Biomass and Molecular Characteristics of Multi-tillering *Miscanthus* Mutants

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Abstract - Compared to wide ranges of genetic variation of natural populations, very limited *Miscanthus* cultivar has been released. This study was the first report on the development of *Miscanthus* cultivar by means of radiation breeding. Seeds of *M. sinensis* were initially exposed to gamma rays of 250 Gy for 24 h, generated from a ⁶⁰Co gamma-irradiator. The irradiated seeds were sown and then the highly tiller-producing mutants were selected for this study. Biomass-related parameters including tiller number, plant height, stem diameter, and leaf number were measured. Ploidy level and internal transcribed spacer (ITS) were investigated to characterize the mutants compared to wild type (WT) *Miscanthus*. Plant height and tiller number were negatively related, where multi-tillering mutants were relatively short after 4 month growth. However stem diameter and leaf number were greater in mutants. All the materials used in this study were diploid, implying that the mutants with greater tiller numbers and stem diameter were not likely related to polyploidization. Based on the sequence of ITS regions, the mutants demonstrated base changes from the gamma irradiation where G+C content (%) was decreased in the ITS1, but increased in ITS2 when compared to WT sequence. ITS2 region was more variable than in ITS1 in the mutants, which collectively allows identification of the mutants from WT. Those mutants having enhanced tillers and allelic variations might be used as breeding materials for enhanced biomass-producing *Miscanthus* cultivars.

Key words - Internal transcribed spacer (ITS), Plant height, Ploidy level, Stem diameter, Tiller number

Introduction

The carbon dioxide released from various industrial processes has been increased at a rate of approximately 3% yearly for the past 50 years. Heaton *et al.* (2003) indicated that this increase would be continued by combustion of fossil fuels, which caused the significant change in climate. Research reports indicated increasing interest in the use of plant material as a direct source of bioenergy and biomaterials (Henry 2010). Renewable energy consumption rose by 6% to over 8 quadrillion BTU (British Thermal Unit) between 2009 and 2010, and the biomass share of the renewable consumption increased from 49 to 53% during the years of 2006 to 2010 (Eynon, 2011). This fact demonstrates that the biomass plays an important role in the renewable energy resources.

The genus *Miscanthus* belongs to the family Poaceae and

most of the species were originated from tropical and sub-tropical or temperate regions of Asia (Greef and Deuter, 1993). Out of more than 20 species, only *M. sinensis*, *M. sacchariflorus*, *M. floridulus*, and *M. x giganteus* (hybrid between *M. sinensis* and *M. sacchariflorus*) are of interest for biomass production or industrial uses (Jones and Walsh, 2001; Sorensen *et al.*, 2008). Three major components of *Miscanthus* ligno-cellulosic feedstock include cellulose, hemicelluloses, and lignin, which range 43-52%, 25-33%, and 9-13%, respectively, depending on species and genotypes (Brosse *et al.*, 2012). Holocellulose content (cellulose+hemicellulose) was generally higher in *M. sinensis* (78~82%) than *M. sacchariflorus* (76~78%) or *M. x giganteus* (75~77%) (Brosse *et al.*, 2012).

Most of the reports on the genus *Miscanthus* attributed to agronomic or physiological studies including yield trials, rhizome propagation, and resource use efficiency (Beale and Long, 1997; Venendaal *et al.*, 1997; Lewandowski *et al.*, 2000; Pyter *et al.*, 2010). Some of taxonomics and phylogenetics

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provided basic biology for the plant (Greef and Deuter, 1993; Hodkinson *et al.*, 2002), and recent molecular studies by using genomics information accelerated development of molecular markers for gene mapping from the complex genome (Rayburn *et al.*, 2009; Swaminathan *et al.*, 2012).

Physical mutagens like gamma ray or fast neutron were reported to induce phenotype change in many species, which was mostly related to base change for point mutation, sequence shift by deletion or insertion of some DNA bases, or even chromosome aberrations (Tindall *et al.*, 1988; Li *et al.*, 2002; Dhanavel *et al.*, 2012). The induced mutants are partly considered in a breeding program for improving some characters including yield, quality, earliness, resistance to pathogens, and tolerance to abiotic stresses (van Harten, 1998). Ribosomal genes consisted of 18S, 5.8S, and 28S were reported to be conserved in the closely related species, while internal trans-

cribed spacers (ITS) between the conserved regions were found to be highly variable, allowing the tool used as taxonomic marker (Chen *et al.*, 2010).

The objective of this study was to evaluate the potential use of the ionizing mutagenesis via a gamma radiation for induction of biomass-enhanced *Miscanthus* mutants. We first compared biomass-related traits between wild type (WT) *Miscanthus* and its mutants, and then characterized the mutants by investigating molecular changes at the DNA and ploidy levels.

