# Metabolic phenotyping of genetically diverged species in Gramineae

**Mochida K[1], Furuta T[2], Ebana K[3], Ogihara Y[4], Shinozaki K[1], Kikuchi J[1,2]**

*[1]RIKEN Plant Science Center, Jpn. [2]Yokohama City Yuniversity, International Graduate School of Arts and Sciences, Japan. [3]National Institute of Agriculture Sciences, Japan. [4]Yokohama City University, Kihara Institute for Biological Research, Japan*

## ABSTRACT

Systematic evaluation of phenotypic variations among cereal breeds based on metabolic profiling is an effective approach to holistic discovery of metabolic markers as well as nutrition targeted breeding. Furthermore, integrated analysis of metabolo-phenotype and genotype of natural variations or cultivars should be useful to find out metabolic markers in association with genotypes. We have applied NMR methods to perform metabolic profiling in Gramineae plants (wheat, barley and rice), in order to develop a novel metabolic-phenotyping procedure by global measurement of metabolite in various samples. Solution samples extracted from seed grains of 21 wheat, 21 barley and 18 rice strains were conducted for the metabolic profiling by 1D-$^{1}$H-NMR and 2D-$^{1}$H-$^{1}$H NMR spectra. The metabolic fingerprint of each of 60 strains by the 1D-$^{1}$H-NMR were analysed using principal component analysis (PCA) and hierarchical clustering analysis (HCA) to compare metabolic profiling among species and to find out metabolic marker by chemical shift values corresponding to metabolite specifically abundant in each strain. Furthermore, 2D-$^{1}$H-$^{1}$H NMR spectral profiling was also applied to quantify major metabolites. In total, 22 metabolites were compared among strains by the 2D-NMR spectra. These major metabolite profiles allow us to evaluate nutritious balance in cereal grains. Furthermore, in order to compare genetic divergence and metabolo-phenotypic variations, we performed genome wide genotyping using AFLP analysis for comparison between genotype and metbolo-phenotype to discover metabolites in association with genetic polymorphisms. NMR based metabolic-phenotyping and that integration with genome wide genotyping should be mostly applicable to systematic exploration of cereal genetic resources as well as to metabolite based breeding for enhancement of cereal productivity.

## INTRODUCTION

Metabolic profiling is becoming a quite useful technology for microscopic and comprehensive phenotyping and diagnostic analyses in plant as a key approach to annotate gene function and systematic evaluation of metabolite component (Schaurer and Fernie 2006, Saito et al .2006). Metabolic phenotyping is applicable to holistic discovery of metabolite markers as well as nutrition targeted breeding based on high throughput profiling of metabolite contents as traits to screen genetic resources. There are now several examples of metabolo-phenotype based breeding including carotenoids contents in tomato (Liu et al. 2003), protein and oil content in maize (Moose et al. 2004) and starch content of rice and potato (Ferine and Willmitzer 2004). Metabolite profiling was applied to identify QTLs in association with metabolites accumulations to dissect the genetic basis of metabolic network in Arabidopsis, tomato, and poplar (Kliebenstein et al. 2001, 2002, Tieman et al. 2006). Integrative analysis of metabolomics and genomics or transcriptomics should facilitate to elucidate plant metabolic systems as well as to explore key loci applicable for crop improvements.

RIKEN Plant Science Center, we have established metabolomics platform including various types of mass spectrometry (MS) and NMRs as well as the informatics technologies namely PRIMe (http://prime.psc.riken.jp/), to boldly carry forward plant metabolomics and to understand metabolic systems for plant productivity (Tian et al. 2007, Kusano et al, 2008,).

