight per plant (-0.52) and dry weight (-0.58). In consequence of these results, the gain for harvest index was also negative. Martin and Futi (2000) reported that the negative gain for yield in the F_3 in a soybean breeding program was observed when strong selection for earliness was applied to this stage.

Conclusion

Heritability of Al tolerance in sorghum was moderately low in both series of crosses. Consequently, low genetic gain of Al tolerance was observed in both populations. High allocation of resources in early generations for Al tolerance and high selection intensity of Al tolerance must be applied if higher gain for Al tolerance is to be obtained. Low genetic correlations in some agronomic traits and inconsistent relationship with the phenotypic correlations were observed.

4.2. Path Coefficient Analysis of Al tolerance and Several Agronomic Characters of Sorghum

Common breeding for Al tolerance was conducted by empirical selection of tolerant plants with little regard for the physiological relationships of both Al-tolerant and susceptible genotypes. The studies on correlated characters are important to know how the improvement of one character will cause simultaneous changes in other characters (Falconer and Mackay, 1996). The relationships among characters were usually measured with correlation coefficients. If more variables are considered in the correlation table, these indirect associations become more complex, less obvious and somewhat perplexing (Dewey and Lu, 1959). At this point, path analysis is used to partition the relative contribution of components via standardized partial-regression coefficient and measures the direct influence of one variable upon another.

Path analysis was often used to identify important yield components on yield in various crops including rice, wheat and soybean (Ball et al., 2001). Using the same idea with yield components, a tolerant plant to Al toxicity can be considered if it has a good phenotypic performance in acid soil stress condition, such as good root system, plant height, dry weight and yield. However, for field-grown plants, root traits are difficult to measure (Flores et al., 1988), thus evaluations from the other traits were commonly used to evaluate field-grown plants for tolerance to soil acidity, such as plant survival, plant vigor, grain yield and mineral element content.

Al tolerance assessed by using hematoxylin staining method correlated with several agronomic traits in sorghum as described in previous chapters. It was reported that Al-tolerant plants had a better phenotypic performance than susceptible plants in

acid soil and the different response of the sorghum agronomic traits in acid soil stress condition was also reported (Flores et al., 1988; Miller et al., 1992). However, there are few reports about direct and indirect effect of agronomic traits on Al tolerance through path analysis.

The different genetic controls of Al tolerance were reported even in the same crop species (Martinez et al., 1999). Therefore, sorghum genotypes from various origins were used as a parent in plant breeding program and two crosses of sorghum were used for this path analysis study.

Materials and Methods

Field experiment and hematoxylin analysis

The materials used in this study are shown in Table 13. The field trial and hematoxylin staining screening method are same with methods in previous sections. Data were obtained from average of two planted seasons (two years).

Calculation

The linear interrelationship among Al tolerance and other agronomic traits is illustrated in the regression model;

Al tolerance in $F_3 = \beta_o + \beta_1$(dry weight) + β_2(length of head) + β_3(days to flowering) +

β_4(plant height) + β_5(harvest index) + β_6(Al tolerance in F_2)

A path coefficient is defined as the portion of the standard deviation of dependent variable that is due to the variation of in independent variable and is simply a standardized partial-regression coefficient (Li, 1956; Dewey and Lu, 1959) as follows;

$$P_{xy} = B\sigma_x / \sigma_y$$

where, P_{xy} is path coefficient for the direct path from X (independent variable) to Y (dependent variable); B is regression coefficient of Y on X; σ_x and σ_y are standard deviation of X and Y, respectively.

A path diagram is shown in Fig. 13, where P_{17}, P_{27}, P_{37}, P_{47}, P_{57} and P_{67} represent path coefficients to Al tolerance in the F_3 from dry weight, length of head, days to flowering, plant height, harvest index and Al tolerance in F_2 population, respectively. The direct effect of each variable component on Al tolerance is a path coefficient from that component to Al tolerance. The indirect effect of one component through a second component is the product of the path coefficient from the second component and the correlation between the two components.

Collinearity diagnostic was performed to increase accuracy of the regression model and screening the unstable variable's regression coefficients. Pearson correlation, multiple linear regression analysis and data validation were analyzed using SPSS 10.1 for Windows software program (SPSS for Windows, 1999).

Results

Correlation and multiple linear regression model

Grain weight plant^{-1} was excluded from the linear regression model because it was almost a linear combination of the other independent variables. Based on collinearity statistic analysis, the tolerance test of grain weight plant^{-1} was very small (near zero- Table not shown). This suggested that the other independent variables could explain the almost 99.9% variability of grain weight plant^{-1} in both C9/H11//ICR3 and C9/H13//ICR3. Therefore, it was no surprising if grain weight plant^{-1} showed significant

correlations with the others independent variables in both series of crosses (Table 18 and 19).

ANOVA of multiple linear regression coefficients were significant for both C9/H11//ICR3 and C9/H13//ICR3 (Table not shown), indicating that there was a linear relation between six independent variables and dependent variable in the model of multiple linear regressions. The multiple linear regression models of C9/H11//ICR3 and C9/H13//ICR3 for six-independent variables explained the variation in Al tolerance with R^2 value 0.501 and 0.555, respectively. It means that six agronomic variables that were included in this model explained more than 50% variability of Al tolerance.

C9/H11//ICR3 cross

Generally, dry weight mainly affected Al tolerance through its direct effect (Table 20). The greatest direct influence was registered by dry weight on Al tolerance in F_3. The indirect effect of dry weight on Al tolerance via days to flowering and plant height were contradictory but relatively small, reflecting the fact that plant height and days to flowering were less correlated with Al tolerance (Table 20).

A small direct effect of harvest index and Al tolerance in F_2 population on Al tolerance in F_3 was registered and a negligible direct effect of length of head on Al tolerance in F_3 was seen in this cross. A low heritability 0.35 of Al tolerance (Table 14) caused a small contribution of Al tolerance in F_2 population to the improvement of Al tolerance in F_3. All indirect effects of Al tolerance in F_2 population on Al tolerance in F_3 were generally negligible. Harvest index and length of head affected Al tolerance mainly through their indirect effect via dry weight.

C9/H13//ICR3 cross

There was the greatest direct influence from plant height on Al tolerance, with harvest index of next magnitude, then Al tolerance in F_2 population (Table 21). Generally high influence was registered by plant height both directly and indirectly on Al tolerance in F_3.

A strong positive effect (0.544) was registered directly by Al tolerance in F_2 population on Al tolerance in F_3 for C9/H13//ICR3 (Table 21). This magnitude was higher than direct effect of Al tolerance in F_2 for C9/H11//ICR3. The indirect effect of Al tolerance in F_2 population on Al tolerance in F_3 via plant height had also positive influence due to the fact that the plant height was correlated with Al tolerance in this cross. The length of head and dry weight mainly affected Al tolerance through its indirect effect of plant height that was higher in magnitude than their direct effect.

