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REVIEW

The Present Status of C_4 Tropical Grasses Breeding and Molecular Approaches

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Abstract

Tropical grasses have been widely utilized as warm-season grasses in the warm temperate zone since the early 20th century because of their high yields as well as for perennial forages in their native tropical areas. The high yield of tropical grasses is mainly due to C_4 photosynthesis. However, the soaring demands for animal production sparked by economic development in tropical countries mean genetic improvement of such grasses is urgently needed. Considerable breeding efforts have been made to create and develop new tropical grass cultivars, although direct selection from natural populations remains the main method used for breeding. Cross-breeding has not proliferated due to a lack of genetic information concerning the polyploidy, high sterility, outcrossing, and apomixis of these grasses, although several of these difficulties are starting to be resolved by advanced research using molecular biology tools. These tools are an effective means of achieving genetically improving of tropical grasses, and further development is anticipated. In this review, achievements in the improved guineagrass, brachiariagrass, sorghum, and zoysiagrass are introduced and discussed.

Discipline: Biotechnology, Genetic resources, Plant breeding
Additional key words: brachiariagrass, forage, guineagrass, sorghum, zoysiagrass

Introduction

Tropical grasses have been widely utilized as warm-season forage grasses in warm temperate regions and as perennial forage grasses in tropical areas since the early 20th century, mainly because of their high yields. Some of these grasses represent familiar species whose value as forage has only recently been recognized (Moser et al. 2004). As a typical example, guineagrass is a valuable forage grass with numerous names given to it by various tribes familiar with it in its natural habitat, and brachiariagrass is still not utilized willingly in its area of origin. Most of these grasses have extremely high yields because of C_4 photosynthesis. Demand for animal production in tropical countries is currently soaring due to economic development there, which is one of the major factors driving the development of new tropical grass cultivars.

Despite considerable breeding efforts to create and develop new tropical grass cultivars, direct selection from natural populations remains the main breeding method used (Moser et al. 2004), with some notable exceptions. Some efforts have also been made to perform crossing in guineagrass (Bhandari et al. 2011) and brachiariagrass (Felismino et al. 2010, Miles et al. 2004) species, but their polyploidy, high sterility, outcrossing, and apomixis hinder the development of new cultivars. Some breeding progress has been made using molecular biology methods in addition to conventional methods (Ebina et al. 2005, Tsuruta et al. 2011). These molecular tools are particularly powerful for obtaining information on their genetic information and inheritance, which has usually been insufficient to improve the key traits of tropical grasses. For these reasons, further developments of molecular tools in these species hold great promise.

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In this review, we report on our progress in breeding some tropical grasses (guineagrass, brachiariagrass, zoysiagrass) and a forage crop species with various varieties (sorghum). In guineagrass, a forecast by designed breeding is hardly prevented by their apomixis trait, but the use of molecular tools means that breeding is no longer hindered by the presence of apomixis. The improved digestibility is one of key areas of focus in guineagrass, while in brachiariagrass, the marker-assisted selection (MAS) method used for guineagrass was applied (Ebina et al. 2005), spawning faster breeding. In sorghum, the lignification mechanisms have been revealed by molecular techniques (Tsuruta et al. 2010) and used to produce higher-quality forage cultivars. In zoysiagrass, a domestic forage grass in Japan, molecular techniques also provide a number of advantages. In this review, we introduce the details of these applied molecular techniques and discuss how they are used to breed tropical grasses.

1. Guineagrasss

(1) Breeding and characteristics of recently released cultivars

Guineagrass (*Panicum maximum* Jacq.), a member of the family Poaceae, subfamily Panicoideae, and tribe Paniceae, forms an agamic complex with two other species, *Panicum infestum* Anders and *Panicum trichocladum* K. Schum. (Muir & Jank 2004). Guineagrass is one of the major forage grasses in tropical and subtropical regions and originated in Africa, where it exhibits much wider climatic adaptation than in cultivated pastures of other tropical and subtropical regions. The largest variations in native guineagrass emerged in accessions from its center of origin in East Africa rather than in West Africa. We attempted to obtain molecular phylogenetic data using simple sequence repeat (SSR) markers, which were consistent with the previous morphologically observed variations (Ebina et al. 2007).

In the 1940s, numerous *Panicum* accessions were collected and the cultivar 'Gatton' was released from the collection (Edye & Miles 1976), while in the 1970s, the Plant Introduction Center in Georgia collected and characterized guineagrass accessions mainly from South Africa (Hanna et al. 1973). French and Japanese scientists discovered sexual materials from East Africa through the diversity center germplasm collections in Kenya and Tanzania, respectively (Pernès 1975, Nakajima et al. 1979, Hojito & Horibata 1982). Recently, an intensive breeding effort using the French collection sparked the release of 'Tanzania', 'Mombasa', and 'Massai' in Brazil. We developed and released 'Paikaji' in Japan using the Japanese collection and 'Paikaji' was developed particularly for subtropical monsoon areas, whereas most other cultivars of guineagrass cultivars have been released for tropical areas (Ebina 2008). A Japanese guineagrass cultivar for use in temperate areas, 'Natsukaze', is relatively vigorous in terms of initial growth and annual yield, but has a considerably lower yield in tropical areas, while another, 'Natsuyutaka', exhibits good persistency. However, 'Natsuyutaka' shows a swift decline in forage quality owing to its early flowering when grown in subtropical areas of Japan.

'Paikaji' is an apomictic cultivar exhibiting good persistence, comparatively high yield, high quality, and slightly later flowering than early-flowering varieties such as 'Gatton' and 'Natsuyutaka' (Table 1). The dry-matter yield (DM) of 'Paikaji' exceeded that of 'Gatton' and resembled that of 'Natsuyutaka'. The *in vitro* dry-matter digestibility (IVDMD), crude protein (CP), and high-digestibility fiber (Oa) of 'Paikaji' exceeded those of 'Natsuyutaka', indicating high forage quality (Kouki et al. 2007). One reason for the high quality of 'Paikaji' is its flowering characteristics, so we examined the segregation

Table 1. Agronomical characteristics of the 'Paikaji' compared with other used cultivars in South-East islands in Japan

	DM t/ha/yr.	Dry matter ratio %	IVDMD %	Flowering date	CP %DM	ADF %DM	NDF %DM	Ob %DM	Oa %DM
'Paikaji'	27.9	21.7	52.5	8-Aug	11.3	41.5	70.5	59.8	11.1
'Gatton'	24.7	23.7	49.6	31-Jul	10.3	39.9	70.1	57.8	11.7
'Natsuyutaka'	28.0	23.7	47.6	6-Aug	8.8	42.3	73.1	62.1	10.1

Measurements were collected two years after establishment under good fertile conditions (Kouki et al., 2007).
Flowering date: the date of 10% emerged from total stems
IVDMD: in-vitro dry matter digestibility
CP: crud protein content
ADF: acid detergent fiber
NDF: neutral detergent fiber
Ob: low-digestibility fiber
Oa: high-digestibility fiber

of flowering time in progeny of 'Paikaji' (Fig. 1), because flowering usually causes forage quality to decline. The maternal parent of the 'Paikaji' and 'Gatton' progeny was tetraploid sexual guineagrass 'Noh PL1' (Nakagawa & Hanna 1992). The progeny of 'Paikaji' and 'Gatton' were checked by examining amplified fragment length polymorphism (AFLP) banding patterns indicative of crossing, whereupon progeny from self-pollination was eliminated. The flowering data were checked NILGS, NARO in Tochigi, JAPAN. The flowering-time segregation of the 'Paikaji' progeny was unusual in not being at the median of the segregating progeny. Instead, 'Paikaji' had the same flowering time as its earliest-flowering progeny. In contrast, the flowering time of 'Gatton' was at the median of flowering time among its progeny. The broad-sense heritability of flowering time, as calculated by the variance of repetition of the apomictic paternal parent plant and its segregating progeny, was 94.8%. This value was considered a slight overestimation due to apomixis. However, almost all of the progeny of 'Paikaji' showed late flowering time. Accordingly, although 'Paikaji' carries genetic factors which delayed flowering, its own flowering time is only slightly later than that of the early-flowering cultivar 'Gatton'. Accordingly, 'Paikaji' must also carry genetic factors for both early and delayed flowering. In the field, 'Paikaji' did not all flower at once and the date when all ears emerged was later than the other cultivars tested (data not shown).