NMR method is a spectroscopy allowing us to elucidate 3D structure and dynamics of biological molecules with high repeatability. Various NMR methods make it possible to gain holistic metabolic profile data not only from solution samples but also from insoluble and/or solid state samples (Kikuchi et al. 2004). Metabolic phenotyping using $^{1}$H-NMR is applicable to acquire holistic profile of metabolites in high throughput and to compare characteristic metabolites accumulating among varieties of species

In this study, we have applied 1D-$^{1}$H-NMR and 2D-$^{1}$H-$^{1}$H NMR methods to measure soluble metabolite of seed grains of wheat, barley and rice to acquire metabolo fingerprints of strains of those crops, and demonstrated effectiveness of NMR metabolic phenotyping for characterization of each strains based on metabolite components. Results of metabolic fingerprints of cereal grains have been conducted to principal coordinate analysis (PCA) and hierarchical clustering analysis (HCA) to compare metabolic profiling among species and varieties as well as to find out chemical shift values corresponding to metabolites specifically abundant in each strain. Furthermore, in order to compare genetic divergence and metabolo-penotypic variations, distance matrix of genotype and those of metbolo-phenotype have been compared to discover metabolites in association with genetic polymorphisms. Herein, we have demonstrated NMR metabolic phenotyping that should become a powerful phenotyping procedure to explore and evaluate genetic resources for metabolite based crop breeding.

## MATERIALS AND METHODS

Plant materials

Seed grains of 21 wheat strains were derived from National Bio Resource Project "KOMUGI", those of 21 barley strains were selected from barley core collections derived from Okayama University, and 18 rice strains were selected from the world rice collection derived from NIAS (Table 1).

NMR-based metabolic profiling

NMR samples were prepared essentially as described previously (Kikuchi and Hirayama 2007). Briefly, 10 mg of the milled seeds was extracted with 600 µl of 0.1 M-KPi buffer at 50 °C for 5 min with gentle vortexing. Aftercentrifugation, the extracted supernatant was transferred into a 5-mm Ø NMR tube for NMR measurements. One-dimensional $^{1}$H NMR spectra were acquired at 298 K on a Bruker DRX-500 NMR spectrometer equipped with a $^{1}$H inverse probe and a triple-axis gradient. The chemical shifts were determined using sodium 2,2'-di-methyl 2-silapentane 5-sulfoxide as a reference. The one-dimensional NMR spectra were integrated between 0.5 and 10.5 ppm over a series of 0.04-ppm integral regions using our custom integration software. After exclusion of the water resonance, each integral region was normalized to the total integral region. The data were analysed by partial least-squares projection based on the spectral bins obtained from one- and two-dimensional spectral analyses using the pls package (version 2.0) with the "simpls" method running on R software (Tian et al. 2007).

Plant Genotyping

Genomic DNAs of individual plant was extracted using DNeasy plant mini kits (QIAGEN), and those were conducted to AFLP analysis using AFLP Core Kit (Invitorogen). AFLP fragment patterns were detected by using a capillary electrophoresis, eGene (eGene). AFLP fragment patterns were scored and applied to calculate genetic distance among plant using restdist, and neighbor of PHYLIP package ver.3.67.

## RESULTS AND DISCUSSION

*$^{1}$H-NMR metabolic phenotyping of cereal grains*

Seed soluble extracts of each strain were conducted by $^{1}$H-NMR experiments in order to acquire metabolic fingerprint to find metabolite candidates contributing to differentiate metabolic phenotypes. Spectral data were digitized and globally normalized to apply HCA and PCA analysis. Expanded region of $^{1}$H chemical shifts corresponding to metabolites of Organic acids or Lipids of wheat grains are shown in Fig1A.

Table 1. Cultivars and strains applied to this work.