Discussion

Generally, two crosses used in this study showed a similar correlation with Al tolerance. Partitioned into each components show different direct and indirect effects on Al tolerance between these crosses. However, the examination of the path analysis revealed that dry weight or plant height exerted the great influence both directly and indirectly on Al tolerance (Table 20 and 21). The different direct effect on Al tolerance between these genotypes might be attributable to: (i) a different genetic control between these genotypes, (ii) an environmental factor that influenced the Al tolerance.

Table 18. Pearson's correlation coefficients for six agronomic traits and Al tolerance of C9/H11//ICR3 sorghum genotype.

	Dry weight	Length of head	Grain weight plant^{-1}	Days to flowering	Plant height	Harvest index	Al-tol. in F_2	n
Length of head	0.514**							138
Grain weight per plant	0.619**	0.450**						138
Days to flowering	0.632**	0.251**	0.639**					131
Plant height	0.467**	0.292**	0.503**	0.237**				138
Harvest index	0.096	0.216**	0.841**	-0.097	0.265**			138
Al tolerance in F_2	-0.119	-0.174	-0.201	-0.232	0.053	-0.201		57
Al tolerance in F_3	-0.505**	-0.267*	-0.488**	-0.207	0.115	-0.350**	0.268*	57

**, * = significant at the 1% and 5% level, respectively.

Table 19. Pearson's correlation coefficients for six agronomic traits and Al tolerance of C9/H13//ICR3 sorghum genotype.

	Dry weight	Length of head	Grain weight per plant	Days to flowering	Plant height	Harvest index	Al-tol. in F_2	n
Length of head	0.611**							119
Grain weight per plant	0.628**	0.377**						115
Days to flowering	0.440**	0.236**	0.002					120
Plant height	0.727**	0.280**	0.624**	0.270**				111
Harvest index	0.128	0.139	0.852**	-0.331**	0.298**			119
Al tolerance in F_2	-0.265*	-0.258	-0.209	-0.382**	-0.376**	-0.183		57
Al tolerance in F_3	-0.387**	-0.408**	-0.370**	-0.385**	-0.661**	-0.254	0.597**	57

**, * = significant at the 1% and 5% level, respectively.

Table 20. Direct (underline) and indirect effect of dry weight, length of head, days to flowering, plant height and Al tolerance in F_2 on Al tolerance for C9/H11//ICR3 genotype.

	Dry weight	Length of head	Days to flowering	Plant height	Harvest index	Al-F_2	*r*
Dry weight	**-0.764**	-0.004	0.166	0.238	-0.093	-0.029	-0.486**
Length of head	-0.334	**-0.010**	0.046	0.140	-0.082	-0.026	-0.265*
Days to flowering	-0.492	-0.002	**0.258**	0.169	-0.069	-0.049	-0.185
Plant height	-0.385	-0.003	0.093	**0.471**	-0.069	0.012	0.119
Harvest index	-0.387	-0.004	0.098	0.178	**-0.183**	-0.030	-0.329*
Al-F_2	0.113	0.001	-0.065	0.030	0.029	**0.195**	0.303*

Number of sample was 57. Variables were transformed by the natural logarithm. Direct and indirect effects of particular components are within a row. **, * = significant at the 1% and 5% level, respectively; *r* = correlation in path analysis.

Table 21. Direct (underline) and indirect effect of dry weight, length of head, days to flowering, plant height and Al tolerance in F_2 on Al tolerance for C9/H13//CR3 genotype.

	Dry weight	Length of head	Days to flowering	Plant height	Harvest index	Al-F_2	*r*
Dry weight	**0.091**	-0.195	0.109	-0.688	0.451	-0.141	-0.372**
Length of head	0.079	**-0.224**	0.074	-0.549	0.372	-0.116	-0.364**
Days to flowering	0.026	-0.044	**0.375**	-0.422	-0.093	-0.238	-0.395**
Plant height	0.070	-0.138	0.178	**-0.888**	0.440	-0.196	-0.535**
Harvest index	0.068	-0.138	-0.058	-0.645	**0.606**	-0.094	-0.261
Al-F_2	-0.024	0.048	-0.164	0.321	-0.104	**0.544**	0.621**

Number of samples is 57. Variables were transformed by the natural logarithm. Direct and indirect effects of particular components are within a row. **, * = significant at the 1% and 5% level, respectively; *r* = correlation in path analysis.

Dry weight was an important component in C9/H11//ICR3 and plant height was an important component in C9/H13//ICR3 (Table 20 and 21). Determining whether this phenomenon is unique to these lines used in the current study or is related to the genetic control of the Al tolerance mechanism will take additional studies. Shuman et al. (1990) reported sorghum plant height decreased linearly with Al^{3+} activity, while plant weight vs. Al^{3+} decreased in curvilinear. Flores et al. (1988) reported that the severe acid soil affected more extensively the dry matter, stover, grain and head yields, seeds per head and seed weight traits than the growth traits of sorghum.

Conclusion

Dry weight and plant height were most closely associated with Al tolerance. Based on the overall correlation data and path analysis data, a way to maximize the Al tolerance in sorghum is proposed as follows. Increasing of dry weight and selection of Al tolerance in F_2 was more appropriate to maximize the Al tolerance in sorghum because correlation of dry weight and Al tolerance was more stable than plant height, and its direct and indirect effect on Al tolerance were high.

V. Future Development of Al-tolerant Sorghum

5.1. Genotypic Difference of Sorghum in Callus Formation and Callus Growth on Al-containing Medium

The use of tissue culture for selection of several Al-tolerant plants has been reported (Ojima and Ohira, 1983; Smith et al., 1983; Duncan et al., 1995; Conner and Meredith, 1985a; Barnabas et al., 2000). However, previous screening by tissue culture was generally made based on the growth of calli derived from the explants of each genotype on Al-containing medium. Even when the variation in Al tolerance was not detected in the original explants, it appeared during the culture process.

Parrot and Bouton (1990) reported that alfalfa plants from the Al-tolerant germplasm rapidly expressed Al tolerance at the callus stage. If the performance of Al tolerance in tissue culture is similar to that at the plant level, tissue culture may be more useful for breeding programs because selection can be earlier and faster than that in the field. Moreover, the selection by tissue culture can be applied to identify Al-tolerant plants in a segregating population.

Although this approach has not been applied to sorghum, previous work with alfalfa showed that Al tolerance of plants was also expressed at the cellular level (Parrot and Bouton, 1990). The present study aims to study the genetic variation of Al tolerance in sorghum and to elucidate the callus performance of the Al-tolerant and Al-susceptible genotypes on the Al-containing medium with high availability Al^{3+} for the callus.

