International Scholars Journals

International Journal of Medicinal Plants Research ISSN 2169-303X Vol. 8 (8), pp. 001-006, August, 2019. Available online at www.internationalscholarsjournals.org © International Scholars Journals

Full Length Research Paper

Ecotypic variation of a medicinal plant *Imperata cylindrica* populations in Taiwan: mass spectrometry-based proteomic evidence

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Accepted 16 April, 2019

Cogon grass [*Imperata cylindrica* L. Beauv. var. *major*] is the only one species of *Imperata*, and one of the medicinal plants in Taiwan. The rhizome can be used for medicinal purposes. In the field alcohol dehydrogenase (ADH) activity, proline and sodium content in tissues of Imperata showed variation between wetland (Chuwei population) and the other two representative non-wetland ecotypes (Neihu and Sarlun populations) . Chuwei ecotype is known tolerant to flood and salt shown in a flood and salt treatment experiment in previous study. A mass spectrometry (MS)-based proteomic analysis identified a fast moving 2-phospho-D-glycerate hydrolase (enolase) isoform only in the wetland ecotype on a native-polyacrylamide gel electrophoresis, whereas a slow moving enolase isoform was identified only in the non-wetland ecotypes. In addition, a mitochondrial malate dehydrogenase and a chloroplast ferredoxin-NADP(H) oxidoreductase were identified only in the wetland ecotype. These proteomic results provide additional evidence of the ecotypic variation among *Imperata cylindrica* ecotypes. The potential use of this novel approach to identify protein markers is discussed in the study.

Key words: *Imperata cylindrica,* ecotype, mass spectrometry, proteomic, enolase, malate dehydrogenase, ferredoxin-NADP(H) oxidoreductase.

INTRODUCTION

In Taiwan *Imperata cylindrica* (L.) Beauv. var. *major* (Nees) Hubb., a top-ten weed (Holm et al., 1977), is widely distributed (Hsu, 1975). This weed particularly can be found in Chuwei mangrove forest in the estuary area located at the river mouth of Tamshui River in north Taiwan. *I. cylindrica* is a well known medicinal plant (Hsu, 1975). Secondary metabolites have been isolated and identified from the rhizome of *I. cylindrica* (Ohmoto et al., 1965; Nishimoto et al., 1965; Matsunaga et al., 1994a; Matsunaga et al., 1994b; Matsunaga et al., 1994c; Matsunaga et al., 1995; Pinilla and Luu, 1999; Sripanidkulchai et al., 2001; Yoon et al., 2006). These secondary metabolites can have medicinal uses (Doan et al., 1992; Matsunaga et al., 1994a; Matsunaga et al., 1994b; Matsunaga et al., 1994c; Matsunaga et al., 1995; Pinilla and Luu, 1999; Sripanidkulchai et al., 2001; Yoon et al., 2006).

Morphological and molecular evidences have shown variation among Imperata ecotypes. In 1997 Cheng and Chou (1997a) utilized scanning electron microscope (SEM) to examine the leaves of *I. cylindrica* of Chuwei population. They found the stele was empty but not solid, and the lower stem was surfaced with wax but not trichomes. The phenotype of the Chuwei plant remained unchanged after transplanting into greenhouse. In addition, the polymorphism of Imperata populations on the molecular level was investigated by use of polymerase chain reaction(PCR) amplified-restriction fragment length polymorphism (RFLP) of the intergenic spacer (IGS) of ribosomal DNA (rDNA) (Chou and Tsai, 1999), and rapid amplify polymorphic DNA (RAPD) (Cheng and Chou, 1997b). Based on these studies, Chuwei population appeared to be a distinct ecotype.

Physiological and biochemical evidences also showed variation among Imperata ecotypes, and the uniqueness of Chuwei ecotype. In 2006 Chang and his co-workers examined the polymorphism of salt and flooding stress responses of *I. cylindrica* between wetland and non-wetland ecotypes by use of ADH activity, proline and sodium content as biochemical markers (Chang and

Chou, 2006). Both field and greenhouse studies of three ecotypes showed that ADH activity, proline content in leaf tissues, and sodium content in *I. cylindrica* tissues were differentially up-regulated in Chuwei ecotype, which is flood and salt tolerant (Chang and Chou, 2006).