| | Cultivar/Strain | Nation |
|---|---|---|
| *Triticum aestivum* | KM06-64 | Afghanistan |
| | KM06-174 | Afghanistan |
| | CX89 | Hsinchiang Uighur |
| | Zenkouji-komugi | Japan |
| | Mochikomugi | Japan |
| | Chihoku-komugi | Japan |
| | Zenith | Swiss |
| | Tundra | Netherlands |
| | Kleiber | Sweden |
| | Runan | Norway |
| | Bounty | United Kingdom |
| | HOPE | United Kingdom |
| | Gaines | United States |
| | Palo Duro | United States |
| | Turkey Red | United States |
| | Jones Fife | United States |
| | Sonora 64 | United States |
| | Neepawa | Canada |
| | Bluebird 4 | Mexico |
| | RL 4137 | Brazil |
| | Chinese Spring | Hungary |
| *Hordeum velegare* | TKB73a | Bhutan |
| | Anbyeon Native | Korea |
| | Prok 1 | Nepal |
| | Tsumje 1 | Nepal |
| | K. 12 | India |
| | Tibba 1 | India |
| | Milgagar | India |
| | Mansinghkanda 4 | India |
| | Mongolia 6-row | Mongolia |
| | Kobdicum Type 20 | Tibet |
| | Chiuchiang | China |
| | Shantung Naked | China |
| | Wuhu | China |
| | Sikangense Type 15 | Tibet |
| | Satsuki Nijo | Japan |
| | Fuji Nijo | Japan |
| | Haruna Nijo | Japan |
| | Akashinriki | Japan |
| | Tokushima Mochimugi 1 | Japan |
| | Ulleri 10 | Nepal |
| | Betzes | Germany |
| *Oryza sative* | Surjamukhi | India |
| | Nepal 8 | Nepal |
| | Jhona 2 | India |
| | Muha | Indonesia |
| | Co 13 | India |
| | Vary Futsi | Madagascar |
| | IR 58 | Philippines |
| | Milyang 23 | Korea |
| | Basilanon | Philippines |
| | Dianyu 1 | China |
| | Kasalath | India |
| | Jaguary | Brazil |
| | Ma sho | Myanmar |
| | Rexmont | United States |
| | Khau Mac Kho | Viet Nam |
| | Khao Nok | Laos |
| | Padi Perak | Indonesia |
| | Nipponbare | Japan |

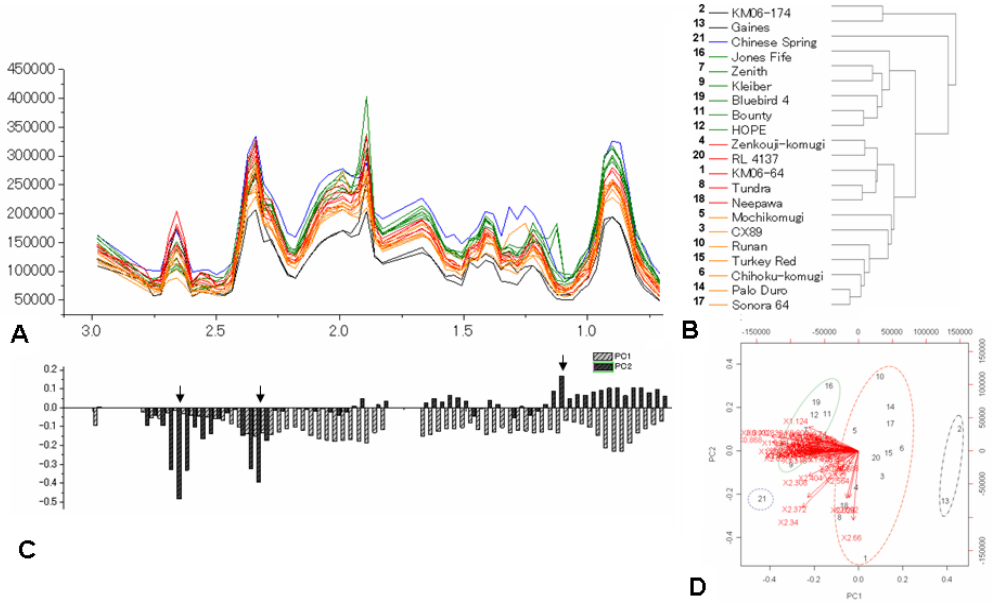


Fig.1 $^{1}$H-NMR spectral phenotype of organic acids and lipid area of wheat seed soluble extract. $^{1}$H-NMR spectra obtained from the 21 wheat strains (A). Hierarchical clustering of wheat strains based on metabolite profiles (B). Loading plots of PC1 and PC2 of the PCA plot (C). The arrows are indicating remarkable peaks for classification of strains in the PCA plot (D).

