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Identification of a constitutive metabolite marker for distinguishing tolerant and sensitive varieties of rice through GC-MS based metabolomics approach

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Abstract
Plants have both induced and constitutive mechanisms to resist environmental stress. Different mechanisms of induced responses to tolerate salinity in rice, a staple food for more than half of the world's population, are in report. The objective of the study was to search for constitutive metabolite(s) for differentiate the grains of salinity tolerant and sensitive rice varieties, on the basis of metabolic profile. Rice grains of total 13 varieties, (8 tolerant and 5 sensitive) were analyzed by gas chromatography coupled with mass spectrometry for identification of aqueous methanol soluble metabolites and cell wall bound phenols. Chemometric study (Orthogonal Partial Least Squares Discriminant Analysis and Partial Least Squares Discriminant Analysis) separated the sensitive and tolerant varieties based on 86 metabolites including 8 wall bound phenols. Four wall bound phenols were identified from the loadings to contribute most for the separation of the two groups, tolerant and sensitive. One cell wall bound phenol 4-hydroxy-3-methoxybenzoic acid (vanillic acid) was detected only in the sensitive varieties but not in the tolerant varieties. This finding suggests that cell wall bound phenol 4-hydroxy-3-methoxybenzoic acid is a constitutive biomarker metabolite to distinguish tolerant and sensitive varieties rapidly.

Keywords: Rice, constitutive metabolites, Chemometric study, vanillic acid

Introduction
Soil salinity is one of the major limiting factors for crop productivity. Rice (*Oryza sativa* L.), a glycophyte, is one of the major staple foods feeding more than half of the world's population [1]. However, there are some salt tolerant varieties which are used for breeding programme. About 90% of world's production and consumption of this agronomically important crop are in Asia [2]. Productivity of rice is substantially reduced in parts of India and other countries due to salinity affected rice field and salinity is largely affected by NaCl [3]. Methods of salinity tolerance screening are important for successful breeding programme [1]. Screening for salinity tolerance in rice, based on agronomical parameters [4,5], physiological parameters [6,7] such as association of salt tolerance with Na^+ and / or Cl^-, sodium concentration [8] in shoot, leaves etc. have been reported. However, there was a negative correlation between sodium (and chloride) accumulation by individual plants and their survival in saline conditions [7]. Zeng *et al*. [6] demonstrated significant correlation between leaf area index and yield components in both salt-tolerant and sensitive genotypes. They suggested that Na-Ca selectivity could be a salt tolerance component and a useful criterion in screening for salt tolerance. Use of morphological and physiological traits in multivariate analyses elucidated significant genotypic variation and correlations among the salt injury score, ion leakage, chlorophyll reduction, shoot length reduction, shoot K+ concentration, and shoot Na+ / K+ ratio in southern USA rice genotypes. DNA profiling using simple sequence repeat markers showed narrow genetic diversity among those genotypes [9]. However, salt responsive genes have been identified by sequencing of rice genome [10]. Some protein coding genes responsive to high salinity were also identified in rice genome [11, 12]. Role of DNA methylation, genome duplication in salinity tolerance have been studied in rice [10]. Metabolomics also helped understanding salinity induced increase or decrease of metabolite levels in rice [13, 14]. NaCl induced osmoprotectants and signaling molecules (seronin and gentisic acid) have been identified by metabolomics technology [15] using both salt tolerant and salt susceptible varieties.

However, most of the screening work has been performed by inducing stress using NaCl. But plants have both constitutive and induced mechanisms to withstand stress. Induced resistance is activated or expressed only after the plant is attacked or otherwise injured. Constitutive resistance is expressed independently of injury [16]. During the present study attempt was made to study the metabolic profile of the grains obtained from different varieties of indica rice growing under similar agroclimatic conditions to find their correlation with their known salt tolerance capability and to identify metabolic markers unique for salt tolerance and to understand metabolite – phenotype correlations.

Materials and Methods

Plant material

Grains of thirteen rice varieties were collected from Rice Research StationChinsurah, West Bengal. Among thirteen varieties, eight were salt tolerant namely, Amalmona, Bhutnath, Dudheswar, Jarava, Lalat, Rupsail, Mohan and Nonabokra and rest five salt sensitive varieties were Kaushalya, MTU 7029, Swarna sub1, Sabita and Sujala. About 5-6 grains were taken from each variety, dehusked and weighed together. Then the grains were crushed into powder with the help of a mortar and pestle. Same procedure was applied for each of the variety to make the grains crushed into powder.

Chemicals

FAME markers, methoxyamine hydrochloride, and ribitol were purchased from Sigma (USA). Authentic amino acids, sugars, fatty acids and organic acids were purchased from SRL (Sisco Research Laboratory, India), authentic phenols O-acetylsalicylic acids was collected from Sigma, gentisic acid from Aldrich, 4-hydroxy-3-methoxybenzoic acid from Himedia, and other phenols were collected from SRL. Authentic samples were injected further for confirmation of the identified peaks. HPLC graded methanol was purchased from Sigma (USA) and all other chemicals were purchased from Merck Specialities Pvt. Ltd., India.