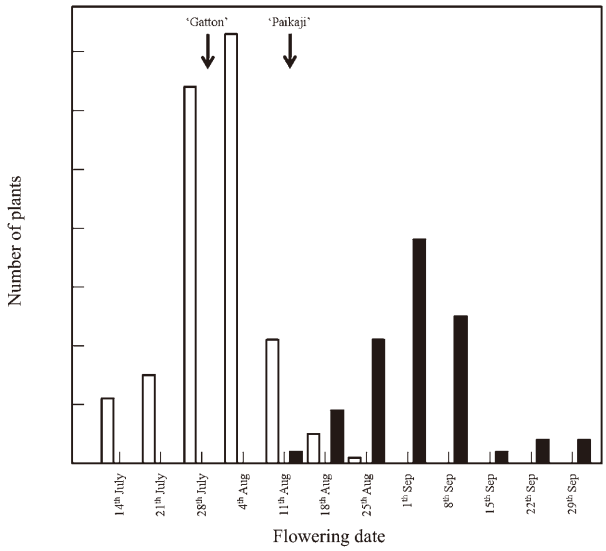

Fig. 1. Comparison of flowering date frequencies between the progeny of guineagrass cultivars 'Paikaji' and 'Gatton', both used as male parents in crosses with 'Noh PL1'. Arrows indicate flowering dates of the paternal apomictic plants. White and black bars show 'Gatton' and 'Paikaji' progeny respectively.

(2) Molecular breeding tools

Apomixis is an interesting and valuable trait (Ozias-Akins & van Dijk 2007) because it enables the propagation of numerous cloned seeds with genotypes identical to those of the maternal plant. Since apomixis also enables more rapid and efficient breeding, if a gene for apomixis were discovered, transgenic apomictic crops would hold considerable promise. Accordingly, many research groups, including our own, have worked to isolation of apomixis genes, both through molecular marker analysis (Ebina et al. 2005) and through gene expression analysis in apomictic flowers (Yamada-Akiyama et al. 2009). Guineagrass is one of the model plants for apomixis in tropical grasses. The linkage analysis of apomixis in guineagrass has sparked more than 250 molecular markers being identified at apomixis loci (Fig. 2). Gene isolation is being carried out using these numerous apomixis markers, while BACs harboring these markers are being used for contig construction and sequencing with next-generation sequencing technology. These activities have been done using progeny of 'Natsukaze'. The progeny of 'Paikaji' have also been tested with STS-AFLP apomixis markers, revealing that there are at least eight markers strongly associated with apomixis loci in the progeny of both 'Natsukaze' and 'Paikaji'. These two cultivars are genetically distant among guineagrass accessions (Ebina et al. 2007). Only a few sexual accessions have been recognized in guineagrass, so the screening and discovery of agronomically superior sexual lines of guineagrass among wild and breeding populations remains a key goal for the further development of guineagrass breeding. A common apomixis marker applicable across guineagrass germplasm could be a helpful molecular tool for the discovery of new sexual guineagrass accessions (Ebina et al. 2013a).

2. Brachiariagrass

(1) Recent breeding efforts

Brachiariagrass is a tropical forage grass that has recently attracted attention because of its high yield and forage quality. Breeding programs for brachiariagrass have been conducted mainly by the International Center for Tropical Agriculture (CIAT) and the Brazilian Agricultural Research Corporation (EMBRAPA). Careful and effective recurrent selection of tetraploid sexual lines for vigor, growth habit, leafiness, and spittlebug resistance have spawned several successful hybrid cultivars: 'Mulato', 'Mulato II', and 'Caiman' (Miles et al. 2004). An old cultivar, 'Basilisk', has been released and recognized for its forage value by the Commonwealth Scientific and Industrial Research Organization (CSIRO) after being selected from brachiaria germplasm introduced in the 1930s. 'Basilisk' spearheaded a 'Green Revolution' over tens of millions of hectares in the Central Brazilian Cerrado in the 1970s. After 10–15

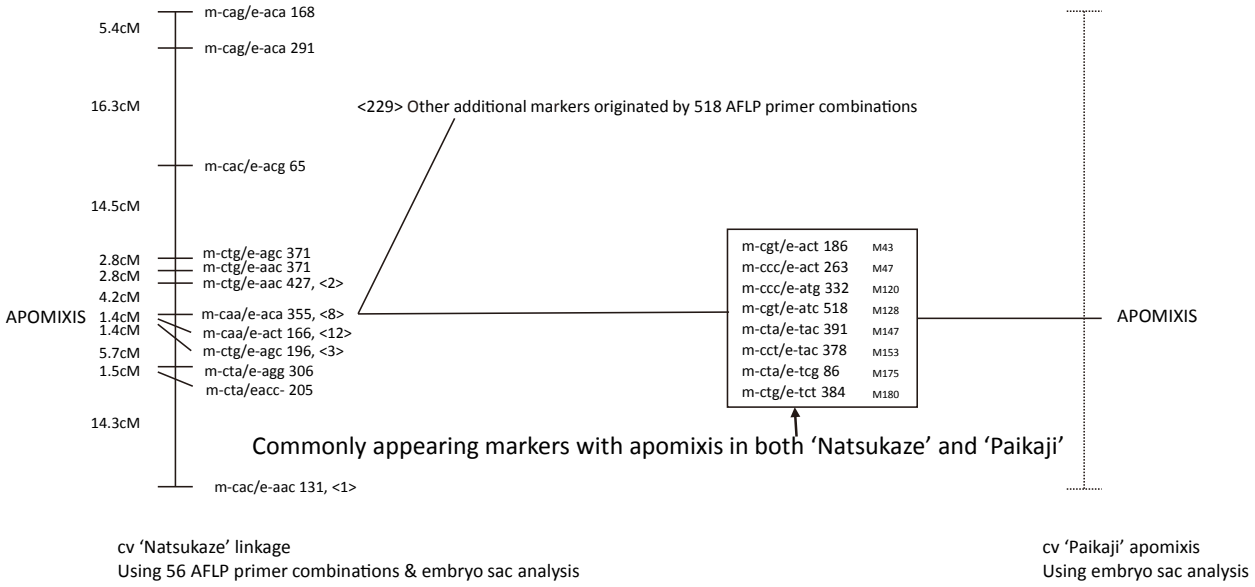

Fig. 2. AFLP markers tightly linked to apomixis in both 'Natsukaze' and 'Paikaji' guineagrass. Primer pairs are indicated to the right of the 'Natsukaze' linkage map at left; for example, "m-caa/e-aca 355" indicates a primer pair with an MseI-adaptor primer plus caa and an *Eco*RI-adaptor primer plus aca sequence and a band molecular size of 355 bp. Numbers within angle brackets (e.g., "<8>") following a primer pair name indicates the number of co-segregating AFLP markers. The box indicates AFLP-STS primers tightly linked to apomixis in both 'Natsukaze' and 'Paikaji'.

years, the spittlebug-resistant cultivar 'Marandu' was also selected from among the accessions and was substituted for the susceptible 'Basilisk'. Most recently, 'Mulato', 'Mulato II', and 'Caiman' have been released for use on tropical grasslands worldwide (Miles et al. 2004).

Important germplasm collections were accumulated in the 1970s and 1980s by CSIRO and the United States Department of Agriculture (USDA). The most intensive collection was amassed in the 1980s by CIAT, which maintained, evaluated, and selected several important cultivars in Colombia. Almost all the available the germplasm has been summarized in a CIAT genetic resources database (CIAT 2014).