Materials and Methods

Plant materials and explant preparation

Ten selected sorghum genotypes were used in this experiment (Table 22). All of

these genotypes except G1 were screened for Al tolerance by hematoxylin staining and the growth on the soil in pots with Al added in the previous chapters. G4, SPA 2, and SPAD were considered as tolerant genotypes and G2, G5, C9/H11, C9/H13 were considered as susceptible genotypes (Table 22). The G1 genotype was chosen because of its high cell proliferation rate in tissue culture (Can and Yoshida, 1999b).

Seeds were surface-sterilized with TWEEN and calcium hypochlorite (60%) and were then soaked in sterile water at room temperature. After small radicles had emerged, seeds were allowed to grow on a solidified hormone-free MS medium and were then kept in an incubator in darkness at 25^{o}C.

Media

Calli were induced on the medium containing Murashige and Skoog (MS) inorganic salts, 2.0 mg L^{-1} kinetin, 1.0 mg L^{-1} IAA and 2.5 mg L^{-1} 2,4-D, from the cut pieces of mesocotyl (called explants, hereafter) at zones III and IV, which were specified by Gendy et al. (1996) (Fig. 14). The culture medium was adjusted to pH 5.8. This medium was referred to as Al-free medium.

To examine the Al toxicity, the standard MS medium modified according to the report by Conner and Meredith (1985b) was used. The pH of the culture medium was adjusted to pH 4, at which Al^{3+} was considered as the dominant form of Al in the solution (Koyama et al., 1990). The phosphate concentration was reduced from 1250 µM to 10 µM and calcium was reduced from 3000 µM to 100 µM to give high effect of Al toxicity on callus (Ojima and Ohira, 1983; Conner and Meredith, 1985b). The Al concentration of the Al-containing medium was adjusted to 400 µM by adding $AlCl_3 \cdot$

Table 22. Sorghum genotypes used in callus selection and their aluminum tolerance score.

Genotype	Al tolerance score		
	Hematoxylin staining	RRL[1)]	Acid soil[2)]
G1	-	-	-
G2	5 (susceptible)	-	susceptible
G3	3 (intermediate)	1 (tolerant)	
G4	1 (tolerant)	1 (tolerant)	
G5	-	-	susceptible
G7	3 (intermediate)	1 (tolerant)	
SPA 2	1 (tolerant)	1 (tolerant)	
SPAD	2 (tolerant)	1 (tolerant)	
C9/H11	4 (susceptible)	3 (intermediate)	
C9/H13	4 (susceptible)	5 (susceptible)	

Score 1 = tolerant; score 5 = susceptible; RRL = relative root length in growth response method; G2 = Hegari; G5=RTx403; [1)] Data were copied from section 3.1. [2)] Data from Duncan et al. (1995).

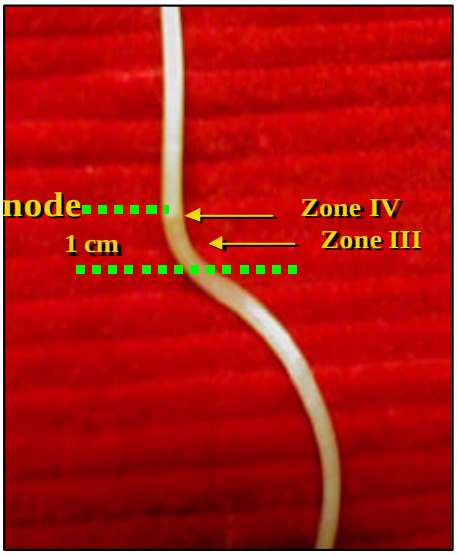


Fig. 14. Sorghum mesocotyl. The transverse thin cell layer (1 - 0.3 mm) was cut from the mesocotyl at zones III and IV. Zone III is the sub-apical zone and zone IV is the base of the apical meristem.

$6H_2O$ to the medium (Taylor, 1995; Conner and Meridith (1985b), after autoclaving but immediately prior to gelling of the medium.

Culture condition

The explants were cultured on 20 mL of the media with and without Al added for the observation of the callus formation. For each treatment, at least 1050 explants were planted over three replications. Petri dishes with explants were placed in an incubator in darkness at 30^oC. Observations were made at two, five and eight weeks after planting.

To examine the effect of Al on the callus growth, calli were subcultured twice on Al-free medium before the treatment with Al. The calli were cut into small pieces as uniform as possible and planted on the medium with or without Al added in petri dishes. At least 30 calli per treatment were used for one replication. The weight and size of each callus was measured at three-week intervals up to the 11th week. Callus was transferred to fresh medium at three weeks intervals to eliminate micronutrient deficiency.

Calculation and experimental design

The percentage of callus formation (CF) is calculated as;

$$CF = (\text{number of explants with calli})(\text{total number of explants})^{-1} \times 100.$$

The relative suppression of CF by Al (RSC) is calculated as;

$$RSC = 100 - \{(\text{CF on Al-containing medium})(\text{CF on Al-free medium})^{-1}\} \times 100.$$

Fresh callus weight was determined as the difference between the weight of a petri dish with callus and that after removal of the callus. The percent growth of callus in fresh weight (GW) is calculated as (Conner and Meredith 1985a);

$$GW = \{100 \times (Wf - Wi)\}(Wi)^{-1}$$

where, Wi and Wf are the initial and final fresh weight of callus, respectively. GW on Al-containing medium relative to that on Al-free medium is expressed by relative growth in weight (RGW) and calculated as;

$$RGW = \{(GW \text{ on Al-containing medium})(GW \text{ on Al-free medium})^{-1}\} \times 100.$$

A multiplication of the longest and the widest axes of callus was used to show callus size. The percent growth in size of callus (GS) is calculated as;

$$GS = \{100 \times (Sf - Si)\}(Si)^{-1}$$

where Si and Sf are the initial and final size of callus, respectively. GS on Al-containing medium relative to that on Al-free medium was shown by relative growth in size (RGS) and calculated as;

$$RGS = \{(GS \text{ on Al-containing medium})(GS \text{ on Al-free medium})^{-1}\} \times 100.$$

The CF, RSC, RGW and RGS of each genotype were then analyzed in randomized complete block design. Three replications were designed both in Al-free medium and Al-containing medium. Those with a similar planting time were regarded as one replication in this experiment.

Results and Discussion

All genotypes used in this experiment formed calli on Al-free medium, but the percentage of the explants that formed calli (CF) significantly varied with the genotypes ($p < 0.01$) either on Al-free or Al-containing medium (Fig. 15). Genotype SPA2, G1 and G3 formed more calli on Al-free medium than the other genotypes, and the SPAD formed the fewest calli on Al-free medium, suggesting the poor callus formation in tissue culture.

