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Genetic Resources and Breeding of Tropical Grasses, Forages - Apomixis - Biofuel Feedstocks

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Farmers select landraces from wild plant populations. Landraces are useful for plant breeding. Genetic erosion is diminishing these valuable genetic resources and steps to retain these materials are required. Therefore, germplasm collection represents an important activity of agricultural scientists. From 1971 to 1973, two Japanese scientists made excursions to East Africa and collected ca. 2,000 accessions of tropical grasses, named the "Africa collection". Within the collection, there are 140 guineagrass accessions, which is primarily a tetraploid (4x) form that reproduces through aposporous apomixis. A single, diploid (2x) sexual accession, GR297, was isolated from the collection and this accession played an important role for the breeding and molecular analysis of apomixis gene(s). During progeny testing of the open-pollinated GR297, a vigorous apomictic 4x hybrid plant, registered as cv. Natsukaze, was identified. Then, chromosome doubling of the GR297 was attempted to facilitate hybridization with 4x lines, and the 4x sexual line, Nekken No. 1 (Noh PL 1), was developed. This line has been utilized as a maternal parent for the development of new varieties, mapping and linkage analysis of apomixis. Even though the locus for apospory was identified, markers linked to the gene(s) exhibited no recombination. This apospory-specific genomic region was physically mapped by FISH to a single chromosome. Sorghum is cultivated as a forage crop in Japan. Germplasm of sorghum were collected with the cooperation of Myanmar and Kenya. Recently, sweet sorghum has attracted the interests of the biofuel industry for use as bioethanol feedstock. Radiation breeding has been applied to induce *bm* and *bmr* mutantions; which would be useful for efficient biofuel production. Mutation breeding elevates the frequency of natural mutation rates and can be efficiently utilized to develop new breeding materials. The importance of Genebank activities and mutation breeding towards biofuel production conducted within our center are discussed.

Key words: apospory, germplasm collection, guineagrass, linkage analysis, sorghum

Introduction

Farmers select cultivated forms (landraces) from wild plant populations. These landraces and wild populations are very useful for plant breeding and the development of superior genotypes. Genetic erosion, caused by the development of the land and cultivation of high-yielding varieties, is diminishing these valuable genetic resources and steps to retain these materials are required.

Germplasm collection represents an important starting point of most breeding programs. Therefore, exploration followed by collection and maintenance of such genetic resources represents one of the most important activities of agricultural scientists.

1. Genetic resources of guineagrass

Guineagrass (*Panicum maximum* Jacq.) is one of the most important tropical forage grasses native to tropical Africa and is cultivated widely in tropical, subtropical, and even in temperate regions. Its reproductive method is based on aposporous apomixis with pseudogamy (Warmke, 1954). Apomixis, an asexual method of reproduction, provides a method for

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cloning plants through the seed. As a result, the progeny are genetically identical to their maternal plant. The advantages of an apomictic form of reproduction, as reviewed by several authors (Hanna and Bashaw, 1987; Vielle *et al*., 1996; Kindiger and Sokolov, 1997; Savidan, 2000), are: 1) the fixation of any genotypic combination; 2) propagation of hybrid seeds through the sowing of open-pollinated seeds harvested from hybrid plants rather than hybridization of parental inbred lines (we term this a permanent hybrid; Nakagawa and Ebina, 2001); 3) the maintenance of unique chromosome aberrations which would ordinarily be lost in a sexual reproductive system; and 4) the mass production of true-bred commercial seeds without separation from the other varieties etc. The most effective use of apomixis is in the commercial seed production of hybrid cultivars, that would exhibit heterosis or advantageous heterogenous gene combinations.

Breeding of guineagrass has been difficult because of difficulty in hybridization prior to the identification of sexual forms. However, sexual plants were found by Combes and Pernes (1970), Smith (1972), and Hanna *et al*. (1973), and the role of obligate sexual plants and facultative apomicts with high sexuality in the evolution of guineagrass through diversification and adaptation to new environments has been previously suggested (Savidan and Pernes, 1982).