Materials and Methods

Mutant induction using a gamma radiation

Seeds of diploid *Miscanthus sinensis* were collected from an isolated Imja island of Jeonnam province in 2010, and then

Fig. 1. One seed-originated plants and initial selection of higher tillering mutant (A) and division of the genotypes into 3 tillered plant in a pot (B) for a conformational experiment in a consecutive year.

were exposed to γ -rays of 100, 125, and 150 Gy for 24 hrs as generated by a ^{60}Co gamma-irradiator (150 TBq capacity; ACEL, Canada) at the Korea Atomic Energy Research Institute, Jeongeup. All treated seeds (M1) were then directly sown in a plastic greenhouse, and the M2 plants were grown in a successive season to select mutants. The germinated plants which showed strong seedling growth were transplanted into an individual pot. Finally two greater tillering mutants exposed to 125 Gy were selected and used in this study (Fig. 1).

Cultivation and growth measurement

In a consecutive year, the selected mutants and WT (no treatment) which were originally grown from one seed which was treated with 125 Gy gamma radiation were divided into three-tillered plants per pot with three replications for measurements of biomass-related traits (Fig. 1). The divided multi-

tillering mutants and WT were investigated monthly from June 1st to October 16th, 2011. The plant growth parameters including plant height, tiller number, stem diameter and leaf number were measured. The plant height was recorded as the perpendicular distance from the soil at plant's base to the highest point reached with all the parts in their natural position. The stem diameter was measured at 5 cm-height from soil surface.

Measurement of nuclear DNA content

The ploidy level of WT (diploid), *M. sacchariflorus* (tetraploid) as a reference, and two mutants was examined by using a flow cytometry (Partec, Germany) equipped in the Institute of Agricultural Science and Technology, Naju city, Jeonnam province, Korea. About 1 cm² segments of fresh leaf tissue was chopped in 500 μl of the nucleus isolation buffer in a petri

Fig. 2. Comparative growth of WT and two mutants based on plant height (A), stem diameter (B), number of tiller (C), and number of leaf (D) by growth stages.

dish. The extracted leaf nuclei were stained in 2 ml of CyStain® UV Precise P250 containing 4',6'-diamidino-2-phenylindole (DAPI). The resultant solution was filtered through 30 µm nylon mesh into a 5 ml collection tube. The relative fluorescence of total DNA of single nuclei was analyzed and recorded. Prior to measuring the nuclear DNA content, the flow cytometry was calibrated with a chicken red blood cell to adjust all the parameters including gain value, flow speed, and an internal standard.

ITS analysis for identifying mutants

To determine the sequence of a 5.8S ribosomal gene and flanking ITS regions, another grass belonging to a Poaceae family, zoysiagrass (*Zoysia japonica*) ITS (NCBI genebank no. HQ600481) sequence was used to amplify the *Miscanthus* ITS region. The forward (ATTGTCGTGACCCTGACCAAAAC) and reverse primer (TGGGGTCGTGGTCCAAGCAATG) sets were designed and the amplicons were successfully obtained and visualized on 1% agarose gel (Fig. 4, top left), and then sequenced directly.

Data analysis

Data collected from growth measurements were analyzed for statistical significance by the SPSS software, version 19. Four growth parameters in three replications were recorded monthly and data were compared among the genotypes (WT and two mutants), which were plotted with a standard error on the graph (Fig. 2).

Results and Discussion

After exposing of *Miscanthus* seeds to gamma ray at 125 Gy, two variants showing higher tillering were selected (Fig. 1A), which were subjected to performance evaluation in the following year (Fig. 1B). At the initial selection, the mutants that were commonly started from one seed and grown in the same growing conditions for one year were washed and compared, which showed distinctive difference in numbers of root and tillers (Fig. 1). In the second year, WT and two mutants started from three tillers commonly exhibited significantly different performance. The higher tillering mutants showed thicker stem diameter and more leaf numbers, but

shorter plant height in comparison with WT under flowering stage at the end of the experiment (Fig. 2). During the heading period in October, the tiller numbers for both mutants, CNU03BR22 and CNU03BR24 were 38.7 per pot, while WT (CNU03A01) produced 29.7, indicating 30% higher to WT. Similarly stem diameters for two mutants CNU03BR22 and CNU03BR24 were 1.3 and 1.5 times higher, respectively when compared with WT. Also leaf numbers were 213 and 190 per pot for CNU03BR22 and CNU03BR24 mutants, respectively, while WT had 136 leaves. In contrast, plant height of WT was 135 cm, while CNU03BR22 and CNU03BR24 mutants were 126 cm and 102 cm, respectively.

Generally dwarfism along with higher tillers was reported to be associated with suppression of apical dominance controlled by auxin and more acropetal movement of cytokinin produced in root (McSteen, 2009). Another phytohormone strigolactone was recently reported to suppress shoot branching (Gong *et al.*, 2012). Two mutants showing higher tiller numbers in this study would be good resources to clarify factors such as genes, hormones, and any environmental conditions involving in axillary bud initiation and outgrowth, because CNU03BR22 remained to be taller as like WT, but CNU03BR24 was shorter. One of the targets will be a *miR393* microRNA which downregulates auxin receptor and transporter genes such as *OsTIR1*, *OsAFB2*, and *OsAux1* and promotes tillering and early flowering by repression of tillering inhibitor, *TBI* (Xia *et al.*, 2012). Interestingly, two contrasting genes which include a reduced culm number 1 (*Rcn1*) gene promoting tillering or culm length and a dominant tillering dwarf (*D3*) gene reducing tiller number were reported to play independently, implying that plant stature is not always associated with tiller numbers as found in two mutants in this study (Yasuno *et al.*, 2007).