Recently, mass spectrometry (MS) technique was applied to identify protein markers (Aebersold and Mann, 2003), and single-nucleotide polymorphism (SNP) Aebersold, 2003; Törjék et al., 2003). This novel approach provides a chance to detect and identify varia- tion among different ecotypes. In this study, a MS-based proteomic analysis by use of liquid chromatography tandem MS (LC-MS/MS) was performed and detected polymorphism of two different enolase isoforms in wetland and non-wetland ecotypes. Besides, a mitochondrial malate dehydrogenase and a chloroplast ferredoxin - NADP(H) oxidoreductase were identified as a polymorphism asso-ciated to wetland ecotype only, which is flood and salt tolerant. Our findings supported the previous discovery of the variation among ecotypes (Cheng and Chou, 1997a), and confirmed that Chuwei ecotype is physiologically dis-tinct. These findings may help us understand more of biodiversity of *I. cylindrica*.

MATERIAL AND METHODS

Sampling sites and plant materials

I. cylindrica (L.) Beauv. var. *major* (Nees) Hubb, Cogon grass, was sampled from Chuwei mangrove salt-marsh wetland site (Hwang and Chen, 1995). This area is periodically flooded by sea water. The other two sites, Sarlun (sandy beach) and Neihu (inland-park), are control sites with no flooding (non- wetland sites). Every two weeks plant leaves were harvested from each site in 2004. During the harvesting, each leaf sample was excised by sterilized scissors and stored in zip-block in ice bucket with dry ice to keep it fresh and brought back to lab immediately before use.

Protein purification and native-polyacrylamide gel electrophoresis

One gram of fresh leaf tissues was chopped into pieces and frozen in liquid nitrogen. Two ml extraction buffer [0.1 M Tris- HCl, pH 8.0, 0.01 M beta-mercaptoethanol, 1 mM dithiothreitol, 0.02 mM phenylmethylsulfonyl fluoride (PMSF)(Sigma)] with 0.5 g sea sand was added for grinding and homogenization. The homogenated sample was centrifuged at 5380 g centrifuge (Sigma, Model 2K15) for 10 min. Supernatant was precipitated by addition of two volumes of 100% (v/v) acetone, incubated on ice for six hr, and centrifuged at 4°C for 15 min at 16000 g. The pellet was washed with 70% (v/v) ethanol, air-dried and re-suspended in 40 l of loading buffer [10 mM Tris (pH 6.8), 1% (v/v) beta-mercaptoethanol, 2.5% (v/v) glyce-rol, 1% (w/v) bromophenol blue]. Ten l of protein samples were loaded into each well of the 12% native-polyacrylamide gel electro-phoresis (PAGE) [12% (w/v) acrylamide, 0.4% (w/v) N', N'-methylene-bis-acrylamide, 0.28 M Tris (pH 8.8), 0.5% (w/v) ammonium persulfate and 0.5% (v/v) TEMED], and electrophored at 100V for two hr. The protein gels were stained by Coomassie blue R-250, and scanned for image analysis. Experiment was conducted with three replicas.

In-gel digestion of proteins and mass spectrometry analysis

MS analysis of proteins was performed by the method as previously described (Chang et al., 2005). Individual gel bands were excised with a sterile scalpel, placed in an eppendorf tube, immersed in 400 μl destain solution [50% (v/v) acetonitrile (ACN), 0.025 M ammonium bicarbonate, pH 8.0] followed by vortex for 15 min at room temperature three times, and vortex in 100% (v/v) ACN for five min. Gel slices were soaked in 60 μl trypsin solution (25 mM ammonium bicarbonate, pH 8.0) containing 0.1 μg trypsin (Promega, Madison, WI). In-gel digestion was performed for 16 h at 37°C, peptides were eluted from the gel slice with 50% (v/v) ACN, 5% (v/v) trifluoroacetic acid (TFA), and the eluate was dried in a vacuum. Digested peptides were resuspended in 0.1% (v/v) TFA. Electro Spray Ionization (ESI)-MS/MS analysis was performed by use of the Quadrupole Time-of- Flight (ESI Q- TOF) on a instrument (Micromass, Manchester, UK) coupled with a capillary high performance liquid chromatography HPLC)-(Agilent) (Mass Spectrometry Facility, Chemistry Department, University of California, Riverside).