1) Collection of genetic resources

From 1971 to 1973, Hojito and Horibata (1982) made excursions to East Africa. Specifically, in Kenya, Tanzania, Uganda, and Ethiopia, they collected ca. 2,000 accessions of tropical grasses. The collection is named as the “Africa collection”. Within the collection, there are 140 accessions of guineagrass, which is primarily a tetraploid (4x) form that reproduces through aposporous apomixis.

2) Evaluation and breeding of genetic resources

Nakajima *et al*. (1979) who intended to develop an apomixis breeding methodology attempted to isolate sexual accessions from this collection by the use of open-pollinated progeny testing in the field and was successful in identifying a diploid (2x) sexual accession, GR297 (Fig. 1a), which exhibited phenotypic diversity in the open-pollinated progeny. Nakajima *et al*. (1979) was also able to identify 4x sexual plants from other introductions. These findings set in motion an approach to utilize apomixis gene(s) in the breeding programs of guineagrass in Japan. During progeny testing of the open-pollinated GR297 accession, Nakajima identified a vigorous hybrid plant that was an apomictic 4x plant which was eventually registered as cv. “Natsukaze (Norin No. 1)” guineagrass, the first apomictic hybrid cultivar, which is generated by a cross between a diploid sexual plant from GR297 and an unidentified apomictic line through open pollination (Nakajima, 1985; Sato *et al*., 1990). Although the cultivar is a tetraploid facultative apomict which produces ca. 11% sexual embryo sacs, it is true breeding and no off-types have appeared in its open pollinated progenies. This behavior is probably due to a level of self incompatibility of the sexual embryo sacs (Nakagawa *et al*., 1993).

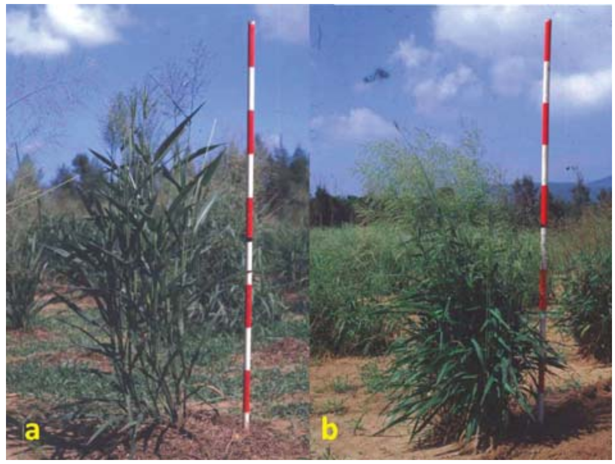

Fig. 1. Diploid sexual GR297 (a) and tetraploid sexual “Nekken No. 1 (Noh PL 1)” guineagrass (b).

Nakagawa and Hanna (1992) successfully double the chromosome number of the GR297 by colchicine treatment of germinating seeds. Hybridization among the newly generated auto-tetraploid plants eventually resulted in the development of the 4x sexual line, “Nekken No. 1 (Noh PL 1: Fig. 1b)” (Nakagawa and Hanna, 1992; Nakagawa, 1993). This line would later be utilized to facilitate hybridization with other 4x lines.” Nekken No. 1” has been utilized as a maternal parent for the development of new guineagrass varieties, as well as to generate mapping population for the linkage analysis of apomixis. Utilizing such materials, Nakagawa (1990) developed an apomixis breeding methodology, including embryo sac analysis and identification of reproductive method (Fig. 2), chromosome counting (Fig. 3), and an effective crossing procedure (Fig. 4).