Addition of Al to the medium with low pH, low Ca and low phosphate resulted

in a severe decrease in the percentage of callus formation (CF) in both Al-tolerant and Al-susceptible genotypes (Fig. 16). Parrot and Bouton (1990) reported that medium modification other than of Al (low pH, low Ca, low phosphate) resulted in a depression of callus growth relative to that on standard medium for both Al-tolerant and Al-susceptible genotypes. However the addition of Al to this modified medium resulted in an additional decrease in callus growth of the Al-susceptible population of Alfalfa, but not in the Al-tolerant population.

In all cases, the genetic variance was higher than the individual variance (Table 23), suggesting that the difference in callus performance among genotypes was genetic. The relative suppression of callus formation by Al (RSC) in the Al-tolerant genotypes was lower than that in the susceptible genotypes (Table 23). This suggested that the Al-tolerant genotypes were more capable of forming callus than susceptible genotypes on Al-containing medium. Some of the susceptible genotypes did not form calli on Al-containing medium and were shown by 100% RSC (Table 23 and Fig. 15). There was a significant negative correlation ($r = -0.664^*$) between RSC and CF on Al-containing medium. Genotypes that showed low RSC also showed high capability of callus formation on Al-containing medium.

The RGW and RGS of tolerant genotypes was still higher than that of the other genotypes with exception of SPAD (Table 23) because SPAD showed a low callus formation even in Al-free medium as was mentioned above and shown in Fig. 15. Al-susceptible population of Alfalfa also showed an additional decrease in callus growth when it was planted in the modification medium supplemented with Al (Parrot and Bouton, 1990).

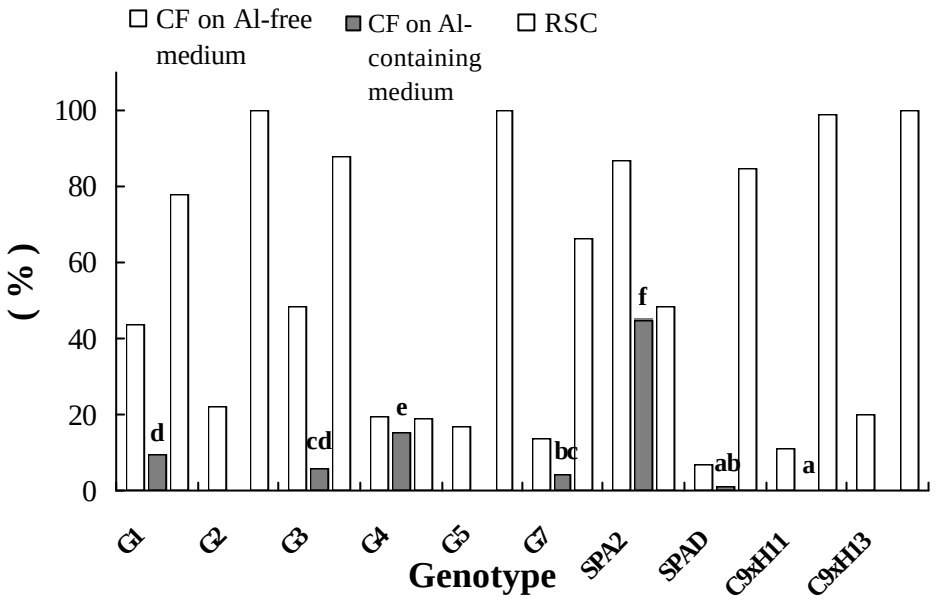


Fig. 15. Percentage of callus formation (CF) on Al-free medium, that on Al-containing medium and percent relative suppression of callus formation by Al (RSC) at eight weeks after planting. A common letter on a graph is not significantly different at the 5% level by Duncan's multiple range test. Genotype G2, G5 and C9xH13 were excluded from the analysis because no callus variation over replications was observed.

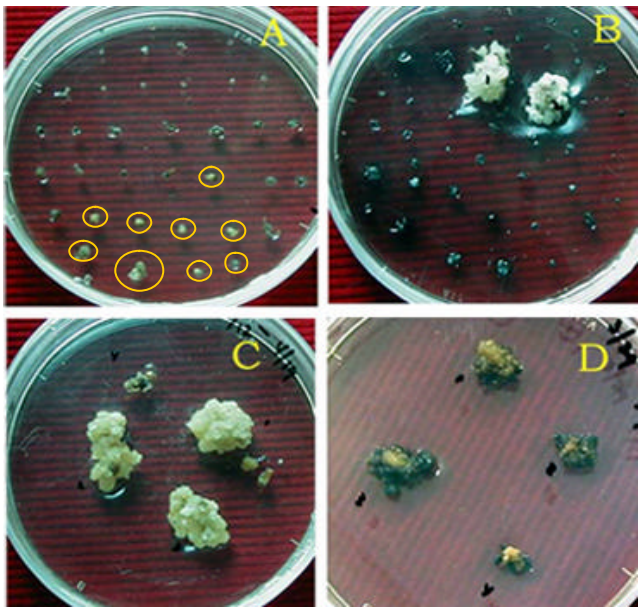


Fig. 16. Differences in callus formation and callus growth of the same genotype (G1) between Al-free medium and Al-containing medium. A, callus formation on Al-free medium (formed callus are enclosed by circle); B, callus formation on Al-containing medium; C, callus growth on Al-free medium; D, callus growth on Al-containing medium.

Table 23. Percent relative suppression of callus formation by Al (RSC), relative growth in fresh weight of callus on Al-containing medium (RGW) and relative growth in size of callus on Al-containing medium (RGS) at 11th week after planting in various sorghum genotypes.

Genotype	RSC (%)	RGW (%)	RGS (%)
G1	78.00 cd	21.115 ab	39.968 cd
G2	100.00	10.655 a	11.831 a
G3	88.15 cd	30.495 b	16.491 ab
G4	18.83 a	50.073 c	70.617 e
G5	100.00	10.243 a	11.509 a
G7	66.39 bc	23.397 b	29.275 bc
SPA2	48.47 b	71.910 d	51.577 d
SPAD	85.00 cd	10.017 a	11.667 a
C9/H11	99.17 d	10.139 a	11.745 a
C9/H13	100.00	10.033 a	11.402 a
Genetic Variance (σ_g^2)	3.71	429.154	420.076
Individual Variance (σ_e^2)	0.68	35.753	63.036

Values followed by a common letter in the column are not significantly different at the 5% level by Duncan's Multiple Range Test. Genotype G2, G5, C9xH13 were excluded from the analysis because of no variation over replications.