Research activities on tetraploid sexual lines of *Brachiaria* spp. have been continued in tropical and subtropical Japanese and Southeast Asia (Ishigaki et al. 2010, Akiyama et al. 2010). Our recent breeding program for *Brachiaria* spp. has also got underway in the tropical monsoon region, particularly Thailand. Preliminary information on dry-matter yields of candidates for new brachiariagrass cultivars is summarized in Table 2. These candidates were selected by assessing isolated plant vigor. This breeding criterion facilitates the selection of high-yield lines, with a broad-sense heritability of 45.0% (calculated according to Caradus & Woodfield 1990). In grass breeding, there is usually little or no relationship between isolated plant vigor and grassland yield, preventing the selection of superior lines (Jameson 1963). However, the strong association between the performance of isolated brachiariagrass plants and grassland enables successful breeding selection based on isolated plant vigor. This method is used in the recurrent sexual line selection program at CIAT (Miles et al. 2004).

(2) Molecular breeding tools

Some techniques that applied to guineagrass spp. as molecular breeding tools for genetic improvement of brachiariagrass must be useful. The breeding population in Thailand was analyzed using AFLP analysis for apomixis, as was done in guineagrass (Ebina et al. 2005). First, seven obvious apomictic plants in the first 40 progeny (among 250 total progeny) were selected. Subsequently, the mode of propagation (i.e. apomictic or sexual) was precisely determined by using the microscopic embryo sac method according to Nakagawa et al. (1990) and shown in Fig. 3. Using 64 AFLP primer combinations, 12 apomixis markers were found (Fig. 4) and MAS was performed on all 250 plants in the breeding population (Table 3). In this way, the apomictic progeny in the breeding population from Thailand were precisely identified. The efficiency of MAS for apomixis is 50% that of direct selection because the apomixis trait is inherited as a single major gene. However, pre-selection has been combined with selection for seed fertility, and MAS for apomixis could enable the selection of the top 10% of superior apomictic plants prior to field selection for agronomic traits. These selection steps enable

Table 2. ANOVA for total of first-year dry matter yield and broad-sense heritability in candidate brachiaria-grass cultivars

Source of variation	df	SS	MS		significance
All	79	1849946.5			
Main-plot	19	115102.2			
Block [B]	3	12864.9	4288.3	1.2470	
Cultivar [V]	4	60971.2	15242.8	4.4325	*
Residual	12	41266.1	3438.8		

Model

Source of variation	df	Components of variation
Block [B]	b − 1	$\sigma^2e + v\sigma^2b$
Cultivar [V]	v − 1	$\sigma^2e + b\sigma^2v$
Residual	(b − 1)(v − 1)	σ^2e

$H_{BS} = \sigma^2v/\sigma^2p = \sigma^2v/\sigma^2v + \sigma^2b + \sigma^2e = 45.0\%$

σ^2p: Phenotypic variance

σ^2v: Genotype variance

σ^2b: Replicate variance

σ^2e: Error variance

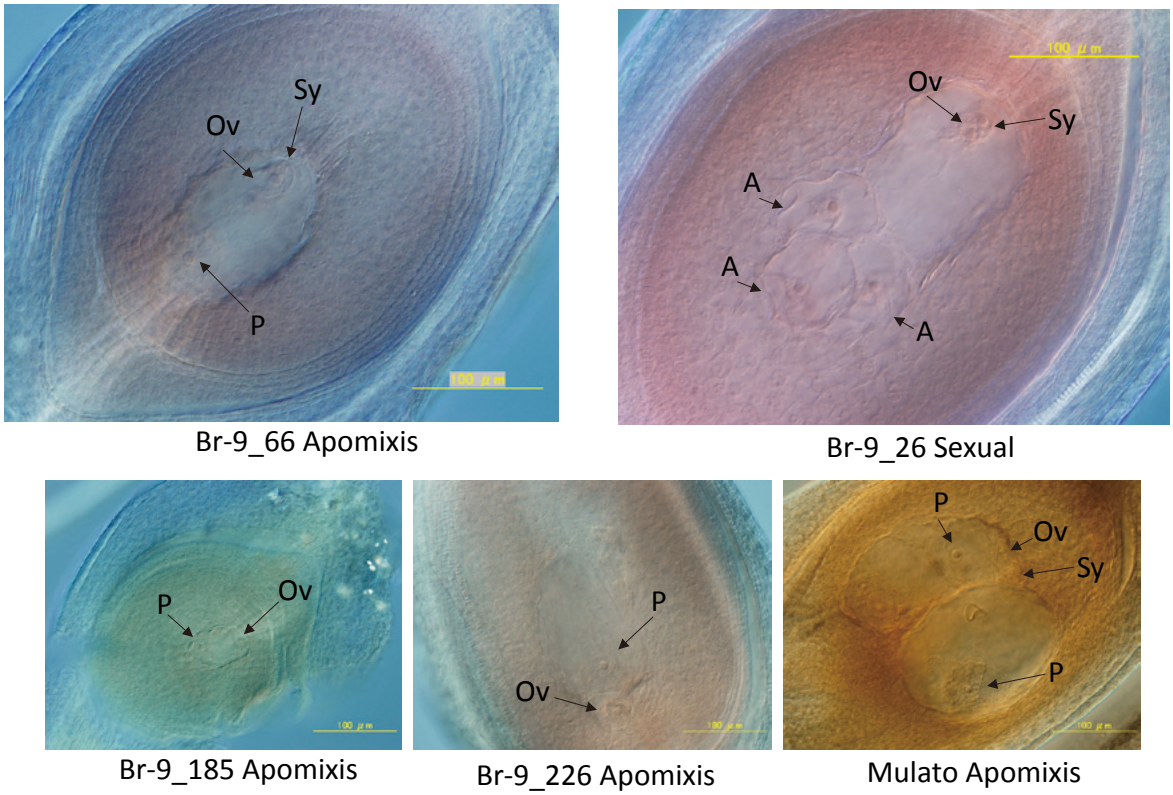

Fig. 3. Brachiaria Br-9 progeny indicating obvious apomictic (Br-9_66) and sexual (Br-9_26) embryo sacs, two apomictic progeny determined by MAS (Br-9_185 and Br-9_226), and the apomictic parent ('Mulato', with twin embryo). P: polar nucleus, Ov: ovule, Sy: synergid, A: antipodal cell.

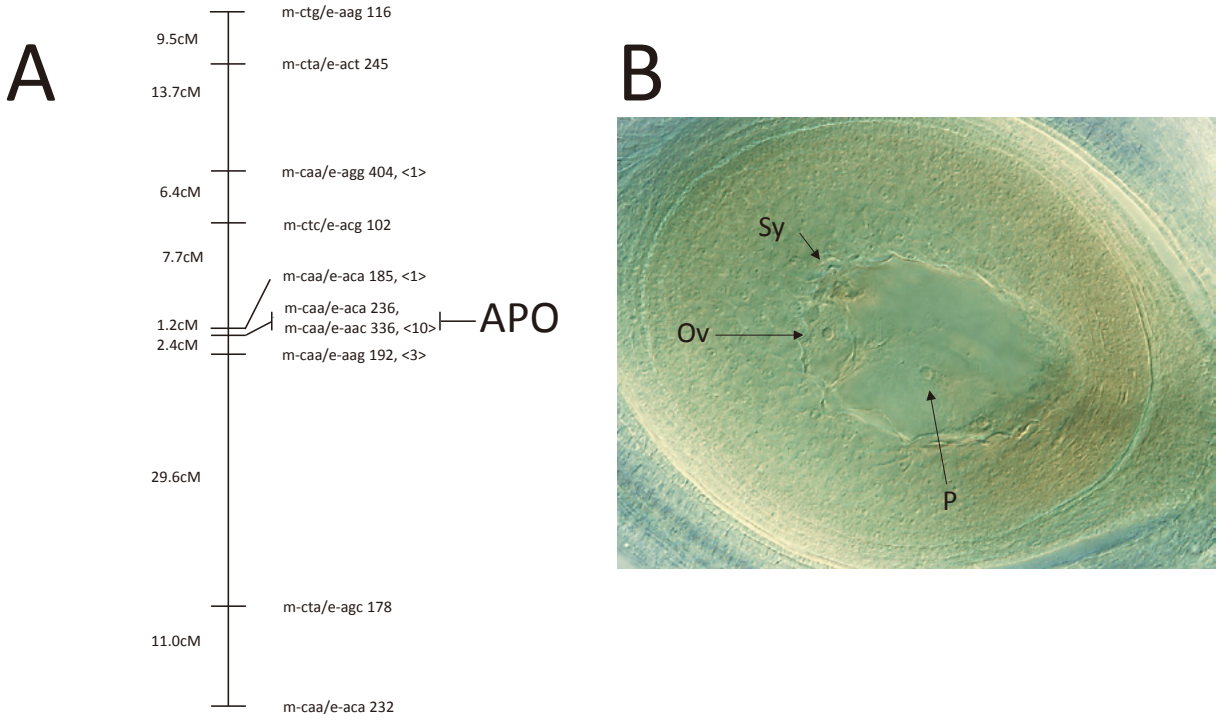

Fig. 4. Brachiaria apomixis linkage map and embryo sac analysis. (A) Linkage map. APO indicates the map location of 12 co-segregating AFLP markers. (B) One of the obvious apomictic embryo sacs from the F_1 progeny of a cross between a tetraploid sexual line and apomictic paternal plant 'Mulato'. P: polar nucleus, Ov: ovule, Sy: synergid.