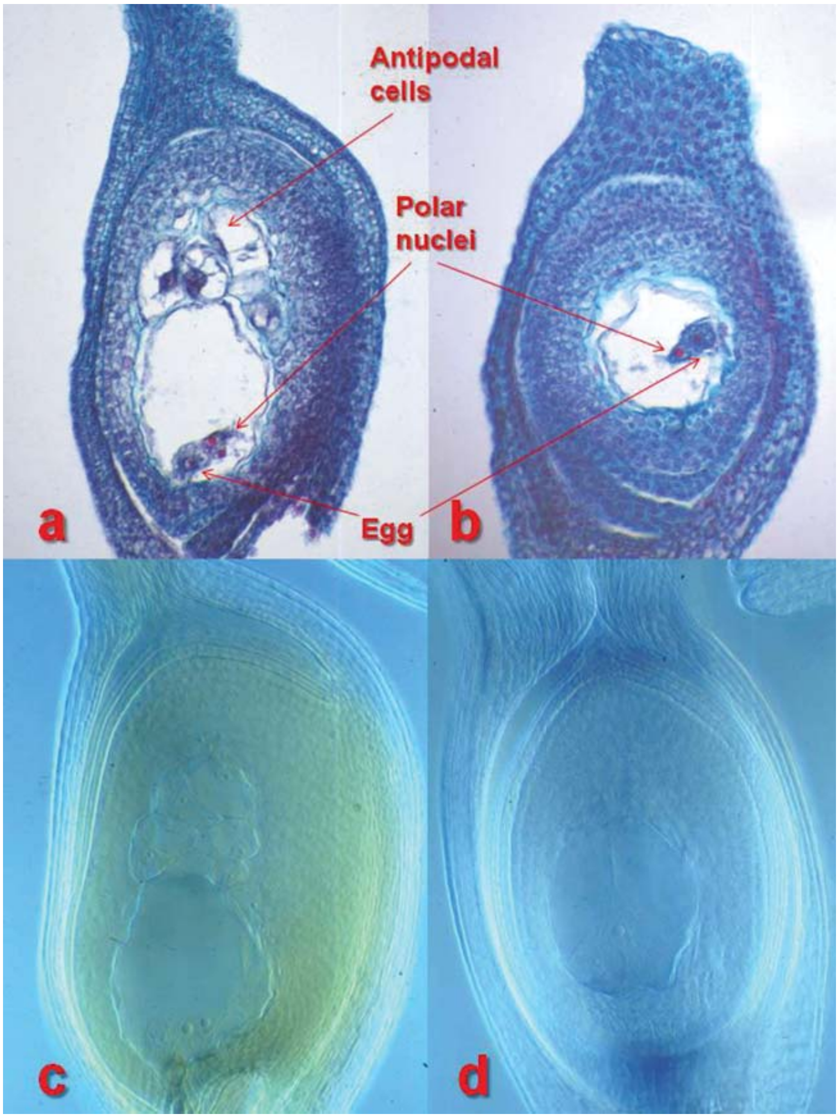

Fig. 2. Sexual (a) and aposporous apomictic (b) embryo sac by paraffin sectioning and sexual (c) and aposporous (d) embryo sac by clearing technique through Nomarsky differential contrast microscope (Nakagawa, 1990).

3) Linkage analysis of apomixis and mapping

There are basically two hypothesis concerning the number of gene(s) controlling aposporous apomixis in guineagrass: (1) two recessive genes control apomixis with epistasis (Hanna *et al*., 1973); (2) one dominant gene controls apomixis and the genotype of apomictic plant is only *Aaaa* and that of sexual plant is *aaaa* (Savidan, 1981); (3) one dominant major gene controls apomixis as reported by Savidan and sexuality is under polygenic control (Nakajima and Mochizuki, 1985). Nakagawa (1993) suggested that sexuality is closely related to the abortion of sexual embryo sacs, typically regarded as female sterility.

The DNA content of the diploid line, “Nekken No. 1”, was determined to be approximately 500 Mbp, which is similar size to *Oryza sativa* and represents a relatively small genome size when compared to other members of the plant kingdom and the 4x cultivars were determined to exhibit a genome size of approximately 1,000 Mbp (Akiyama *et al*., 2008). This information suggested mapping and linkage analysis of apospory were possible in guineagrass. Mapping and linkage analysis of guineagrass were conducted through the utilization of a hybrid population of “Nekken No.