Complemented with the above morphological traits which are more likely influenced by environmental factors, identification of mutants at DNA or cellular level will be a desirable strategy. A ploidy modification caused by nuclear DNA content was investigated in this study. However, no change in ploidy level was detected in two mutants when compared with diploid *M. sinensis* (Fig. 3A) and tetraploid *M. sacchariflorus* (Fig. 3B). Some morphological and physiological variations were reported to be associated with changes in DNA content, in which the ploidy level was not significantly different in

banana when exposed to gamma radiation (Shadia *et al.*, 2002). Due to increase in DNA content in banana mutants when exposed to low to higher doses of the gamma ray, more precise measurement of nuclear DNA content might be useful to identify mutants in *Miscanthus*. Additionally, target genes that regulate phenotypes of interest can be cloned and sequenced, resulting in verification of deletion or insertion of the allele affected by the ionizing mutagenesis.

Identification of accessions or ecotypes within a species or at the inter-specific level has been achievable at the molecular level since many types of molecular markers have been

applicable (Chen *et al.*, 2010; Kim *et al.*, 2007). Among many reported DNA fingerprinting tools, the ITS region were found to be suitable due to its PCR efficiency and sequence divergence. Sequences of the ITS regions and intervening 5.8S region of two *Miscanthus* mutants exhibiting higher number of tillers were compared with those of WT (Table 1). Total length of three regions was 611 bp, which was consisted of ITS1, 5.8S, and ITS2 with 211, 164, and 227 bp, respectively (Fig. 4). All the accessions had the same size for ITS1, 5.8S, and ITS2 regions, but the sequences were different as seen in G+C percentage, where the G+C content of two mutants

Fig. 3. Flow cytometric analysis of DAPI-stained DNA nuclei from diploid *M. sinensis* (A; WT), tetraploid *M. sacchariflorus* (B) and two mutants (C, CNU03BR22; D, CNU03BR24) which were induced by gamma ray treatment at 125 Gy.

Table 1. Sequence length and G+C content (%) of ITS1, 5.8S and ITS2 regions of WT and the derived two mutants of *Miscanthus*

Genotype	ITS1		5.8S		ITS2	
	Length (bp)	G+C (%)	Length (bp)	G+C (%)	Length (bp)	G+C (%)
CNU03A01 (WT)	211	135 (63.9)	164	93 (57.0)	226	152 (67.2)
CNU03BR22	211	134 (63.5)	164	93 (57.0)	226	154 (68.1)
CNU03BR24	211	133 (63.0)	164	93 (57.0)	226	153 (67.7)

showed less in ITS1 and more in ITS2 (Table 1). There was no sequence change in 5.8S subunit for three genotypes, while five bases were changed in both ITS1 and ITS2 of the mutants compared to WT. The base changes were occurred more in the 5' side of ITS1, while the event was relatively evenly spaced from 500 to 541 bp in ITS2 (Fig. 4). This result demonstrates that ITS regions can be used to identify the *Miscanthus* higher tillering mutants which were induced by a gamma radiation as suggested in other reports (Kress *et al.*,

2005; Chen *et al.*, 2010).

One of the advantages using the ITS regions for mutant identification in this study is its bi-parental inheritability, which may distinguish those mutants from any artificial hybrids. Because limited genomic sequences of the genus *Miscanthus* are publicly available, the ITS regions were amplified by designing PCR primers of other zoysiagrass sequence (NCBI genebank no. HQ600481), indicating the potential application of the ITS sequences to other grasses for phylogentic divergence.

Fig. 4. Successful PCR amplicons on 1% agarose gel (top left; m = 100 bp DNA marker, wt = wild type, m1 = CNU03BR22, m2 = CNU03BR24), simplified structure and designated size (bp) of ITS regions (top right), and sequence variations (arrows) of both spacers linked to 5.8S region (bottom) of *Miscanthus* ribosomal DNA.

Size of plant ITS regions ranges from 500 to 700 bp long and the ribosomal gene repeats are abundant (hundreds to thousands) in plant genome, which facilitates the tool for convenient PCR amplification compared to other DNA barcode system (Kress *et al.*, 2005; Chen *et al.*, 2010).

Mutation breeding through diverse (physical, chemical, or biological) mutagens has been widely applied to various plant species. One limitation of the breeding method is to identify mutants, which mostly relies on phenotypic variations feasible to environmental extremes. As instrumental advance and genome sequences become more available and accessible, relatively efficient and precise identification tools such as STS (sequence tagged site) markers and universal ITS barcode can be good alternatives as shown in this study.

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