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Construction and classification of a cDNA Library from *Miscanthus sinensis* (Eulalia) treated with UV-B

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Abstract

The establishment of EST database is important to study classification and function of gene groups in *Miscanthus*. We isolated total RNA at 48 h after UV-B treatment to develop a gene expression profile of the entire *Miscanthus* plant (except for the root). A cDNA library was prepared from mature mRNAs, and 1000 cDNA clones were partially sequenced using a DNA sequence kit (BigDye TM Terminator Cycle Sequencing Ready Reaction mixture). A total of 827 unigenes, including 72 contigs and 755 singletons, were identified after assembly of the expressed sequence tags (ESTs). To analyze the ESTs from the UV-induced cDNA library, we grouped the sequences and classifications based on the best homology match of BLASTX searches against various plant protein sequences. The functional classification sequences belonged to 15 categories including metabolism, energy, cell cycle, transcription, protein synthesis, protein fate, protein with binding function, regulation of metabolism and protein function, cell transport, signal transduction, cell defense, interaction with the environment, cell fate, biogenesis of cellular components, and subcellular localization, which were considered to be of interest for UV-response signaling.

Keywords: Blast, cDNA library, ESTs, UV-B.
Abbreviations: Blast_Basic local alignment search tool; cDNA_complementary DNA; ESTs_ expressed sequence tags; UV-B_ultraviolet B.

Introduction

Biomass is attracting increasing interest as a new source for fuel production or useful chemicals, and it is an important component of national energy supplies in many countries (Adams et al., 2006; Demiral and Sensoz, 2006; Antonakou et al., 2006). The practical potential of biomass residues is equivalent to one-third of the commercial energy consumed worldwide in 1990 (Yamamoto et al., 2001). Crops have many advantages as biomass resources for energy production, and numerous crops have been proposed or are being tested for commercial energy farming (Lewandowski et al., 2000; Lewandowski et al., 2003). *Miscanthus* plants have previously been used as a genus of C4 perennial rhizomatous grasses identified for potential use as biomass plants for renewable energy (Lee, 1995). *Miscanthus* grows rapidly, has a high biomass yield, and is genetically variable (Lewandowski et al., 2003). The stem of *Miscanthus* may be used as fuel or ethanol for producing heat and electric power (Lewandowski et al., 2000; Minkova et al., 2001). UV-B irradiation affects plant development, morphology, and physiology (Frohnmeyer and Staiger, 2003). Treatment with UV-B irradiation in higher plants causes DNA photolesions, and plants have developed defense mechanisms against UV-B irradiation (Rozema et al., 1997). Furthermore, secondary metabolites such as flavonoids absorb these wavelengths (Caldwell, 1979; Karabourniotis and Liakopoulos, 2005). Continuous UV-B irradiation causes the accumulation of polyphenolic compounds in cucumber cotyledons. UV-B exposure induces specific morphological changes and increases lignin content in this tissue (Ryu et al., 2006). The classification of genes from a plant cDNA library is very important not only for understanding their physiology, but also for implementing breeding programs. Researchers utilize sequence information as a tool for manipulating the plant system. Monocot cDNA libraries have been constructed from various tissues (Hisada et al., 1996; Omura et al., 2000). However, a *Miscanthus* cDNA library has not yet been constructed. Therefore, we present the digital gene expression profiles for 1000 *Miscanthus* genes across all tissues. We focused on the classification of expressed sequence tags (ESTs) from an enriched cDNA library of *Miscanthus* leaves and stems exposed to UV-B irradiation.

Results

Sequencing and grouping of ESTs

We constructed a cDNA library from *Miscanthus* leaves. A total of 1000 cDNAs were randomly selected for partial sequencing. Subsequent vector and low-quality sequence trimming resulted in 947 high-quality sequences, with an average EST read length of 422 nucleotides. In this library, 72 contigs clustered from 1000 clones and 755 singletons were formed. The set of contigs and singletons resulted in 947 assembled sequences that represented the putative transcripts found in *Miscanthus* leaves. We have displayed this library analysis in Table 1.

Annotation and classification of known functional genes

A homologous similarity analysis of the cDNAs was conducted with a BLASTX program to assign functions to 757 unigenes. Examination of the initial BLASTX matches showed that these could be categorized into three categories: 36% of unigenes matched to proteins with known function in the public databases; 55% of unigenes matched to proteins with unknown functions; and 9% of unigenes matched to proteins with no hit in the NCBI database (Fig. 1A).