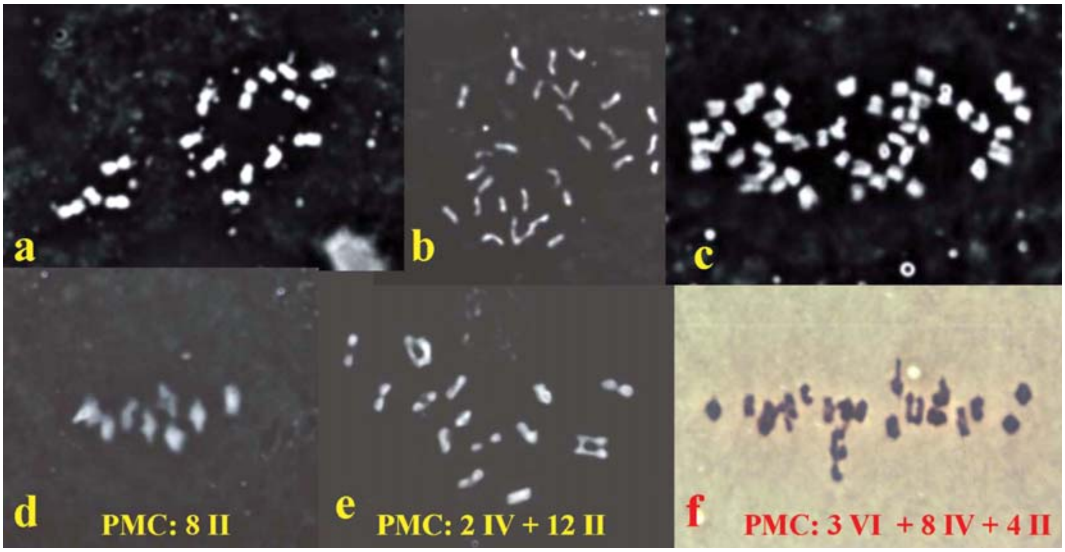

Fig. 3. Chromosome of diploid, (2n=2x=16; a: root tip; d: PMC), tetraploid (2n=4x=32; b: root tip; e: PMC) and hexaploid (2n=6x=42; c: root tip; f: PMC) (Nakagawa, 1993).

Fig. 4. Hybridization of guineagrass.
a: Emasculation by a plastic bag method; b: Collection of pollen from pollen parents; c: Hybridization in the field.