Table 24. Correlations among relative suppression of callus formation (RSC), relative growth in fresh weight of calli on Al-containing medium (RGW), relative growth in size of calli on Al-containing medium (RGS), hematoxylin staining score on Al medium (HS) and score of growth response to Al added in the soil in pot (ASP).

	RGW	RGS	HS[1)]	ASP[1)]
RSC	– 0.820**	– 0.956**	0.852**	0.583
RGW		0.824**	– 0.787*	– 0.514
RGS			– 0.792*	– 0.472
HS				0.666**

*, ** = significant at the 5% and the 1% level, respectively; [1)] Data were copied from section 3.2. CF was measured at eight week after planting; GS and GW were measured at 11th week after planting.

The RGW and RGS showed a significant correlation (r = 0.824**) at the 11th week after planting (Table 24). RGW and RGS also showed a significant negative correlation (r = –0.820** and r = –0.956**) with RSC. Genotypes that showed a high RGW and RGS also showed low RSC, as was shown for Al-tolerant genotypes such as G4 and SPA2 (Table 23).

The variations in Al tolerance among these genotypes were observed by hematoxylin staining and the growth on the soil with Al in pots as described in the previous chapters. In all cases, the callus performance (RGS, RGW, RSC) showed a significant correlation with the Al tolerance score in hematoxylin staining (Table 24), which suggested that Al might be the major limiting factor that affected the callus growth in Al-containing medium in this experiment. Genotypes that showed a low RSC (G4 and SPA2) showed high tolerance score 1 by the hematoxylin staining screening method.

Callus performance was not significantly correlated with the score of growth response to Al in the soil in pot (Table 24). The evaluation of tolerant genotype by a short-term (15 days) growth response to the Al by the pot method might curtail the accuracy of the screening method. However, the growth-response to Al in pot method showed a significant correlation with the score as described in the previous chapters.

Conclusion

The percentage of callus formation and the callus growth on Al-containing medium varied with the genotypes, indicating that the tissue culture could be used to

screen Al-tolerant genotypes. The differences in callus growth and the percentage of callus formation among genotypes were in agreement with those in the hematoxylin staining.

5.2. Direct Gene Transfer to Anther and Floret-derived Callus of Sorghum by Particle Bombardment

Traditional plant breeding methods have mostly been used to combine the desirable characters in one sorghum plant. This approach is laborious and time-consuming compared with genetic engineering (Liu et al. 2003). However, improvements through genetic engineering have been slow in sorghum because of sorghum recalcitrance to genetic transformation (Emani et al., 2002).

The biolistic system, particle bombardment, is widely used for delivery of genetic materials directly into intact cells and tissues. Microprojectile bombardment represents a powerfull method for the transformation of various organisms and tissue (Kruse et al., 2002; Liu et al., 2003). Transformed tissue can be easily visualized using green fluorescent protein (GFP) or staining of GUS expression. However, transformation efficiency of the bombardment method in sorghum is still low (0.2~0.5%) compared with *Agrobacterium* method (Emani et al., 2002).

The promoter plays a crucial role in successful transformation studies. The viral Cauliflower Mosaic Virus 35S (CaMV 35S) promoter has been widely used in the transformation of many dicot and monocot crops (Liu, et al 2003). Some promoters showed different activity in sorghum (Emani et al., 2002), sugarcane (Liu et al., 2003) and tall fescue (Takamizo, 1996). Most of these promoters were rice (actin and polyubiquitin) promoter, maize ubiquitin promoter and Cauliflower Mosaic Virus 35S promoter.

The preliminary microprojectile bombardment experiment was carried out in

this study to evaluate the utility and effect of different DNA constructs on transient GUS expression in anther and floret-derived callus of sorghum.

Materials and Methods

Plant materials and culture media

Sorghum genotype SPA2, C9/H11//C9/H13, C9/H11//SPA2, C9/H13//SPA2, C8/H2 were used for anther and floret callus induction. Tall fescue suspension cultures were used as a control in this experiment. Tall fescue callus induction media was MS medium with 3% sucrose (pH 5.8) and was supplemented with 5 mg L^{-1} 2,4-D and 500 mg L^{-1} casein hydrolysate.

Plants were grown in pots (20 cm in diameter) at a greenhouse and were fertilized with N, P_2O_5 and K_2O at the rate of 1.44g per pot. Anther culture method was carried out according to the protocol of Nakamura et al. (1997). Anthers and floret were harvested from panicle when about 2cm of flag leave sheath arose from culm. Spikelets at one-third from the upper part were excised and sterilized with 60% EtOH for 3 minutes. Anthers and florets were washed three times with sterilized distilled water prior inoculation. Thirty anthers and 15 florets were inoculated onto Murashige and Skoog medium supplemented with 2.0 mg L^{-1} kinetin, 1.0 mg L^{-1} IAA and 2.5 mg L^{-1} 2,4-D. Selective medium for stable transformation was supplemented with 100 mgL^{-1} hygromycin.

DNA transformation constructs.

Three DNA constructs, namely, pAct1-D (rice actin promoter), pWI-GUS (double CaMV 35S promoter), pEX7113 (improved CaMV 35S promoter) were used to

drive transient β-glucuronidase (GUS) expression in sorghum calli. All apparatus and plasmids for particle bombardment in this experiment were kindly arranged and provided by Dr. Tadashi Takamizo, National Grassland and Research Institute. The plasmid pAcH1, kindly provided by Dr. G. Spangenberg (La Trobe University, Australia) carrying the selectable marker gene for hygromycin resistance (*hpt*), was used for stable transformation experiment under the control of rice actin 1 gene promoter (Spangenberg et al., 1995).

Microcarrier preparation (1~1.6 µM gold particles)

Thrirty milligram gold particles were washed first with 1 mL 100% EtOH and particle suspension was sonicated using ultra sonic wave for 60 minutes. The gold suspension was allowed to settle under gravity at room temperature for about 15 minutes and suspension was then transferred into a new sterile microtube (hard particles were removed). The gold suspension was centrifuged at 9000 rpm for 10 seconds and supernatant (ethanol) were then removed. Pellet was added with 1 mL sterile water and then mixed using vortex and settled under gravity for a minute. Washing process was repeated three times with centrifugal interruption for about 10 second at the end of each step. The gold was finally resuspended in 600 µL 50% glycerol.