Extraction of free metabolites

Crushed grain particles of thirteen varieties were extracted with 50% methanol and 50% water at 60 °C for 30 min along with 20 µl of internal standard ribitol. The extraction was done repeatedly for each variety, and then centrifuged at 10,000 rpm for 10 min, supernatant containing all the metabolites were collected and distributed in separate micro centrifuge tubes with 150µl of extracts. Then the extracts were evaporated into dryness and the metabolites in the micro centrifuge tubes were undergone derivatization for GC-MS based metabolite determination.

Extraction of wall bound Phenolics

Extraction of wall bound phenols was done following the modified method of Azuma *et al*. [18]. Powdered grain materials were taken in micro centrifuge tubes and methanolic extraction was done repeatedly for each variety separately. Starch was removed by stirring the material continuously in 20% DMSO for 24 hours and complete hydrolysis was done by amylase for 3 hours. All the hydrolyzed materials were washed thoroughly by water, acetone, methanol:chloroform (1:1 v/v), methanol and water. The material was extracted with 20 mM ammonium oxalate (pH: 4) at 70 °C for 2 h to remove pectic substances. The residue was suspended in 1M NaOH solution containing 0.05 mg/ml $NaBH_4$and stirred for 24 h with magnetic stir bar. After complete hydrolysis, the mixture was centrifuged, and supernatant was acidified to pH 2.0 with HCl and then extracted with ethyl acetate.

Gas Chromatography - Mass Spectrometry (GC-MS) analysis

All the extracts were derivatized for 90 min at 28 °C in 5 µl of 20 mg/ml methoxyamine hydrochloride in pyridine followed by a 30 min shaking in 45 µl of N-Methyl-N-(trimethylsilyl) trifluoroacetamideat 37 °C for trimethylsilylation of acidic protons to increase volatility of metabolites. 1µl of fatty acid methyl ester (FAME) markers [a mixture of internal retention index (RI) markers was prepared by using fatty acid methyl esters of C8, C10, C12, C14, C16, C18, C20, C22, C24 and C26 linear chain length] dissolved in chloroform was added [18]. GC-MS analysis [Agilent 7890 A gas chromatography, software driver version 4.01(054) equipped with 5975C inert MSD with triple axis detector] was performed with HP-5MS capillary column [Agilent J & W; GC column (USA) of dimensions 30 m X 0.25 mm X 0.25 µm]. Analysis was done following the method of Kind *et al*. [18] with oven ramp at 60 °C followed by 1 min hold, to 325 °C (at 10 °C/min) with a 10 min hold before cooling down for a 37.5 min run. Injection temperature, MSD transfer line and ion source were set at 250 °C, 290 °C and 230 °C respectively. Helium gas was used in mobile phase with a flow rate of 0.723 ml/min (carrier linear velocity 31.141 cm/sec). Sample injection was done via split mode (split ratio 10:1). Mass spectra from 50 to 500 m/z were recorded. The analysis for identification of the compounds were carried out following users' guide of automated mass spectral deconvolution and identification system (AMDIS) calibrated with the method, to deconvolute GC-MS results to identify chromatographic peaks. Mass spectral fragmentation pattern and retention time (RT) in Agilent Fiehn GC-MS Metabolomics RTL Library (Agilent Technologies, USA, 2008) were compared for peak identification. For many of the metabolites, RT, RI, and MS were compared with authentic samples.

Statistical analysis

Compound peak area was normalized and divided with the peak area of ribitol and sample fresh weight to obtain response ratio. For wall bound phenols, peak area was divided only by sample fresh weight to obtain response ratio. Data were subjected to Partial Component Analysis (PCA), Partial Least Squares Discriminant Analysis (PLSDA), Orthogonal Partial Least Squares Discriminant Analysis (OPLS-DA), and Sparse Partial Least Squares Discriminant Analysis (sPLS-DA) using MetaboAnalyst 3.5, a comprehensive tool for metabolomic data analysis.

Results and discussion

Present study was an attempt to identify metabolic phenotypes of salt tolerant and sensitive varieties, based on the constitutive metabolites, from the already established varieties of indica rice growing under same agroclimatic field conditions. Thirteen varieties of grains (eight were salt tolerant namely, Amalmona, Bhutnath, Dudheswar, Jarava, Lalat, Rupsail, Mohan, and Nonabokra; and rest five salt sensitive varieties were Sujala, Sabita, Swarna sub1, MTU 7029, and Kaushalya) were considered during the study. Ripe grains, after removal of the husks, were analysed by GC-MS for their aqueous methanol soluble metabolites as well as cell wall bound phenols. Eighty-six metabolites could be detected from the aqueous methanol extracts of rice grains (Table 1). Identified metabolites belonged to classes like phenols, organic acids, amino acids, fatty acids, sugars, and polyols.