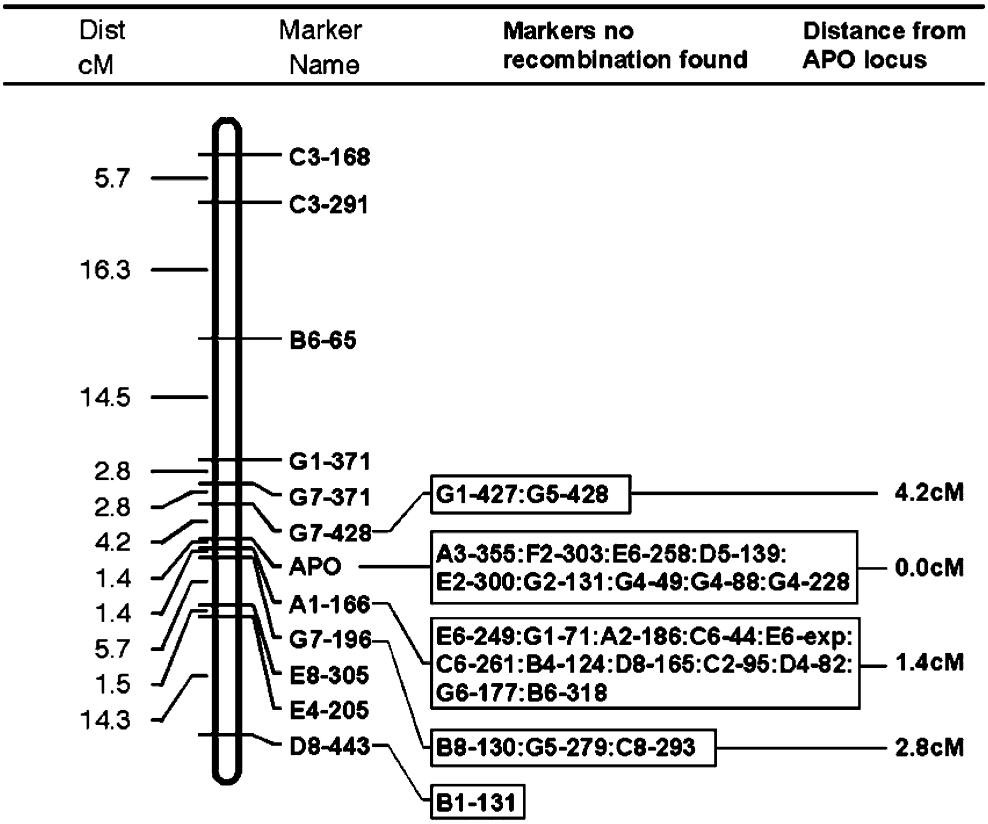

Fig. 5. Maping of apomixis locus (APO), in which recombination of AFLP markers are restricted (Ebina *et al*., 2005).

1" guineagrass and cv. "Natsukaze", which resulted in 71 individuals. A genetic linkage map of guineagrass was generated with AFLP markers found to be associated with apospory (Fig. 5) (Ebina *et al*., 2005). In addition, utilizing 56 AFLP primer combinations and 41 RAPD primers, 39 linkage groups and 360 simplex marker loci were assigned to the genetic map of cv. "Natsukaze". These markers covered 1703.5 cM of auto-tetraploid guineagrass genome (2n=4x=32).

Even though the locus was identified, the nine AFLP markers linked to apospory exhibited no recombination between them, acting as a block of linked markers. This has also been confirmed in *Pennisetum* and another aposporous apomictic tropical grasses (Goel *et al*., 2006). These recombinationally inert regions linked to apospory have been given the terminology apospory-specific genomic region (ASGR). After the continuous research, one hundred and two AFLP markers linked to apomixis were identified from F_1 progeny segregating for apomictic or sexual phenotypes. Among them, 58 markers were successfully converted to STS markers. BAC clones isolated from these materials, using STS markers from the apomicts, were physically mapped by fluorescence *in situ* hybridization (FISH) to a single chromosome in the genome of the aposporous accession. The site was absent in sexual individuals. Expression analyses using a small scale RT-PCR and Q-PCR microarray detected three genes specifically expressed in aposporous tissues (Yamada-Akiyama *et al*., 2009). Another approach toward isolating apomixis gene(s) in these materials involved mutation induction at the ASGR region of apomictic lines through gamma-ray irradiation with the purpose of generating a knock-out gene mutation (Nakagawa *et al*., 2010; Takahara *et al*., 2010).

2. Genetic resources of sorghum

Sorghum (*Sorghum bicolor* (L.) Moench) is the world's fifth leading cereal grain, after wheat, rice, corn, and barley, and the third leading cereal grain in the United States, following corn and wheat. Sorghum is a crop with a great genetic diversity, with its major advantage being its tolerance toward heat and drought. Sorghum, which was domesticated in Africa, introduced through India to China and other Asian regions. In Japan, cultivation of sorghum has a long history. The cultivation of sorghum is similar to cereal crops such as the millets, which were rotated with buckwheat and azuki bean by slash and burn agriculture in the mountainous regions until recently. Today, it is one of the most important summer annual crops for forages of beef and dairy cattle, being similar to maize production in Japan.

Recently, sweet sorghum has attracted the interests of the biofuel industry for use as a bioethanol feedstock crop. As a consequence, new genetic resources are required depending on these new biofuel objectives.

1) Germplasm collection

Recently, two explorations for sorghum germplasm collection were made by Japanese breeders cooperating with researchers in Kenya (1997) with Kenya Agricultural Research Institute (Tsurumi *et al*., 1997) and in Myanmar (2002) with Myanma Agricultural Service (Nakagawa *et al*., 2002). During these explorations, 142 and 78 accessions of *Sorghum* spp. were collected in Kenya and in Myanmar, respectively. The evaluations revealed that many of these materials were found to be of late maturity when grown in Japan.

2) Mutation breeding of sorghum

Radiation breeding has been applied to induce the following *bm* and *bmr* mutations, which are anticipated to be useful for the efficient biofuel production of sorghum (Nakagawa *et al*., 2011a). Mutation breeding elevates the frequency of natural mutation rates in nature and can be efficiently utilize to develop new breeding materials which have not been identified in germplasm collections.