The ESTs with known function were assigned to a functional category based on BLASTX matches at the NCBI homepage (Fig. 1B). In that analysis, the 757 unigenes were manually classified into 15 functional categories: metabolism, energy, cell cycle, transcription, protein synthesis, protein fate, protein with binding function, regulation of metabolism, cellular transport, signal-transduction mechanism, cell rescue, defense and virulence, interaction with the environment, cell fate, biogenesis of cellular components, and subcellular localization. The *Miscanthus* EST categories were protein synthesis and processing, followed by metabolism (15%), protein with binding function (36%), and subcellular localization (12%). The transport category consisted of 5% of the ESTs and regulation of metabolism contained 6%. Finally, the ESTs comprised 4.0% for energy, protein synthesis, and cell rescue-defense and virulence, 0.5–2% for cell cycle, transcription, signal-transduction mechanism, interaction with the environment, cell fate, and biogenesis of cellular components. These functional classifications are summarized in Table 2.

Expressed genes in the Miscanthus cDNA library

The range of gene expression depends on the abundance of mRNA. The cDNA clones in a contig represent the degree of mRNA transcription and expression. Here, we analyzed individual genes based on the number of cDNA clones. Among these, 179 cDNA clones were similar to known genes; their matched function and E values are displayed in Supplementary data. Response to UV treatment occurs through phenylpropanoid metabolism, cell rescue, cell defenses, and signal transduction (Loyall et al., 2000; Peng et al., 2003). To analyze the ESTs from the UV-induced cDNA library, we grouped the sequences using the NCBI database. The classification was based on the best homology match of BLASTX searches against various plant protein sequences. In the functional classification, sequences belonging to 15 categories, including metabolism, energy, cell cycle, transcription, protein synthesis, protein fate, protein with binding function, regulation of metabolism and protein function, cell transport, signal transduction, cell defense, interaction with the environment, cell fate, biogenesis of cellular components, and subcellular localization, were considered to be of interest for UV-acclimation (Tables 2 and Supplementary Table 1). Many UV-related proteins were among the most abundant ESTs (Supplementary Table 1).

Discussion

We reported that exposure of *Miscanthus* to UV-B irradiation induced various functional genes. The rice RE2 is part of the ubiquitin system and may not interact directly with the UV-light *cis*-element (Leverson et al., 2000). The rice RE2 may respond to UV light by regulating flavonoid metabolism (Peng et al., 2003), but we could not obtain the genes related to flavonoid metabolism. The metabolism grouping contained amino acids, nitrogen and selenium, carbohydrate and lipid, and fatty acid metabolism (Table 2). The *RAD6* gene is sensitive to UV light (Madura et al., 1990). RAD6 contains a C3HC4 sequence motif, known as the RING finger motif, which may be utilized for DNA binding (Prakash, 1989; Gmachl et al. 2000). The RING finger proteins were expressed in the cDNA library of UV-treated *Miscanthus* (Supplementary Table 1) and function in DNA binding and protein transcriptional regulation (Salghetti et al., 2001). From these results, 947 cDNAs from *Miscanthus sinensis* revealed 179 high-quality ESTs, with an average sequence length of 200 bp. A total of 827 assembled sequences (sum of contigs and singletons) were identified, including 72 contigs of two or more ESTs and 755 singletons (Table 1). A cluster analysis of these 1000 sequences was conducted using a CAP3 sequence assembly program. Major genes involved in development, metabolism, and stress have been identified in several energy-producing crops (Li et al., 2010). To analyze the genes related to development, a cDNA library of Barbados nut was constructed, and sequences of genes involved in fatty acid metabolism, signal transduction, and others were obtained (Wang et al., 2007). The cDNA clones were isolated from UV-B-irradiated apple skin and characterized based on their sequence similarities to other plants (Li et al., 2010). It is generally known that UV-B-responsive genes occur in various plants (Christiem et al., 1994). However, a report on the UV-B-responsive genes of *Miscanthus* has not been published yet; thus, our results are meaningful. In the current study, a homology search of 947 unigenes was conducted with the BLASTX program against the NCBI database, and the deduced amino acid sequences of 755 unigenes (75%) were matched to protein sequences in that database (Fig. 1). This percentage was higher than that for an EST project with *Citrus* (Boo et al., 2007). The 755 unigenes matched to proteins with known function were classified into 15 cellular functional categories (Fig. 1). The largest category was protein with binding function (36%). The second and the third most prominent were related to metabolism and subcellular localization, with 15% and 12%, respectively. These findings indicate that the distributions of functional categories differ from those following treatment of *Miscanthus*. In the *Citrus* EST project, Omura et al. (2000) found that the largest category pertained to cell growth and structure, followed by environment response, metabolism, and protein synthesis/processing. The last included protein regulation/signal transduction, transcription, and translation (Omura et al., 2000).