DNA coating (for 10 shooting)

100 µL of gold suspension, 10~20 µL of plasmid DNA, 100 µL of 2.5 M $CaCl_2$ and 40 µL 0.1 spermidine were added sequentially to the particle suspension. After thorough mixing by short spurts of vortexing (about 3 minutes), the gold-DNA suspension was settled under gravity at room temperature for about 30 minutes. The

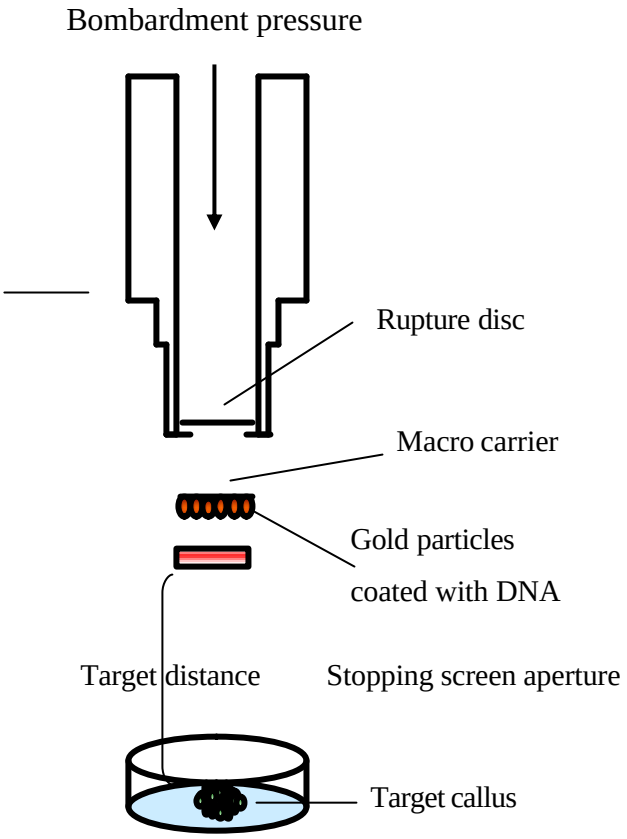
supernatant was carefully removed and pellets were then washed with 200 µL 70% ethanol. The gold-DNA suspension was settled under gravity (or slightly centrifuge) for a minute. Washing process was repeated two times and finally resuspended in 110 µL 100% ethanol. Ten µL of this suspension was spread onto sterile macrocarrier discs for bombardment. The macrocarrier discs were air-dried prior setting for bombardment.

Particle bombardment and plant transformation

Anther calli and floret-derived calli of sorghum were subculture on fresh MS medium with 2% (w/v) sucrose in 2~9 weeks after induction supplemented with 2 mgL^{-1} kinetin and 2.5 mg L^{-1} 2,4-D. All anther-derived calli were divided into small pieces (0.5~0.8 cm in diameter) before bombardment. Calli were transferred on to fresh medium and placed at the center of a petri dish a day prior to bombardment. More than 30 anther calli were placed in a petri dish. Floret-derived calli were not divided into small pieces prior to bombardment and were bombarded in bigger calli size (0.7~1.5 cm in diameter). About 10~15 florets were planted in a petri dish. Regeneration medium for tall fescue was MS medium with 3% (w/v) sucrose supplemented with 0.2 mgL^{-1} kinetin.

A biolistic PDS-1000/He (Bio-Rad) was used for transformation. The standard bombardment parameters were used for routine microprojectile bombardment (Fig. 17). The distance from rupture dish to microcarrier, from microcarrier to stopping screen aperture and from stopping screen aperture to target tissue was 2.5, 1.0 and 9.5 cm, respectively. Bombardment was conducted twice per petri dish with 1100psi bombardment pressure.

B.



C. Bombardment parameters:

Apparatus PDS-1000/He (Bio-Rad)

a. Gold particle: 1.5–3.0 mm (Aldrich).
b. Gold load per bombardment: 0.6 mg.
c. DNA load per bombardment: 2 mg.
d. Bombardment pressure: 1100 psi.
e. Number of bombardment: 2

Bombardment distance:

a. Gap distance: 2.5 cm.
b. Stopping screen aperture: 1 cm.
c. Target distance: 9.5 cm.
d. Vacuum pressure: 125 kPa.

Fig. 17. PDS-1000/He (Bio-Rad) apparatus was used for particle delivery (A); Diagram of PDS-1000/He (Bio-Rad) apparatus showed component parts in detail (B); Bombardment parameters used in this study (C).

Histochemical GUS assay and stable transformation

Following bombardment, the calli were incubated for three days on the same medium and GUS gene expression was assayed histochemically with 5-bromo-4-chlorol-3-indolylglucuronide (X-Gluc, Gold Biotechnology, Inc) substrate according to Jefferson et al. (1987). Bombarded calli were incubated over night at 37^{o}C in an assay solution containing phosphate buffer (150 mM $NaPO_4$, 0.1% (v/v) Triton X-100, pH 7.0) with 1.0 mM X-Gluc and 20% (v/v) methanol, and examined under a dissecting microscope for blue precipitate in a cell. Each distinct spot was counted as one expression event.

For stable transformation, calli were transferred onto MS medium supplemented with 200 mgL^{-1} hygromycin. This hygromycin concentration absolutely repressed the number of colonies and fresh weight of tall fescue protoplast (Takamizo, 1996). Calli were incubated at 25^{o}C for one month for identification of hygromycin resistant callus. Only the survived calli were considered for transformation result.

Results and Discussion

The overall percentage of anther and floret-callus induction varied among genotypes. The percentage of anther-callus induction ranged from 10% to 35% and floret-callus induction ranged from 68% to 80% from the total induced explants (Fig. 18). Embryogenic calli were observed in some florets and anther-derived callus and browning was also observed in anther-derived calli (Fig. 18c,d).

Activity of GUS genes were observed for all DNA plasmids in both sorghum callus and tall fescue suspension cultured cells. Presence of any blue spots in sorghum

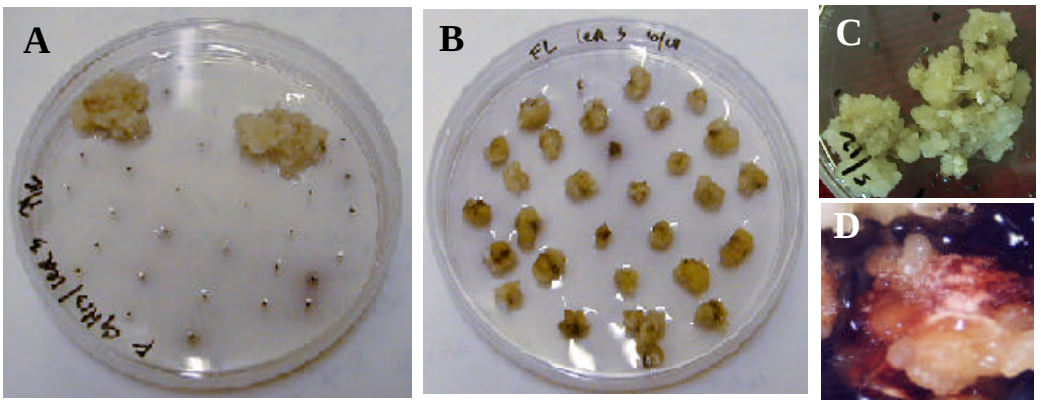


Fig. 18. Anther-derived calli (A) and floret-derived calli (B) of srghum. Embryogenic calli of floret-derived sorghum callus (C) and browning in anther-derived sorghum callus (D).