Database search for protein identification

After LC-MS/MS analysis, the mass values of y and b ions and their associated precursor ions were obtained and implemented in a peak list (pkl) file by use of ProteinLynx (Micromass, Manchester, UK). Protein identification was performed using the Mascot algorithm (Perkins et al ., 1999; http://www.matrixscience.com/cgi/index.pl?page=/) to search against the National Center for Biotechnology Information (NCBI) protein database. The Mascot score with at least 25 was considered as a match of proteins. The fragmentation profile was manually inspected to make sure the right identification.

RESULTS AND DISCUSSION

Mass spectrometry detected polymorphism of proteins among ecotypes

In order to detect polymorphism on protein level, a native-polyacrylamide gel electrophoresis on proteins of three ecotypes was performed. In the gel, two polymorphic bands a (slow moving), and b (fast moving), were detected among three ecotypes (Figure 1A) . All six individuals from each ecotype showed the same pattern (data not shown). Band a was detected in both Sarlun and Neihu ecotypes, whereas band b was found only in Chuwei ecotype. In order to identify proteins in bands a, and b, a MS analysis by use of LC-MS/MS was performed. A peptide matched to an enolase of fungus was both identified in a, and b (Table 1). Therefore, we detect two enolase (GenBank accession # P84208) isoforms in band a, and b (polymorphism). The tandem MS (MS/MS) spectrum is shown in Figure 1B. Some other peptides matched enolase orthologous of other organism were identified in band b (Table 1). Besides, a mitochondrial malate dehydrogenase (GenBank accession # P84209) and a chloroplast ferredoxin-NADP(H) oxidoreductase (GenBank accession # P84210) orthologous to other eukaryotes were identified in band b but not band a (Table 1). Our result showed the MS-based detection of