Table 3. MAS for apomixis progeny of brachiariagrass using AFLP mapping

Plant material	Cultivar/ genotype	Mode of reproduction	AFLP Marker genotype 1: present 0: absent	
			m-caa/e-aac 336	m-caa/e-aca 236
Maternal sexual plants	'Miyaokikoku'	Sexual	0	0
Paternal apomixis plant	'Mulato'	Apomixis	1	1
Obvious apomixis progeny	Br-11	Apomixis	1	1
	Br-16	Apomixis	1	1
	Br-23	Apomixis	1	1
	Br-27	Apomixis	1	1
	Br-28	Apomixis	1	1
	Br-30	Apomixis	1	1
	Br-37	Apomixis	1	1
Other progeny (identified using MAS)	Br-185	Apomixis	1	1
	Br-203	Apomixis	1	1
	Br-226	Apomixis	1	1

1 Obvious apomixis progeny exhibited a typical apomixis embryo, and at least 100 such embryos were observed in each plant. by embryo sac analysis and confirmed by apomixis tightly linked AFLP marker genotypes.

2 Other progeny are identified by MAS of apomixis tightly linked AFLP markers and confirmed by the limited number of observations of embryo sac analysis.

faster, more compact and more efficient breeding.

The effectiveness of MAS in analysizing *Brachiaria* spp. has allowed 3000 brachiariagrass SSR markers to be developed using next-generation sequencing. These SSRs will be useful markers for brachiariagrass breeding by MAS after establishing their linkage relationships with important agronomic traits, such as yield and flowering time with high heritability.

3. Sorghum

(1) High-quality cultivar development

The main objectives of forage sorghum breeding include to increase yield, quality, and pest resistance (Pedersen & Rooney 2004). However, effort to improve forage quality through traditional breeding have been hindered by the complexity of this trait and its regulation by many genes, although the sorghum brown midrib (*bmr*) mutants induced by Porter et al. (1978) sparked a major breakthrough in breeding for forage sorghum quality. Forage digestibility, one of the major limitations of ruminant productivity, is negatively correlated with lignin content and is affected by the lignin composition in plant cell walls, whereby lignin causes forage quality to decline (Barrière et al. 2003). The brown midrib mutation in sorghum affects lignin content and composition in cell walls and provides high forage digestibility compared with the normal counterpart (Cherney et al. 1991). In addition to their biochemical properties, these mutants show a reddish-brown pigment in the leaf midrib and stem. Porter et al. (1978) reported that three of the mutants (*bmr*-6, *bmr*-12, and *bmr*-18) appeared most promising to improve forage quality. Since the brown midrib trait is inherited as a simple recessive trait and is closely associated with improved forage digestibility (Barrière & Argillier 1993, Bittinger et al. 1981), forage quality can be improved by fixing *bmr* alleles. Fritz et al. (1981) reported that both grain sorghum and sudangrass (*Sorghum sudanense* (Piper) Staft), each of which backcrossed with *bmr*-6, *bmr*-12 and *bmr*-18, expressed brown midrib traits and had less lignin content and more digestibility than normal genotypes. Recently, several cultivars bred to contain brown midrib traits have been released (Table 4).

(2) Molecular analysis of brown midrib mutants

Biochemical analysis has suggested that the alteration of lignin content or composition in the brown midrib mutants is due to reduced activities of enzymes involved in lignin biosynthesis, such as phenylalanine ammonia-lyase (PAL), caffeic acid *O*-methyltransferase (COMT), and cinnamyl alcohol dehydrogenase (CAD) (Bucholtz et al. 1980, Pillonel *et al*. 1991, Tsuruta et al. 2003, Palmer et al. 2008). The relationships between reduced enzyme activity and gene structure have been studied by molecular characterization of lignification-related genes. The brown midrib mutants in sorghum were chemically induced via the mutagen diethyl sulfate (DES) (Porter et al. 1978), a monofunctional alkylating agent that acts primarily as a base-substitution mutagen and has specificity for G/C-to-A/T transitions (Hoffman 1980). In previous reports, base substitutions resulting in premature stop codons were identified in the coding regions of the genes encoding COMT (Bout & Vermerris 2003) and CAD (Saballos et al. 2009, Sattler et al. 2009, Tsuruta et al. 2010) in sorghum brown midrib mutants (Table 5). In these mutants, the binding site of the enzyme has been changed, resulting in a loss of enzymatic activity.

Table 4. Sorghum cultivars with high digestibility; developed by fixing bmr genes in the homozygous recessive condition

Cultivar	Release or registration	Fixed *bmr* gene	Source	Reference
Hazuki	2002	*bmr*-18	Nagano Anim. Inds. Exp. Stn., JPN	Kasuga, 2002
Akidachi	2004	*bmr*-18	Nagano Anim. Inds. Exp. Stn., JPN	Kasuga et al., 2003
Atlas *bmr*-12	2005	*bmr*-12	USDA-ARS and Univ. of Nebraska, USA	Pederson et al., 2006
Kazetaka	2009	*bmr*-18	Nagano Anim. Inds. Exp. Stn., JPN	Takai, 2009
Suzukaze	2009	*bmr*-18	Nagano Anim. Inds. Exp. Stn., JPN	Takai, 2009

Table 5. Characters of candidate genes for brown mid rib in three bmr mutants in sorghum

Line	Mutant gene	Site of mutation	Type of mutant	Marker	Reference
bmr-6	CAD	Exon III	Transition (G→A)	CAPS	Saballos et al., 2009, Sattler et al., 2009
				SNPs	In this review
bmr-12	COMT	Exon I	Transition (C→T)	CAPS	Bout and Vermerris, 2003
bmr-18	COMT	Exon I	Transition (G→A)	SNPs	Bout and Vermerris, 2003

Based on the molecular characteristics of sorghum brown midrib mutants, DNA marker–identified *bmr* genotypes have been developed for each mutant. Bout & Vermerris (2003) have developed cleaved amplified polymorphic sequence (CAPS) markers to identify the *bmr*-12 and *bmr*-18 genotypes, while we have developed single-nucleotide polymorphism (SNP) markers to identify the *bmr*-6 genotype. A comparison of the sequences derived from N-6 and *bmr*-6 revealed a C-to-T transition in the third exon of *bmr*-6, leading to a single amino acid alteration (glutamine to a termination codon; Tsuruta et al. 2010). To verify the single base-pair change observed in *bmr*-6, SNP markers were developed to distinguish the wild-type and mutant CAD alleles (Fig. 5a). The mutant alleles were identified using a modified allele-specific PCR method, which is based on the presence or absence of a PCR amplification product from allele-specific primers (Hayashi et al. 2004). Eight primers with mismatches incorporated into the last two bases were also designed and tested to determine the effect of the mismatches on primer specificity. Among the four primers (NTG, NAG, NGG, and NCG) designed to detect only the wild-type allele, NTG and NCG amplified both normal and mutant alleles. Although NAG and NGG revealed amplification products with the expected fragment size only in N-6, as intended, the band obtained with NGG was weak. The mutant-allele-specific primer BAA was successful in generating an amplification product in *bmr*-6 that was absent from wild-type N-6, but bands obtained with BTA, BGA, and BCA were weak or undetected in *bmr*-6 (Fig. 5b). From the results of these tests, we selected NAG and BAA as the reverse primers for allele-specific PCR analysis. PCR amplification of each genotype with these primers produced clear results, and the base substitution in the *bmr*-6 mutant can be readily detected by PCR using these primers (Fig. 5c). This assay could be efficiently incorporated into breeding programs to improve forage digestibility and bioenergy feedstock using *bmr*-6. Because brown midrib mutations in sorghum are inherited as simple recessive alleles (Bittinger et al. 1981), allele-specific PCR analysis would be useful to identify heterozygous plants at any development stage. More experimental data and genetic studies on SNP markers are needed to develop these as a valuable tool for genetic studies and practical breeding in sorghum.