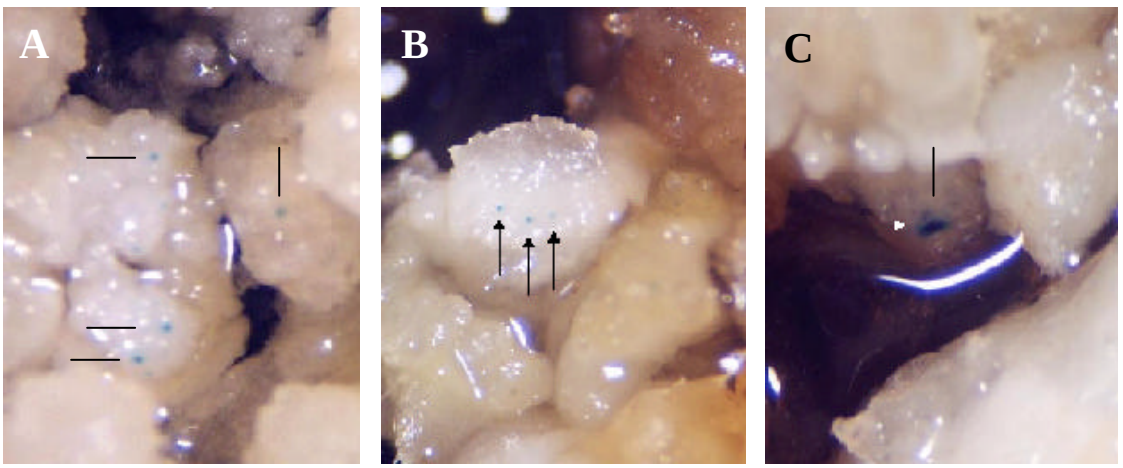


Fig. 19. The transient GUS expression (blue spots by arrow-marked) in floret-derived sorghum calli bombarded with pAct-D (A), pWI-GUS (B), pEX7113 (C).

callus indicated transient expression of the *gus* gene (Fig. 19). This suggested that three DNA constructs (pAct1-D, pWI-GUS and pEX7113) could deliver introduce-genes into sorghum cells and could be used in gene transformation technique. GUS expression was detected three days after bombardment indicating that division and growth of the cell to which DNA was delivered has occurred (Casas et al., 1993). The pWI-GUS construct carrying double CaMV 35S promoter gave the highest gene transformation in sorghum callus (Fig. 20). This DNA construct (pWI-GUS) also gave the highest number of blue spots in tall fescue compared with five others DNA constructs (Takamizo, 1996).

The number of blue spots per petri dish in sorghum callus (2~8 blue spots) was lesser than blue spots per petri dish in tall fescue suspension cell culture (57 blue spots). Tall fescue was used as a control because all protocols and bombardment parameters were routinely used for tall fescue, thus it was not surprising if the number of blue spots in tall fescue was much higher than in sorghum callus. Takamizo (1996) reported difference of transient GUS expression in suspension cultured cells among species, in which the number of blue spots in tall fescue (104 spots) was much higher than meadow fescue (1.7 spots) and Italian ryegrass (8.0 spots). The optimization of bombardment parameters; i.e. vacuum condition, distance between microparticles and target cell, size and density of the microparticles and driving power, were needed for more efficient gene transfer.

No GUS activities were observed in anther-derived callus because dividing of sorghum anther-derived callus prior bombardment caused browning of all anther callus.

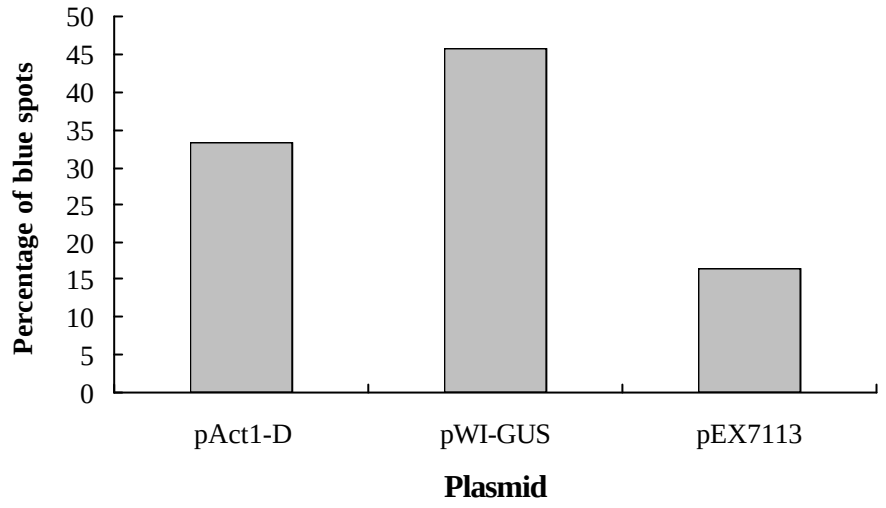


Fig. 20. Transient expression of GUS gene in floret-derived sorghum callus bombarded with 3 different plasmids.

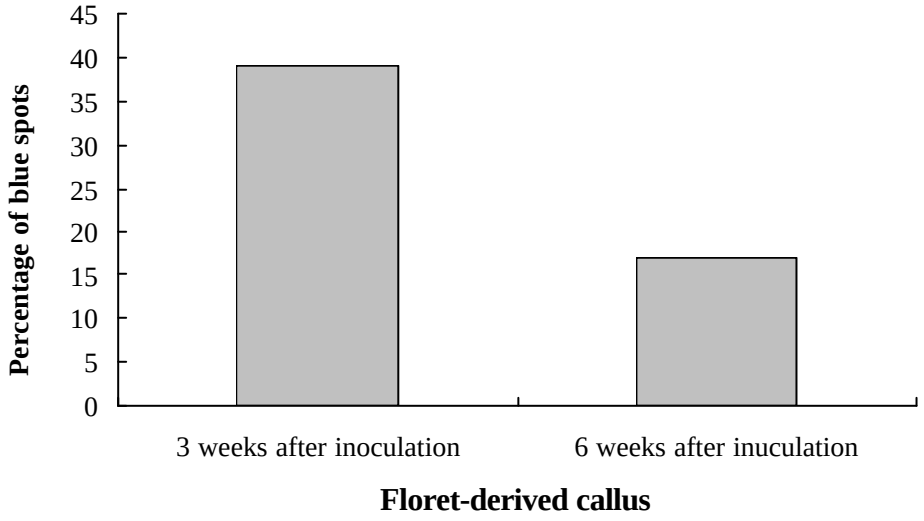


Fig. 21. Effect of callus age on transient expression of GUS gene in floret-derived sorghum callus bombarded with 3 different plasmids. GUS activity were evaluated over plasmids.