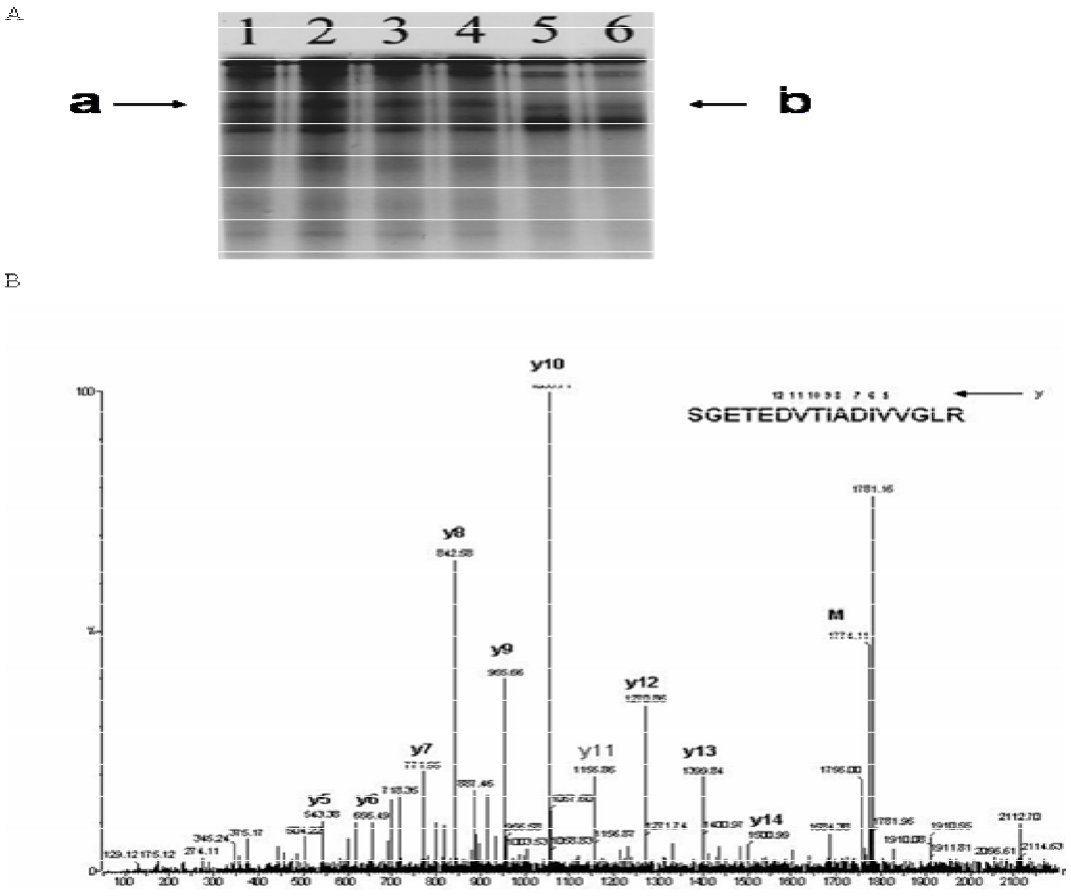

Figure 1. Polymorphism of proteins in leaves of three ecotypes. (A) 1-2: Neihu ecotype; 3-4: Sarlun ecotype; 5-6: Chuwei ecotype. Band **a** and band **b** are two polymorphic bands on a Native- PAGE. (B) Tandem mass spectrometric profile of enolase peptide identified in both band **a** and band **b**. The mass spectrometric profile of peptide was obtained by ESI Q-TOF LC-MS/MS analysis. The sequence of the peptide with mass value 1772.9 as parental peak is SGETEDVTIADIVVGLR, which matched to a fungus enolase. The y ions generated by collision induced dissociation (CID) analysis were shown in the spectrum.

polymorphism on enolase isoforms and identification of a distinct eno-isoforms in Chuwei ecotype. We also showed that a mitochondrial malate dehydrogenase and a chloroplast ferredoxin-NADP(H) oxidoreductase were identified only in Chuwei ecotype.

Mass spectrometry identification of polymorphic proteins associated with the Chuwei ecotype

Although Imperata genome is not completely sequenced, the identification of orthologues from other organisms allowed us to determine the identity of proteins in Imperata. Enolase is highly conserved in eukaryotes (van der Straeten et al., 1991). It catalyzes the late step of glycolytic pathway, which converts 2-phosphoglycerate to phosphoenolpyruvate. It was identified as one of the anaerobic proteins in maize (Sachs et al., 1980). A proteomic analysis showed that a maize enolase increase protein synthesis under hypoxia condition (Chang et al., 2000). Under low oxygen stress, tricarboxylic acid (TCA) cycle is shut down but glycolytic pathway is still functional to produce energy (Bray et al., 2000; Webster, 2003; Drew, 1997). Plants generate 38 ATPs when oxygen was sufficient. But during anaerobiosis, there will be no generation of ATP and plants will be starved to death. However, there are minor ATPs generated either from glycolysis or alcoholic fermentation pathways so that plants can temporarily survive (Fukao and Bailey-Serres, 2004).

It was found that enolase mRNA was up regulated in rice (Umeda and Uchimiya, 1994) and maize seedlings (Andrews et al., 1994), and the enolase activity was up regulated in flood-tolerant Echinochloa under anaerobic condition (Fox et al., 1995; Fukao et al., 2003). Differential expression of mRNAs of enolase isoforms maize was up regulated in response to anaerobic treatment (Lal et al., 1998). Besides, an enolase bind to the DNA in humans (Feo et al., 2000; Subramanian and Miller, 2000), and an arabidopsis enolase, LOS2, binds to DNA

Table 1. Mass spectrometry analysis of polymorphic bands on native-PAGE by LC-MS/MS. The identification of proteins after LC-MS/MS analysis was performed by use of Mascot algorithm. The maximum missed cleavage number was set as 1. The NCBI protein database was chosen to search against.