To classify wheat strains based on metabolic phenotype, HCA and PCA analyses were performed. Wheat strains were classified into 4 major clusters by the both clustering methods (Fig. 1B, 1C). Furthermore, the chemical shifts corresponding to metabolites contributed to the classification of wheat strains have been calculated by using loading plot of PCA. The PCA and the loading plot suggest that the seed metabolite allocated in the $^{1}$H chemical shift 2.660, 2.340 and 1.124 (ppm) in contributions to the metabolophenotypic divergence shown in Fig.1D, and those classified pattern have been also supported by the hierarchical clusters illustrated in Fig.1B.

*Profiling of major metabolite using 2D-NMR*

Major metabolites accumulating in soluble seed extracts of cereal grains can be annotated by using 2D-$^{1}$H-$^{1}$H NMR method to acquire major metabolite profile and that relative abundance to be compared among strains (Fig. 2). In total, 22 compounds have been annotated and profiled. Example of comparison of major metabolites is shown in Fig.2 (B-D).

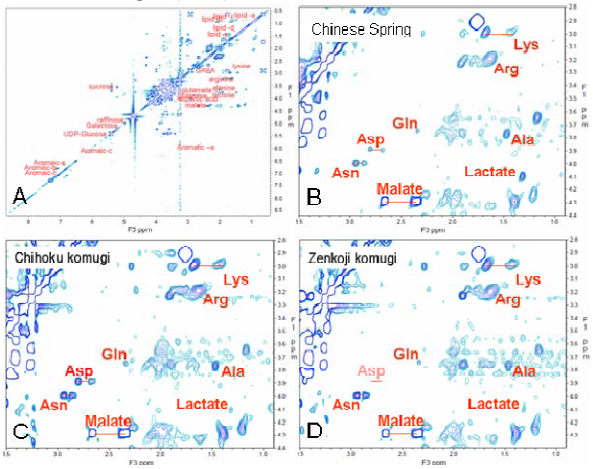


Fig. 2. Major metabolite profiling by using 2D $^{1}$H NMR method. An entire region of 2D NMR spectrum of seed soluble metabolite in wheat (A). The expanded aliphatic regions of Fig2A, each of which are derived from 3 wheat strains, Chinese Spring (B), Chihoku-komugi (C), and Zenkoji-komugi (D).

*Genetic diversity vs. Metbolophenotypic diversity in cereals*

Genetic diversities based on the genetic distance among strains calculated by scored polymorphisms have been compared with metabolophenotypic diversities in each cereal; wheat, barley and rice. Overall comparison between the matrix of genetic distance and those of metabolo profile has not showed significant correlations in each cereal species. To discover chemical shift region of metabolites whose profiles were correlating to genetic distance matrix, we calculated correlation coefficient between each distance matrix of metabolite profile in a sliding region within a various window size of chemical shift range and those of genetic distance. Several candidate regions of $^{1}$H chemical shift have been detected, which have been showing correlation between metabolic profile and genetic distance matrix.

In this study, we have demonstrated metabolic phenotyping using NMR spectroscopy to evaluate genetic resources of cereals. Our results could suggest NMR methods are applicable to evaluate metabolite components of seed grains of cereals. Because the $^{1}$H-NMR method is high throughput and holistically covering metabolite, it should be a novel method as a microscopic phenotyping based on metabolome for cereal phenotyping. We also have demonstrated a novel trial to discover metabolites whose profiles are correlating with genetic relationships. This combination approach between loading plot of metabolite profiles and genetic distance matrix might allow us to find anonymous metabolite which should be allocated onto the chromosome region by association mapping by using natural accessions by population analysis. NMR metabolic phenotyping therefore should be an effective phenotyping method and applicable to metabolite targeted breeding of cereals.

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