4. Zoysiagrass

(1) Characteristics and recently released cultivars

Zoysiagrass has been used as a lawn, turf, and pasture grass. In Japan, the use of zoysiagrass was recorded in the *Sakuteiki*, a Japanese gardening book published in 1156, and its commercial use began in the 1700s (Kitamura 1970). However, the selection and breeding of zoysiagrass is a relatively recent practice.

Zoysiagrass was introduced to the US from Japan in 1902 (Meyer & Funk 1989). *Zoysia japonica* 'Meyer' was the first variety to be jointly developed by the USDA and the US Golf Association. 'Meyer' was originally selected

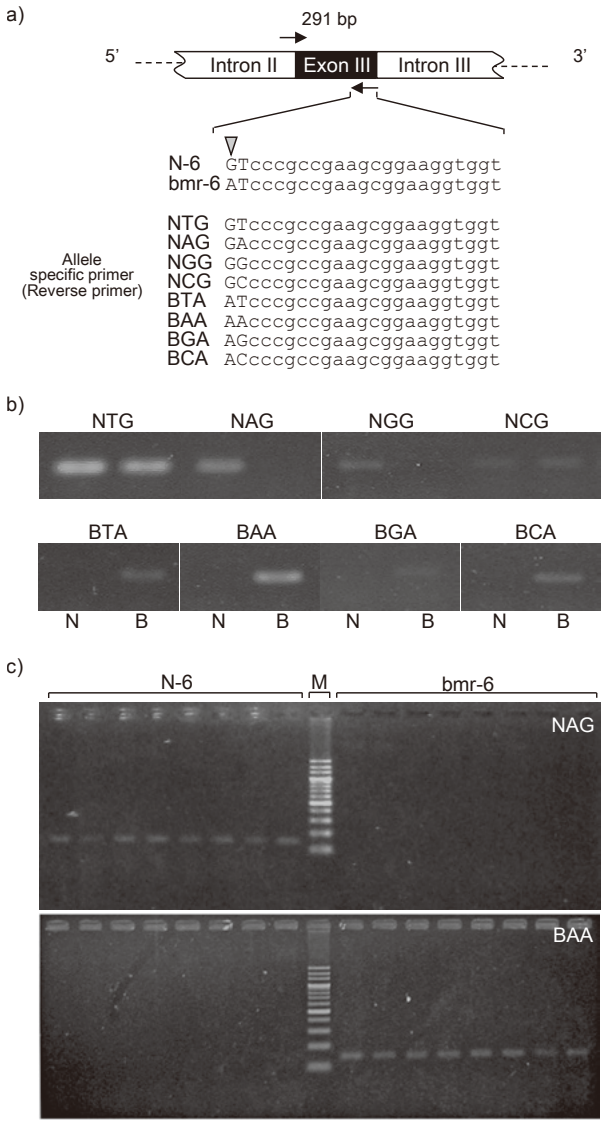

Fig. 5. Development of SNP assay using a modified allele-specific PCR method. (a) Primer sequences tested during the screening for allele-specific primers. The allele-specific primers each contained a mismatch of two nucleotides of the 3′ terminus, corresponding to the mutation site and one artificially mismatched nucleotide. The mismatched nucleotides in each primer sequence are boldfaced. (b) Screening of allele-specific primers. The constructed primers were tested for the effect of incorporating mismatches in the two nucleotides at the 3′ terminus of each primer. N, N-6; B, *bmr*-6. (c) Analysis of SNP markers using sorghum N-6 and *bmr*-6 genomic DNA. PCR amplification with both allele-specific reverse primers (NAG and BAA) and a common forward primer performed in eight individuals of each genotype. M: molecular size marker.

from a population of plants grown from seeds in 1941 and was released in 1951 (Grau & Radko 1951). Today its major use is as a turfgrass cultivar in the US. Subsequently, some commercial zoysiagrass cultivars such as 'Midwest' (released in 1963), 'El-Toro' (released in 1986), and 'Belair' (released in 1987) have been selected from collected genetic resources and plant populations grown from seeds. These cultivars are mainly used in parks and sports fields as turf grass.

At least five *Zoysia* species, including three which are commercially important (*Z. japonica, Z. matrella* and *Z. tenuifolia*), are native to Japan and they exhibit morphological and ecological differences (Ishida 1990). Accordingly, Japan is considered one of the origins of diversity of the genus *Zoysia*. With the aim of identifying materials suited for certain breeding programs (e.g. to stay green longer for late autumn and for low-input sustainable grazing), two large-scale projects involving the germplasm collection of native ecotypes in Japan have been performed. The first collections were performed on all of the Japanese islands in the 1980s and included all five native species. This collection was evaluated at the Japanese National Agricultural Research Center for the Tohoku Region and conserved in the Japanese National Livestock Breeding Center (Fukuoka 2000, Fukuoka et al. 2009). The second collection was organized by the Japanese Ministry of Agriculture, Forestry and Fisheries: more than 1200 accessions of *Z. japonica* were collected and their morphological traits were evaluated by the authors (Ebina et al. 2000a). These accessions are maintained at the National Institute of Livestock and Grassland Science, supported by the Genebank of the National Institute of Agrobiological Science in Japan. In Japan, breeding based on these collections started in the 1980s. Since 'Winter Carpet' (*Zoysia matrella*) and 'Miyako' (a natural interspecific hybrid between *Z. japonica* and *Z. matrella*) were released in 1998, many cultivars have been developed and released under an amendment of the Plant Variety Protection system (Asano & Aoki 1998). Currently, 41 zoysiagrass cultivars have been released in Japan, most of which have improved turf quality to a level comparable to that of 'Miyako' and the US cultivars. Zoysiagrass cultivars used as forage grass have also been developed in Japan: four of the 41 cultivars released are forage-type zoysiagrass. All the cultivars were developed via selection from the genetic resources collection and have characteristics such as long, wide leaves, high runner density, and rapid establishment (Table 6).

(2) Molecular analysis

Recently, several molecular marker studies focusing on characterization and genetic variability assessment of these zoysiagrass accessions have been attempted to preserve the local genetic resources and to understand the evolution of the genus *Zoysia* in Japan. Since SSR or microsatellite markers are generally more efficient for genotyping than other DNA-based marker systems (Morgante & Olivieri 1993, Powell et al. 1996), they are increasingly being developed for zoysiagrass (Tsuruta et al. 2011). We were interested in the relationship between genetic and taxonomic groupings of *Zoysia* species in Japan, particularly in clarifying the species boundaries between the three major zoysiagrass species (*Z. japonica, Z. matrella*, and *Z. tenuifolia*). Using 108 markers, a set of 193 representative zoysiagrass accessions from the three species were genotyped, and 20 microsatellite loci revealed a total of 360 alleles with polymorphism and good reproducibility. Based on these data, model-based Bayesian clustering analysis was performed using the STRUCTURE software (Pritchard et al. 2000). The results indicated that zoysiagrass accessions in Japan

Table 6. Zoysiagrass cultivars for grazing forage in Japan

Cultivar	Species	Release	Source[1]	Characters[2]	Reference
Akemidori	*Z. japonica x Z. matrella*	1999	GAFSA	Less no. of heading but long-wide leaf and increasing seed weight than the variety Mayer.	Nakayama, 2002
Inahikari	*Z. matrella x Z. japonica*	1999	GAFSA	Long-wide leaf, higher density of runner and increasing seed weight than the variety Mayer.	Nakayama, 2002
Asagake	*Z. japonica*	2002	NARO-ILGS	Long-wide leaf, higher density of runner, good to excellent winter hardiness.	Kobayashi et al., 2013
Asamoe	*Z. japonica*	2004	NARO-ILGS	Long-wide leaf, erect type, higher density of runner, rapid establishment, good to excellent winter hardiness.	Kobayashi et al., 2013