GUS activity was observed in 2~3 weeks age of all floret-derived calli and were higher than GUS gene activities in 6~7 weeks age floret callus (Fig. 21). These results suggest that young, vigorously growing fresh callus is more suitable for direct gene transfer. In addition, callus without physical interference at least a day before bombardment was important in efficient gene transfer.

Hygromycin resistant calli could not be selected in this study because number of escaped callus in hygromycin medium was too high (more than 90% callus survival rate was observed). Takamizo (1996) reported that callus size affected the successful selection of callus and number of escaped callus in the hygromycin-containing medium.

Conclusion

Activities of GUS genes were observed in both sorghum floret-derived callus and tall fescue suspension cultured cells that were bombarded with three DNA plasmids (pAct1-D, pWI-GUS and pEX7113). GUS activity was observed in all 2~3 weeks age floret-derived calli and were higher than GUS gene activities in 6~7 weeks age floret callus. The pWI-GUS construct carrying double CaMV 35S promoter gave the highest gene transformation in sorghum callus.

VI. General Conclusion

For effective Al-tolerant sorghum breeding program, (i) well understanding in genetic diversity and genetic background of the intended germplasm; (ii) accurate Al screening technique; (iii) understanding how the Al tolerance is inherited; and (iv) efforts to try the new breeding method of genetic engineering are needed. All these problems were subjected in this thesis.

High phenotypic and genotypic variations were observed among the sorghum breeding germplasm used in this study. Generally, harvest index, 1000-grain weight, length of head, plant height, stalk diameter and dry weight showed high variation. The highest phenotypic and genotypic variations were shown by 1000-grain weight, with harvest index of next magnitude, then plant height, length of head, dry weight and stalk diameter. High phenotypic variation among plants in germplasm will provide an opportunity in selection of superior plants that can be used as a parent in a cultivar development program.

High phenotypic and genotypic variations in field experiments were also supported by their genetic diversity assessed using SSR markers. Phenotypic or genotypic variation that closely related to the gene diversity would increase the effectiveness of selection. All breeding materials were distinctly placed at different clusters in a dendogram. Japanese and almost USA populations were separately clustered from ICRISAT populations. These populations were basic populations for development of sorghum tolerance to Al toxicity in this study. Further crossings between the materials and germplasm of separated groups were proposed for high yield progenies.

From the markers technology side, using nine SSR markers out of 15 SSR markers, which were usually used for genetic diversity study in multiplex PCR process, satisfied enough for genetic diversity study in sorghum. It was shown by relatively high diversity indices of each SSR marker and polymorphism of all SSR markers. Diversity indices of each locus ranged from 0.70 (Sb6-34) to 0.94 (Sb1-10).

Hematoxylin staining screening method was applied to select Al-tolerant genotypes in this study because it was simpler than other Al screening techniques and had a significant correlation with Al screening technique in Al-added soil in pot method. Hematoxylin staining screening method was also in agreement with performance information at field with high Al saturation. This gave a basis of accurate Al tolerance evaluation and was used all through this study.

Rapid evaluation of Al-tolerant genotypes at the cellular level could be detected by tissue culture. The differences in callus growth and the percentage of callus formation among genotypes were also in agreement with those in the hematoxylin staining screening method, indicating that cell culture should be feasible to develop a strategy based on the use of callus culture to assist in identification of Al-tolerant or susceptible genotypes for use as parents in a breeding program.

Crossings of breeding lines that genetically showed a different genetic background were conducted for development of Al-tolerant genotypes and for genetic study of Al tolerance. Some breeding lines were chosen as parents in the cultivar development program based on the results revealed in this study: (i) their performance in field trials; (ii) their relationship in diversity study using SSR markers technology and (iii) their performance in hematoxylin staining screening technique.

Moderately low heritability (H = 0.35 and H = 0.43) of Al tolerance was

observed in two crosses of sorghum and low genetic gain of Al tolerance was also observed. It shows that high allocation of resources in early generations for Al tolerance must be applied if higher gain for Al tolerance is to be obtained.

Indirect selection, which is believed to be effective for improvement of Al tolerance, might be possible to achieve more rapid progress in breeding of Al-tolerant genotypes. Based on the overall correlation data and path analysis data, dry weight was the important component in the relationship with Al tolerance. Therefore, it was suggested that increasing of dry weight and selection of Al tolerance in early generations were more appropriate to maximize the Al tolerance in sorghum.

As it was mentioned above that cell culture was possible to assist in identification of Al-tolerant genotypes, direct gene transfer into sorghum callus using particle bombardment should be an alternative for development of Al-tolerant cultivars through gene transformation. Transient expressions of the GUS gene were observed in floret-derived sorghum calli bombarded with three DNA plasmids (pAct1-D, pWI-GUS and pEX7113). The pWI-GUS construct carrying double CaMV 35S promoter gave the highest gene transformation in floret-derived sorghum calli. The young and vigorously growing fresh callus was more suitable for direct gene transfer using particle bombardment. However, more studies are necessary for optimization of bombardment parameters; i.e. vacuum condition, distance between micro particles and target cell, size and density of the micro particles.

Finally, since the gene control of Al tolerance in sorghum is not well understood, the conventional plant breeding program is still important for development of Al-tolerant sorghum. Improvement of efficiency of the conventional plant breeding program that was judged from results obtained in this study might be gained through: (i)

precise selection of parents that will be used for development of Al-tolerant population; (ii) adequate number of plants in early generations for selection of Al tolerance; (iii) use of indirect selection via increasing of dry weight that was closely correlated with Al tolerance; (iv) use of cell culture assay procedure to assist identification of Al-tolerant genotypes. Further development of DNA markers, including SSR markers for Al tolerance and understanding of gene control of Al tolerance that can be utilized in direct gene transfer will more improve the efficiency of the conventional plant breeding program.

This thesis includes new findings about: (i) genetic relationship and genetic background of the Japanese cultivated sorghum and the elite breeding germplasm; (ii) association between SSR markers and agronomic traits in sorghum; (iii) efficient and reliable Al screening technique in sorghum; (iv) genetic gain of Al tolerance and direct or indirect effect of agronomic traits on Al tolerance. All of this information, which I believe, should contribute to the improvement of efficiency in the sorghum-breeding program and support to the gain in world food production for mankind welfare.

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