The UV irradiation generates oxidative stress through reactive oxygen species in plants (Green and Fluhr, 1995). GST gene activity participates in the detoxification of products created by oxidative damage (Dixon et al., 1998). These observations indicate that GSTs are expressed in plants treated with UV light. *Arabidopsis* responds to low UV light by transc-

Table 1. Result of EST sequencing and contigs assembly.

EST sequencing	Number of cDNAs sequenced	947
Contig assembly results	Number of EST assembled	947
	Number of contigs	72
	Number of singletons	755

Table 2. Functional category of ESTs matched to known genes

Metabolism	Regulation of metabolism and protein Function
Amino acid metabolism	regulation by
Nitrogen, sulfur and selenium metabolism	CELLULAR TRANSPORT, TRANSPORT FACILITIES AND TRANSPORT ROUTES
C-compound and carbohydrate metabolism	transported compounds (substrates)
Lipid, fatty acid and isoprenoid metabolism	transport facilities
ENERGY	transport routes
Glycolysis and gluconeogenesis	CELLULAR COMMUNICATION/SIGNAL TRANSDUCTION MECHANISM
Glyoxylate cycle	cellular signalling
Pentose-phosphate pathway	transmembrane signal transduction
tricarboxylic-acid pathway (citrate cycle, Krebs cycle, TCA cycle)	CELL RESCUE, DEFENSE AND VIRULENCE
photosynthesis	stress response
CELL CYCLE	detoxification
DNA processing	INTERACTION WITH THE ENVIRONMENT
cell cycle	cell motility
TRANSCRIPTION	CELL FATE
RNA synthesis	cell death
RNA processing	BIOGENESIS OF CELLULAR COMPONENTS
PROTEIN SYNTHESIS	cytoskeleton/structural proteins
translation	extracellular / secretion proteins
aminoacyl-tRNA-synthetases	SUBCELLULAR LOCALIZATION
PROTEIN FATE (folding, modification, destination)	cytoplasm
protein folding and stabilization	cytoskeleton
protein targeting, sorting and translocation	endoplasmic reticulum
protein modification	Golgi
assembly of protein complexes	nucleus
protein/peptide degradation	mitochondrion
PROTEIN WITH BINDING FUNCTION OR COFACTOR REQUIREMENT	vacuole or lysosome
protein binding	plastid
nucleic acid binding	
structural protein binding	
lipid binding	
C-compound binding	
metal binding	
nucleotide/nucleoside/nucleobase binding	
complex cofactor/cosubstrate/vitamine binding	

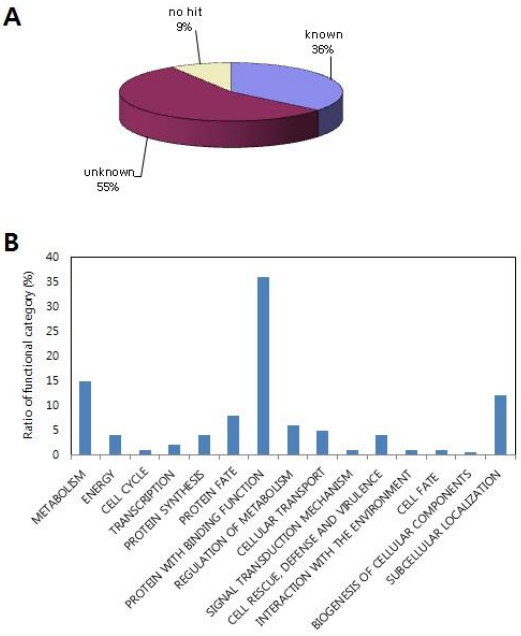

Fig 1. Representations for functional annotation and classification of unigenes. (A) Functional annotation of unigenes with BLASTX program in NCBI. (B) Distribution of ESTs using functional categories.