1 GAFSA: Japan Grassland Agriculture and Forage Seed Association (388-5 Higashi-akada, Nasushiobara, Tochigi, Japan), NARO-ILGS: National Agricultural and Food Research Organization, Institute of Livestock and Grassland Science (768 Senbonmatsu, Nasushiobara, Tochigi, Japan)

2 Description by the inventor

that were classified in the cluster analysis as originating from different species follow phenotypically recognized species boundaries. Twenty-eight (14.5%) of the 193 accessions were inferred to represent admixed individuals. The F_{st} value in *Z. matrella* was weak (0.122-0.074), although the values varied significantly between species. Most of these individuals were collected in the Southwest islands of Kyushu and in the Okinawa Archipelago in Japan. These areas contain a mix of all three *Zoysia* species, supporting the assumption that admixed individuals may be produced by interspecific hybridization. However, the divergence time between *Z. japonica* and *Z. matrella* (as calculated from the substitution ratio in a partial chloroplast genome sequence) is relatively recent: 0.8 to 1.0 Mya compared with 1.4 to 2.3 Mya between *Z. tenuifolia* and other species (*Z. japonica* and *Z. matrella*). In addition to the presence of interspecific hybridization, the relatively recent speciation of *Z. matrella* may be one of the factors leading to its unclear classification (Ebina et al. 2013b).

As noted above, DNA-based molecular markers such as RFLPs, AFLPs, and SSRs are being increasingly employed to construct genetic linkage maps of zoysiagrass. We have conducted a genetic linkage analysis of several important traits such as leaf width, salt tolerance, and freezing hardiness using F_1 and F_2 populations derived from an interspecific hybrid of *Z. japonica* and *Z. matrella* (Fig. 6a). Leaf width is used to discriminate among *Zoysia* species and is one of the most visible determinants of turf quality (Kitamura 1970, Turgeon 1996). According to quantitative trait locus (QTL) analysis of several morphological traits, leaf width is controlled by two major QTLs and one minor QTL. The major QTLs, which are located in linkage groups 7 and 15, have significant effects, particularly the region around the AFLP marker D2-433 (Fig. 6b). These results suggest that the genetic factors controlling leaf width in zoysiagrass might be relatively simple, although most morphological traits for turf species show continuous phenotypic variation and are controlled by QTLs. In addition, a linkage peak associated with leaf width was identified at a position similar to that of one associated with leaf length, runner length, and stem length at the heading stage, suggesting that these traits are at least partially controlled by the same QTL.

Although salt tolerance segregated in the F_1 population, no QTLs with LOD scores exceeding 3.0 were detected (Ebina et al. 2000b). Two QTLs with LOD scores exceeding 2.0 were detected in linkage groups 15 and 28 (Fig. 6c). In addition, a peak associated with leaf width in linkage group 15 (between markers A6-383 and D3-172) is at a similar position as a peak for salinity tolerance (Fig. 6b, c). In a study of barley, Mano and Takeda (1997) reported that the QTLs for the most effective abscisic acid response, which is correlated with leaf growth, were located very close to those for salt tolerance. Accordingly, genetic linkage between salinity tolerance and leaf width may be a common characteristic among members of the Poaceae. This finding should facilitate the elucidation of salt tolerance mechanisms in *Zoysia* and related genera.

Zoysiagrass has comparatively high freezing tolerance among the C_4 turf grasses. Freezing tolerance is often evaluated by measuring electrolyte leakage or regrowth of plant tissues after freezing. A QTL for freezing tolerance of zoysiagrass was detected in linkage group 25 in the F_2 population, but no significant QTL was detected in the F_1 population (Ebina et al. 2000b). This result suggests that freezing tolerance in zoysiagrass is conferred by a dominant gene that was homozygous in the female parent, *Z. japonica*. The QTL was located in the region of B1-227 region in linkage group 25, with a –0.294 contribution rate.

More recently, high-density genetic linkage maps have been constructed for zoysiagrass using AFLP and SSR markers (Cai et al. 2004, 2005, Li et al. 2009). In zoysiagrass, morphological characteristics that can be visually assessed are important factors when determining turf quality, while QTLs for tolerance of environmental stressors such as salinity and freezing may be widely distributed among species. In the near future, breeders may be assisted by DNA analysis, which has confirmed the degree of genetic variation in zoysiagrass.

Transgenic approaches to genetic manipulation offer an opportunity to generate unique genetic variation. Plant regeneration systems for zoysiagrass have been established from protoplasts (Asano 1989, Khayri et al. 1989, Inokuma et al. 1996) and embryogenic calluses (Bae et al. 2001). Recently, transgenic zoysiagrass plants were produced by polyethylene glycol–mediated (Inokuma et al. 1998) and *Agrobacterium*-mediated transformation (Toyama et al. 2003, Ge et al. 2006, Zhang et al. 2007). In our research program, Rahman et al. (2003) established a plant regeneration system from calluses derived from the apical meristems of seedlings and successfully produced transgenic zoysiagrass plants using *Agrobacterium*-mediated transformation. Subsequently, some useful genes have been introduced into zoysiagrass by *Agrobacterium*-mediated transformation to improve their resistance to various biotic and abiotic stressors (Li et al. 2006, Zhang et al. 2007). The transgenic plants can be grown by the vegetative propagation of stolons and rhizomes. Despite some concern that large-scale releases of transgenic plants may trigger serious ecological and environmental problems, genetic engineering represents a new avenue for the rapid production of unique *Zoysia* varieties.

References

Akiyama, Y. et al. (2010) Morphological diversity of chromosomes bearing ribosomal DNA loci in *Brachiaria* species.

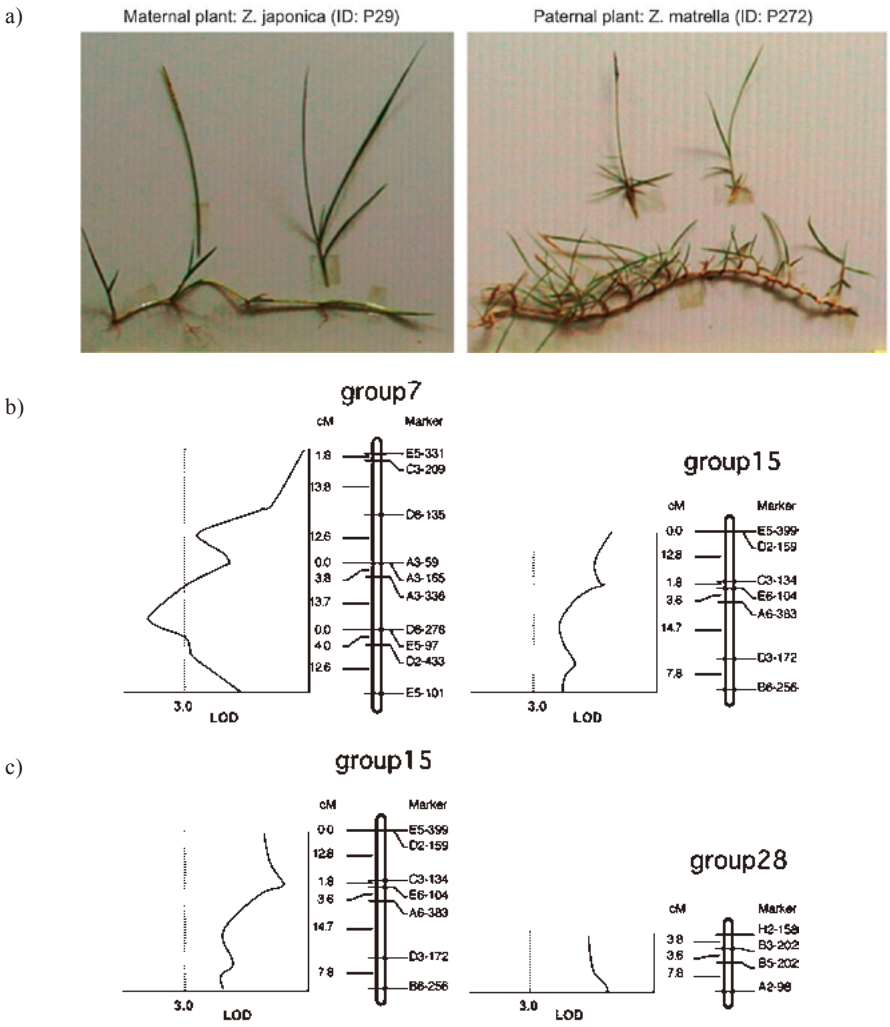

Fig. 6. Linkage analysis of leaf width and salt tolerance using F_1 and F_2 populations derived from an interspecific hybrid of zoysiagrass. (a) The parents of the F_1 progeny. (b) Linkage groups that contain leaf width QTLs and tightly linked AFLP markers. (c) Linkage groups that contain salt tolerance QTLs and tightly linked AFLP markers.