Recent data indicate that gamma-rays induce both a higher percentage of small deletions (1–6 bp (base pair)) and a lower percentage of large deletions (10,000–100,000 bp) (Morita *et al*., 2009).

Gamma-ray irradiation is one of the most powerful tools for the induction of mutations and favorable mutations are valuable for genome analysis, gene isolation, and the development of new varieties. A spontaneous, naturally occurring, recessive mutation called bloomless (*bm*: bloomless, no epicuticular wax on the stem surface) and brown midrib (*bmr*: brown mid-rib, low lignin content) have been successfully utilized in sorghum breeding for forage use in Japan. These characteristics are also suggested to improve sorghum bioethanol production through fermentation of cellulose of stems as well as the digestibility of the foliage. The induction of similar mutants in sweet sorghum varieties, such as cv. “SIL-05” and “Italian”, were attempted through acute gamma-ray irradiation in the Gamma Room and chronic gamma-ray irradiation in the Gamma Field, Institute of Radiation Breeding, National Institute of Agrobiological Sciences (Hitachi-Omiya, Ibaraki, Japan). Large scale acute gamma-ray irradiation at 180Gy and 200Gy to the seed and chronic irradiation in the Gamma Field (planting and irradiating the plants from germination to the seed harvest 10 m to 20 m from the ^{60}Co source) were applied to these varieties. In each population *bm* and *bmr* mutants were successfully obtained. Following pollination with “Nakei No. 3” sorghum, which is a cytoplasmic male sterile line and possesses both naturally occurring *bm* and *bmr* genes, these mutations were identified to be located at a different locus than the naturally occurring spontaneous mutation in “Nakei No. 3”. The *bm* mutants of “SIL-05” and “Italian” exhibited less damage from aphids than *Bm* wild types (Nakagawa *et al*., 2011a) suggesting that these new sweet sorghum materials would be more useful in a biofuel feedstock production field.

3. Future biomass researches and genetic resources

New breeding programs utilizing new biomass feedstocks have been initiated all over the world. Some are focused on ethanol production through fermentation of its stalk or straw. Some are directed toward biodiesel production from seed. Others are emphasizing biomethanol production or electricity generation through gasification of all the part of plant (Nakagawa *et al*., 2011b)

In Japan as well as the other countries, some of the most acute restrictions regarding the production and utilization of biofuel are the cost of feedstocks and its transformation into biofuels or electricity. Therefore, low-cost cultivation technologies relative to harvest, preservation, and pretreatment are required to attain a competitive price level, as well as a general improvement in more efficient transformation systems. Previously, we have maintained our objectives on breeding and cultivation of starch or sugar crops, such as potato, sweet potato, sugar beet, sugarcane, and sweet sorghum, for bioethanol production with the extraction of useful materials from these feedstocks. However, current challenges with biomass and its sustainable management are providing high-yielding perennial wild grass spices, such as *Erianthus* spp. and *Miscanthus* spp. As the diversity of our genetic resources is limited, plant collection of *Erianthus* is being conducted in South Asia with the researchers of these countries. A similar collection project for new *Miscanthus* species is being conducted in Japan (Anzoua *et al*., 2011).

4. Conclusion

"We can learn a lot by studying the genetic architecture of landraces that have evolved over centuries and millennia. We can learn much from traditional farmers who have developed the arts of survival." (Harlan, 1995). Also, discussed in this book, "Still, the question remains; who owns the germplasm and who should benefit from its use? I take it that the wrangling still goes on by people who otherwise are little involved, but a sort of consensus has emerged that the germplasm is a national resource but that should be freely shared to all legitimate users."

Germplasm collection and associated Genebank activities are important because germplasm is priceless and without new materials for agricultural research no development of new varieties or no isolation of new genes is possible. Germplasm would relieve famine through development of new varieties, as the "Green Revolution" demonstrated in India and others (Hesser, 2006). It is very important to all concerned to share the germplsm and its benefits by resolving legal issues relative to ownership and patent.

We, as caretakers of the planets agricultural germplasam resources, need to promote the maintenance and rejuvenation of collections for all humanity.

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