riptional stimulation of *CHS* (Christie and Jenkins, 1996). The GSH and PcGST1 act directly through removal of oxidative stress products and have a different function during UV-dependent signal transduction (Loyall et al., 2000). Our data provide evidence for GST expression, such as GST4, GST17, GST22, GST30, and GST42 involvement in UV-mediated signal transduction in *Miscanthus*. The E-values of these genes matched to known function were identified from 1.00E to 5.00E (Supplementary Table 1). UV light activates *PcGST1* in parsley, and remarkable parallels are found in signal transduction (Loyall et al., 2000). cDNAs have been isolated from UV-B-irradiated apple skin and characterized based on their sequence similarities to other plants (Ban et al., 2007). UV-B-responsive genes in various plants are associated with the flavonoid biosynthetic pathway (Hasegawa et al., 2001). The expression of a putative UDP-glucose 4-epimerase gene related to the flavonoid biosynthetic pathway is higher in apple skin (Ban et al., 2007). The present study showed that the sequences of UDP-glucose 6-dehydrogenase and UDP-glucose 4-epimerase have similarities with UGEs from other species (Supplementary Table 1). UGE catalyzes the reversible epimerization of UDP-galactose and UDP-glucose. It is one of the essential enzymes for the *de novo* biosynthesis of UDP-galactose. The UGE4-deficient mutant shows an approximately 20% decrease in cell-wall-bound galactose in *A. thaliana* (Seifert et al., 2002). The UGE plays an important role in the biosynthesis of cell-wall polysaccharides in potato tuber (Oomen et al., 2004).

Materials and Methods

Preparation of Miscanthus

Miscanthus sinensis (Eulalia) was used to this work. *Miscanthus* was grown in a growth chamber at 25 ± 3°C under a photocycle of 16 h dark/8 h light (600–700 μmol photons m^{-2} s^{-1}) and then treated with UV-B for 48 h. Whole *Miscanthus* plants grown from rhizome for 6 weeks were used as the plant material for cDNA library construction.

Construction of the cDNA library

Total RNA was isolated from whole *Miscanthus* plants using Trizol solution (Invitrogen, Carlsbad, CA, USA), and poly(A) RNA was purified with an Oligotex kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. A GeneRacer kit (Invitrogen) was used for the cDNA library construction. The PCR products were cloned into the pBluescript SK(-) vector, and the plasmids were transformed into *E. coli* cells. Our cDNA libraries were plated onto LB-ampicillin plates containing IPTG and X-gal, and the white colonies were picked into 96-well blocks.

cDNA Sequencing and Sequence Analysis

Bacterial culturing and plasmid extractions were conducted in a 96-well format. The sequencing reactions were performed using a DNA sequence kit (BigDye TM Terminator Cycle Sequencing Ready Reaction mixture; Applied Biosystems, Foster City, CA, USA). Reaction products were analyzed with an ABI 3700 automatic DNA sequencer (Applied Biosystems). Vectors and ends were trimmed and removed along with sequences shorter than 150 nucleotides. The remaining ESTs (high-quality sequences longer than 150 bp) were then subjected to database searches. Database searches were performed using BLASTX software with default parameters. Homologies with E values of $\leq 10^{-5}$ were divided into known functions, putative functions, and unknown functions; the other unigenes (no hit or an E value >10−5) were classified as non-significant matches. Unigenes matching proteins with known functions were categorized manually according to a BLASTN and BLASTX program searches at the homepage of the NCBI database. Individual gene expression levels were examined based on the redundancy of cDNA clones found in each contig comprising more than 10 ESTs.

Conclusion

In conclusion, the EST comparative analysis described here was successful in identifying UV-signaling related genes in *Miscanthus*. This information may be useful for further characterization of data in a *Miscanthus* library. From these outcomes, we will continue to study the precise biological roles of such genes in *Miscanthus*, utilizing specific approaches such as sense and anti-sense experimentation, proteomics, and functional genomics research.

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