Grassl. Sci., **56**, 217-223.

Asano, Y. (1989) Somatic embryogenesis and protoplast culture in Japanese lawngrass (*Zoysia japonica*). *Plant Cell Rep*., **8**, 141-143.

Asano, Y. & Aoki, K. (1998) Turf grasses and the Cultivars. Soft Science, Tokyo, pp. 387. [In Japanese]

Bae, C. H. et al. (2001) Efficient plant regeneration using mature seed-derived callus in zoysiagrass (*Zoysia japonica* Steud.). *Kor. J. Plant Tiss. Cult*., **28**, 61-67.

Barrière, Y. & Argillier, O. (1993) Brown-midrib genes of maize: a review. *Agronomie*, **13**, 865-876.

Barrière, Y. et al. (2003) Genetic variation and breeding strategies for improved cell wall digestibility in annual forage crops. A review. *Anim. Res*., **52**, 193-228.

Bhandari, H. S. et al. (2011) *Panicum. In* Wild Crop Relatives: Genomic and Breeding Resources, ed. Kole, C., Springer-Verlag, Berlin Heidelberg, 175-196.

Bittinger, T. S. et al. (1981) Allelism tests of the *brown midrib* mutants of sorghum. *J. Hered*., **72**, 147-148.

Bout. S. & Vermerris, W. (2003) A candidate-gene approach to clone the sorghum *brown midrib* gene encoding caffeic acid *O*-methyltransferase. *Mol. Gen. Genom*., **269**, 205-214.

Bucholtz, D. et al. (1980) Lignin biochemistry of normal and *brown midrib* mutant sorghum. *J. Agric. Food Chem*., **28**, 1239-1241.

Cai, H. et al. (2004) An AFLP-based linkage map of Zoysiagrass (*Zoysia japonica*). *Plant Breed*, **123**, 543-548.

Cai, H. et al. (2005) Isolation, characterization and mapping of simple sequence repeat markers in zoysiagrass (*Zoysia* spp.). *Theor. Appl. Genet*., **112**, 158-166.

Caradus, J. R. & Woodfield, D. W. (1990) Estimates of heritability for, and relationships between, root and shoot characters

of white clover. I. Replicated clonal material. *Euphytica*, **46**, 203-209.

Cherney, J. H. et al. (1991) Potential of *brown-midrib*, low-lignin mutants for improving forage quality. *Adv. Agron*., **46**, 157-198.

CIAT (2014) Genetic resources program. [http://isa.ciat.cgiar.org/urg/main.do?language=en]

Ebina, M. et al. (2000a) Phylogenetic analysis of genus *Zoysia* for improvement of indigenous grazing grassland. *In* Proc International Workshop Integration of Biodiversity and Genome Technology for Crop Improvement. NIAS, Tsukuba, Japan, 133-134.

Ebina, M. et al. (2000b) Molecular mapping of zoysiagrass for some QTL analysis. *In* Plant Anim Genome VIII Conf, San Diego, 204.

Ebina, M. et al. (2005) Co-segregation of AFLP and RAPD markers to apospory in Guineagrass (*Panicum maximum* Jacq.). *Grassl. Sci*., **51**, 71-78.

Ebina, M. et al. (2007) Genetic relationship estimation in guineagrass (*Panicum maximum* Jacq.) assessed on the basis of simple sequence repeat markers. *Grassl. Sci*., **53**, 155-164.

Ebina, M. (2008) Prospects for breeding tropical grass, *in special feature*, Characteristics of self-supplying feed production grounds by tropical grasses. *Jpn. J. Grassl. Sci*., **54**, 271-275 [In Japanese].

Ebina, M. et al. (2013a) Collaborative Exploration for Panicum maximum Genetic Resources in Kenya, March, 2012. *AREIPGR* (*Annual Report on Exploration and Introduction of Plant Genetic Resources*), **28**, 153-167.

Ebina, M. et al. (2013b) Genetics, Genomics and Breeding of Tropical Grasses. *In* Genetics, Genomics and Breeding of Crop Plants, ed. Kole, C. Springer, Berlin Heidelberg New York, In press.

Edye, L. A. & Miles, J. F. (1976) A comparison of sixty *Panicum* introductions in south eastern Queensland. *Trop. Grasslands*, **10**, 79-88.

Felismino, M. F. et al. (2010) Meiotic behavior of interspecific hybrids between artificially tetraploidized sexual *Brachiaria ruziziensis* and tetraploid apomictic *B. brizantha* (Poaceae). *Sci. Agric*., **67**, 191-197.

Fritz, J. O. et al. (1981) Brown midrib mutants in sudangrass and grain sorghum. *Crop Sci*., **21**, 706-709.

Fukuoka, H. (2000) Breeding of zoysia grass. 1. Collection of genetic resources and general view of their characteristics. *J. Jpn. Soc. Turfgrass Sci*., **29**, 11-21 [In Japanese with English summary].

Fukuoka, H. et al. (2009) Breeding of zoysia grass. 2. A breeding method for seeded type variety. *J. Jpn. Soc. Turfgrass Sci*., **37**, 91-97 [In Japanese with English summary].

Ge, Y. et al. (2006) Transgenic *Zoysia* (*Zoysia japonica*) plants obtained by *Agrobacterium*-mediated transformation. *Plant Cell Rep*., **25**, 792-798.

Grau, F. V. & Radko, A. M. (1951) Meyer (Z-52) *zoysia*. *USGA J Turf Manag*, **4**, 30-31.

Hanna W. W. et al. (1973) Cytology of obligate sexual plants in *Panicum maximum* Jacq. and their use in controlled hybrids. *Crop Sci*., **13**, 695-697.

Hayashi, K. et al. (2004) Development of PCR-based SNP markers for rice blast resistance genes at the Piz locus. *Theor. Appl. Genet*., **108**, 1212-1220.

Hoffman, G. R. (1980) Genetic effects of dimethyl sulfate, diethyl sulfate, and related compounds. *Mutation Res/Review in Genet Toxicology*, **75**, 63-129.

Hojito, S. & Horibata, T. (1982) Plant exploration, collection and introduction from Africa. *In* Nekken Shiryo **58**, Tropical Agriculture Research Center, Tsukuba, 1-120 [In Japanese].

Inokuma, C. et al. (1996) Plant regeneration from protoplasts of Japanese lawngrass. *Plant Cell Rep*., **15**, 737-741.

Inokuma, C. et al. (1998) Transgenic Japanese lawngrass (*Zoysia japonica* Steud.) plants regenerated from protoplasts. *Plant Cell Rep*., **17**, 334-338.

Ishida R (1990) General remarks on the research works of Japanese lawn grass (*Zoysia japonica* Steud.) and zoysia type grasslands in Japan. *J. Jpn. Grassl. Sci*., **36**, 210-217 [In Japanese with English summary].

Ishigaki, G. et al. (2010) Estimation of genome size in *Brachiaria* species. *Grassl. Sci*., **56**, 240-242.

Jameson, D. A. (1963) Responses of individual plants to harvesting. *Bot. Rev*., **29**, 532-594.