Protein identity	Gelband #	Calculatedmass (D)	Ion charge	Missed cleavage	Amino acid sequence	Mascot score	Gen Bank Accession #	Organism
Enolase	a	1772.92	2	0	SGETEDVTIADIVVGLR	72	Gi|1086120	*Cladosporium herbarum*
	b	1772.92	2	0	SGETEDVTIADIVVGLR	71	Gi|1086120	*Cladosporium herbarum*
	b	1534.75	2	0	IEEELGDAAVYAGAK	44	Gi|119355	maize
Mitochondria malate dehydrogenase	b	1317.69	2	0	DDLFNINAGIVK	40	Gi|21388550	*Solanum tuberosum*
	b	1808.06	2	0	VAILGAAGGIGQPLSLLMK	71	Gi|21388550	*Solanum tuberosum*
Ferredoxin-NADP(H) oxidoreductase	b	806.43	2	0	LDFAVSR	37	Gi|20302473	*Triticum aestivum*
	b	1259.64	2	0	GIDDIMVDLAAK	50	Gi|20302473	*Triticum aestivum*
	b	1629.79	2	0	LYSIASSAIGDFGFGDSK	65	Gi|119905	Pea

bi-functional) to activate transcription under cold condition (Lee et al., 2002). Since enolase is post-trans-translational (phosphorylation) regula-ted in response to salt stress in Mysembryan-*themum crystallinum* (Forsthoefel et al., 1995), we proposed that enolase isoforms identified in two separate bands may be due to either post- trans-lational modification or evolutionarily distinct gene family members. However, whether these two enolase isoforms encoded by two different genes and whether there is a post-translational modifica-tion of enolase, and whether the distinct isoform identified in Chuwei ecotype has distinct function required further studies. The malate de-hydroge-nase generates NADH, and ferredoxin-NADP(H) oxidoreductase generate NADPH as reducing power for ATP synthesis. A recent proteomic ana-lysis identified a salt-induced protein, ferredoxin - NADP(H) oxidoreductase, in *Dunaliella* (Liska et al., 2004). Therefore, further studies to correlate the stress treatment and gene expression of these proteins are required.

Traditionally, the detection of polymorphism within a species (inter-populational) was frequently assayed by use of isozymes (Guadagnuolo et al., 2001). Substrate specific to certain enzyme was applied onto a native-PAGE. After enzymatic reac-tion, polymorphic isozyme bands emerge. How-However, the whole process is time-consuming. Our approach showed that MS technology could be a novel approach that is easy and efficient in detecting polymorphism on protein level in a single species (van den Boom et al., 2007). A similar approach was performed by use of MS to classify eight ecotypes of *Arabidopsis thaliana* (Chevalier et al., 2004). In conclusion, this study detected polymorphism of Imperata ecotypes on protein level. We demonstrated the potential application of MS in detecting poly- morphism. (among ecotypes in a single species. Whether these proteins are really involved in flooding or salt stress tolerance requires further studies. The clarification of the correlation relationship may help us understand more of flood and salt stress physiology in planta.

The value of proteomic analysis in medicinal plant study

The identification of variation of proteins among Imperata ecotypes suggests potential protein markers specific to individual ecotype. MS-based proteomic analysis may help us discover the variation of ecotypes on the protein level. The advantage of this survey is that we may identify proteins that are differentially expressed in diffe-rent ecotypes. In fact, this approach has recently been widely introduced to study the variation among different ecotypes of a specific species in planta. For example, a proteomic analysis was performed to compare protein differences in two ecotypes of tomato during ripening (Rocco et al., 2006). Another study used MALDI-TOF and found protein difference between two Arabidopsis ecotypes (Schlesier et al., 2004) . We can design biological experiments to test if the proteins identified are essential to the survival of a particu-lar ecotype. In addition, protein markers may have

additional values. It provides us a chance to screen for ecotypes with high quality or quantity secondary metabolites. For example, we may use proteomic approach to screen for Imperata ecotypes of high-quality or high-quantity secondary metabolites. Once all the polymorphic genes are identified, transgenic strategy can be applied to modulate the biochemistry or physiology of Imperata to improve its medicinal effect. Therefore, the ecotype that was identified will be of great value for drug discovery.

ACKNOWLEDGEMENT

We are grateful to National Taiwan University, Taiwan (Grant # 96R0039-08, 96R0039-01) for funding support. We greatly appreciated Dr. Kangling Zhang, Mass Spectrometry in Analytical Chemistry Instrumentation Facility at University of California, Riverside, CA, USA for LC-MS/MS analyses.

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