Kasuga, S. (2002) Growing of high digestibility sorghum variety and utilization as feed (2).2. Growing of sorghum new breed "Hazuki" using high digestibility gene. *Animal Husbandry*, **56**, 465-469.

Kasuga, S. et al. (2003) A new cultivar "Akidachi" with high digestibility. *The Hokuriku Crop Sci*., **38**, 76-78 [In Japanese with English summary].

Khayri, J. M. et al. (1989) Plant regeneration of zoysiagrass from embryo-derived callus. *Crop Sci*., **29**, 1324-1325.

Kitamura, F. (1970) Studies on the horticultural classification and development of Japanese lawn grasses. Bull Kemigawa Arboretum, Fac. Agric. Univ. Tokyo, **3**, 1-60 [In Japanese].

Kobayashi, M. et al. (2013) Breeding of Japanese Lawngrass "Asagake" and "Asamoe". *Bull NARO Inst Livest Grassl Sci*, **13**, 11-22 [In Japanese with English summary].

Kouki K, et al. (2007) Characteristics of a guineagrass new cultivar 'Paikaji'. Bull Okinawa Pref. Livest. Res. Cent., **44**, 95-102 [In Japanese].

Li, M. et al. (2009) Construction of a high-density SSR marker-based linkage map of zoysiagrass (*Zoysia japonica* Steud.). *Euphytica*, **170**, 327-338.

Li, R. F. et al. (2006) Development of highly regenerable callus lines and *Agrobacterium*-mediated transformation of Chinese lawngrass (*Zoysia sinica* Hance) with a cold inducible transcription factor, *CBF1*. *Plant Cell Tiss. Org. Cult*., **85**, 297-305.

Mano, Y. & Takeda, K. (1997) Mapping quantitative trait loci for

salt tolerance at germination and the seeding stage in barley (*Hordeum vulgare* L.). *Euphytica*, **94**, 263-272.

Moser, L. E. et al. (2004) Warm-Season (C_4) Grass Overview. *In* Warm-Season (C_4) Grass, in the series AGRONOMY, Number 45, eds. Moser L. E. et al., American Society of Agronomy, Crop Science Society of America, Soil Science Society of America, Madison, Wisconsin, 1-14.

Meyer, W. A. & Funk, C. R. (1989) Progress and benefits to humanity from breeding cool-season grasses for turf. *In* Contribution from Breeding Forage and Turf Grasses, eds. Sleeper, D.A. & Asay, K.A., Crop Sci Soc Am, Madison, 31-48.

Miles, J. W. et al. (2004) Brachiariagrass. *In* Warm-Season (C_4) Grass, in the series AGRONOMY, Number 45, eds. Moser L. E. et al., American Society of Agronomy, Crop Science Society of America, Soil Science Society of America, Madison, Wisconsin, 745-783.

Morgante, M. & Olivieri, A. M. (1993) PCR-amplified microsatellites as markers in plant genetics. *The Plant J*., **3**, 175-182.

Muir, J. P. & Jank, L. (2004) Guineagrass. *In* Warm-Season (C_4) Grass, in the series AGRONOMY, Number 45, eds. Moser L. E. et al., American Society of Agronomy, Crop Science Society of America, Soil Science Society of America, Madison, Wisconsin, 589-621.

Nakagawa, H. et al. (1990) Embryo sac analysis and crossing procedure for breeding apomictic guineagrass (*Panicum maximum* Jacq.). *JARQ*, **24**, 163-168

Nakagawa, H. & Hanna, W. W. (1992) Induced sexual tetraploids for breeding guineagrass (*Panicum maximum* Jacq.). *Jpn. J. Grassl. Sci*., **38**, 152-159.

Nakajima, K. et al. (1979) Isolation of diploid and tetraploid sexual plants in guineagrass (*Panicum maximum* Jacq.). *Jpn. J. Breed*., **29**, 228-238.

Nakayama, S. (2002) Forage yield and compositions of zoysiagrass cultivars. Grass & Seed, **7**,14-18 [In Japanese only. Translated title by the present authors.].

Ozias-Akins, P. & van Dijk, P. J. (2007) Mendelian genetics of apomixis in plant. Annu. Rev. Genet., **41**, 509-537.

Palmer, N. A. et al. (2008) Genetic background impacts soluble and cell wall-bound aromatics in *brown midrib* mutants of sorghum. *Planta*, **229**, 115-127.

Pedersen, J. F. et al. (2006) Registration of 'Atlas bmr-12' forage sorghum. *Crop Sci*., **46**, 478.

Pedersen, J.F. & Rooney, W. (2004) Sorghums. *In* Warm-Season (C_4) Grass, in the series AGRONOMY, Number 45, eds. Moser L. E. et al., American Society of Agronomy, Crop Science Society of America, Soil Science Society of America, Madison, Wisconsin, 1057-1079.

Pernès, J. (1975) Organization évolution d'un groupe agamique: la section des *Maximae* du genre. *Panicum* (Graminées). *In* Mémoires ORSTOM, ORSTOM, Paris, 1-105. http://onlinelibrary.wiley.com/doi/10.1111/j.1744-697X.2007.00086.x/pdf

Pillonel, C. et al. (1991) Improvement of cinnamyl-alcohol dehydrogenase in the control of lignin formation in *Sorghum bicolor* L. *Moench. Planta*, **185**, 538-544.

Pritchard, J. K. et al. (2000) Inference of population structure using multilocus genotype data. *Genetics*, **155**, 945-959.

Porter, K. S. et al. (1978) Phenotype, fiber composition, and in vitro dry matter disappearance of chemically induced *brown-midrib* (*Bmr*) mutant of sorghum. *Crop Sci*., **18**, 205-208.

Powell, W. et al. (1996) The comparison of RFLP, RAPD, AFLP and SSR (microsatellite) markers for germplasm analysis. *Molecular Breeding*, **2**, 225-238.

Rahman, S. M. L. et al. (2003) Transient gene expression in *Zoysia japonica* using *Agrobacterium tumefaciens. Subtropic Plant Sci*., **55**, 11-17.

Saballos, A. et al. (2009) A genomewide analysis of the cinnamyl alcohol dehydrogenase family in sorghum [*Sorghum bicolor* (L.) Moench] identifies SbCAD2 as the *Brown midrib* 6 gene. Genetics, **181**, 783-795.

Sattler, S. E. et al. (2009) A nonsense mutation in a cinnamyl alcohol dehydrogenase gene is responsible for the sorghum *brown midrib* 6 phenotype. *Plant Physiol*., **150**, 584-595.

Takai T (2009) Sorghum cultivars. *Grass & Seed* **24**, 35-38 [In Japanese only. Translated title by the present authors.].

Toyama, K. et al. (2003) Production of herbicide-tolerant zoysiagrass by *Agrobacterium*-mediated transformation. *Mol. Cell*, **16**, 19-27.

Tsuruta, S. et al. (2003) Enzymatic activities on the phenylpropanoid metabolism and their relationships with an accumulation of lignin in *brown-midrib*-6 (*bmr*-6) mutant of sorghum (*Sorghum bicolor* (L). Moench). J*ap. J. Grassl. Sci*., **49**, 379-383 [in Japanese with English summary].

Tsuruta, S. et al. (2010) Structure and expression profile of the cinnamyl alcohol dehydrogenase gene and its association with lignification in the sorghum (*Sorghum bicolor* (L.) Moench) *bmr*-6 mutant. *Breed. Sci*., **60**, 314-323.

Tsuruta, S. et al. (2011) Zoysia. *In* Wild Crop Relatives: Genomic and Breeding Resources, ed. Kole, C., Springer-Verlag, Berlin Heidelberg, 297-309.

Turgeon, A. J. (1996) Turfgrass management, 4th ed. Prentice-Hall, Upper Saddle River, NJ, USA.

Yamada-Akiyama, H. et al. (2009) Analysis of expressed sequence tags in apomictic guineagrass (*Panicum maximum*). *J. Plant Physiol*., **166**, 750-761.

Zhang, L. et al. (2007) *Agrobacterium*-mediated transformation of Japanese lawngrass (*Zoysia japonica* Steud.) containing a synthetic cryIA(b) gene from *Bacillus thuringiensis*. P*lant Breed*., **126**, 428-432.