Eight phenolic compounds were detected as wall bound metabolites. The metabolites, identified from grains of 13 varieties of rice (five biological replicas for each variety) were subjected to different multivariate analysis. PCA could not distinguish the tolerant varieties from the sensitive varieties. However, PLS-DA and OPLS-DA could separate the two types (Figures 1, 2). Thus, the tolerant and sensitive groups may be considered as two metabolic phenotype groups. The important contributory metabolites for separation of the tolerant and sensitive varieties, as observed from the loading plot of OPLS-DA were wall bound phenols such as, 4-hydroxy-3-methoxybenzoic acid [p(corr) value -0.910372], 4-hydroxybenzoic acid [p(corr) value -0.532881], and 3,4-dihydroxybenzoic acid [p(corr) value 0.420516]. Five most important contributory metabolites of component 1 obtained from VIP scores were also the wall bound phenols (4-hydroxy-3-methoxybenzoic acid, 4-hydroxybenzoic acid, and 3,4-dihydroxybenzoic acid), proline and galactose. The most important metabolite was 4-hydroxy-3-methoxybenzoic acid based on VIP scores of PLS-DA and OPLS-DA loadings. Based on t-test, only 4-hydroxy-3-methoxybenzoic acid was found to be significantly different (p value 2.0169E-24) in the salt tolerant and sensitive varieties.

The relative response ratios of the above-mentioned metabolites were plotted. The figures1, 2 indicate that except 4-hydroxy-3-methoxybenzoic acid, other metabolites were not played any significant role to differentiate the tolerant and sensitive varieties. It was observed from the Figure 3 that wall bound 4-hydroxy-3-methoxybenzoic acid (vanillic acid) was detected only in the sensitive varieties and was absent in the tolerant varieties.

So, based on the present observations in rice grains, cell wall bound 4-hydroxy-3-methoxybenzoic acid is considered as a biomarker compound for identification of sensitive or tolerant varieties. This is a cell wall bound constitutive metabolite detected only in the grains of the sensitive varieties.

In rice, Zuther *et al*. [17] demonstrated presence of metabolite phenotypes, based on level of salt tolerance, under control conditions without salt treatment which may be indicative of salt tolerance or sensitivity. Such metabolic classification was clearer in the root than in the leaf. However, such metabolic differences were quantitative rather than presence or absence of marker metabolite. In the previous study [19], it was observed that wall bound phenolic metabolites of leaf played a significant role in salt tolerance in rice. The leaves of tolerant varieties (Nonabokra and Bhutnath) contained significant lower level of wall bound ferulic acid and 4-hydroxycinnamic acid than that in the sensitive varieties (MTU 7029 and Sujala) under control condition. But after salt treatment the level of such phenolic metabolites increased in tolerant varieties and decreased in sensitive varieties.

The present study would helpful in identification of tolerant varieties from the grains based on presence or absence of a cell wall bound biomarker metabolite 4-hydroxy-3-methoxybenzoic acid.

Table 1: Metabolites identified from rice grains

Phenols	**Sugars and Derivatives**	**Organic acids**
O-Acetylsalicylic acid*^	N-Acetyl-D-mannosamine*	Trans-Aconitic acid*
Benzene-1,2,4-triol*	Alloinositol*	Capric acid*
Benzoic acid*	Allose*	Caprylic acid*
3,4-Dihydroxybenzoic acid*	Altrose*	Citric acid*^
Ferulic acid*	Beta-glycerophosphate*	Fumaric acid*
Hydroquinone*	Fructose*	Glyceric acid*
4-Hydroxybenzoic acid*^	Galactinol*	Glycolic acid*
4-Hydroxycinnamic acid*^	Galactose*	4-Guanidinobutyric acid*
4-Hydroxy-3-methoxybenzoic acid*^	Galacturonic acid*	3-Indole-acetic acid*
4-Isopropylbenzoic acid*	Gluconic acid*	L-(+) Lactic acid*
Quinic acid*^	Glucosaminic acid*	D-Malic acid*^
Shikimic acid*	Glucose*	Mucic acid*
Amino acids	Glycerol*	Nicotinic acid*
Beta-Alanine*^	Glycerol-1-phosphate*	Oxalic acid*^
L-Alanine*^	Isomaltose*	Pantothenic acid*
Aspartic acid*^	D-Lyxose*	Phosphoric acid*
L-Asparagine*^	D-Lyxosylamine*	Pipecolic acid*
Beta-cyano-L-Alanine*^	Maltose*^	Succinic acid*^
L-Glutamic acid*^	D-Mannitol*^	
Glycine*^	Mannose*	**Others**
DL-Isoleucine*^	Melezitose*	Adenine*
Leucine*^	Raffinose*	Adenosine*
L-Lysine*^	Sophorose*	Allantoin*
Methionine*^	D-Sorbitol*^	Cysteine*
Mimosine*	Sucrose*^	O-Phosphocolamine*
L-Proline*^	Sucrose-6-phosphate*	Porphine*
Pyroglutamic acid*	Tagatose*	Uracil*
L-Serine*^	Talose*	Urea *
L-Threonine*^	D-(+) Trehalose*^	
L-Tryptophan*^		
L-Tyrosine*^		
L-Valine*^		

* RT, RI, MS of Fiehn Library; ^RT, RI, MS of authentic compound

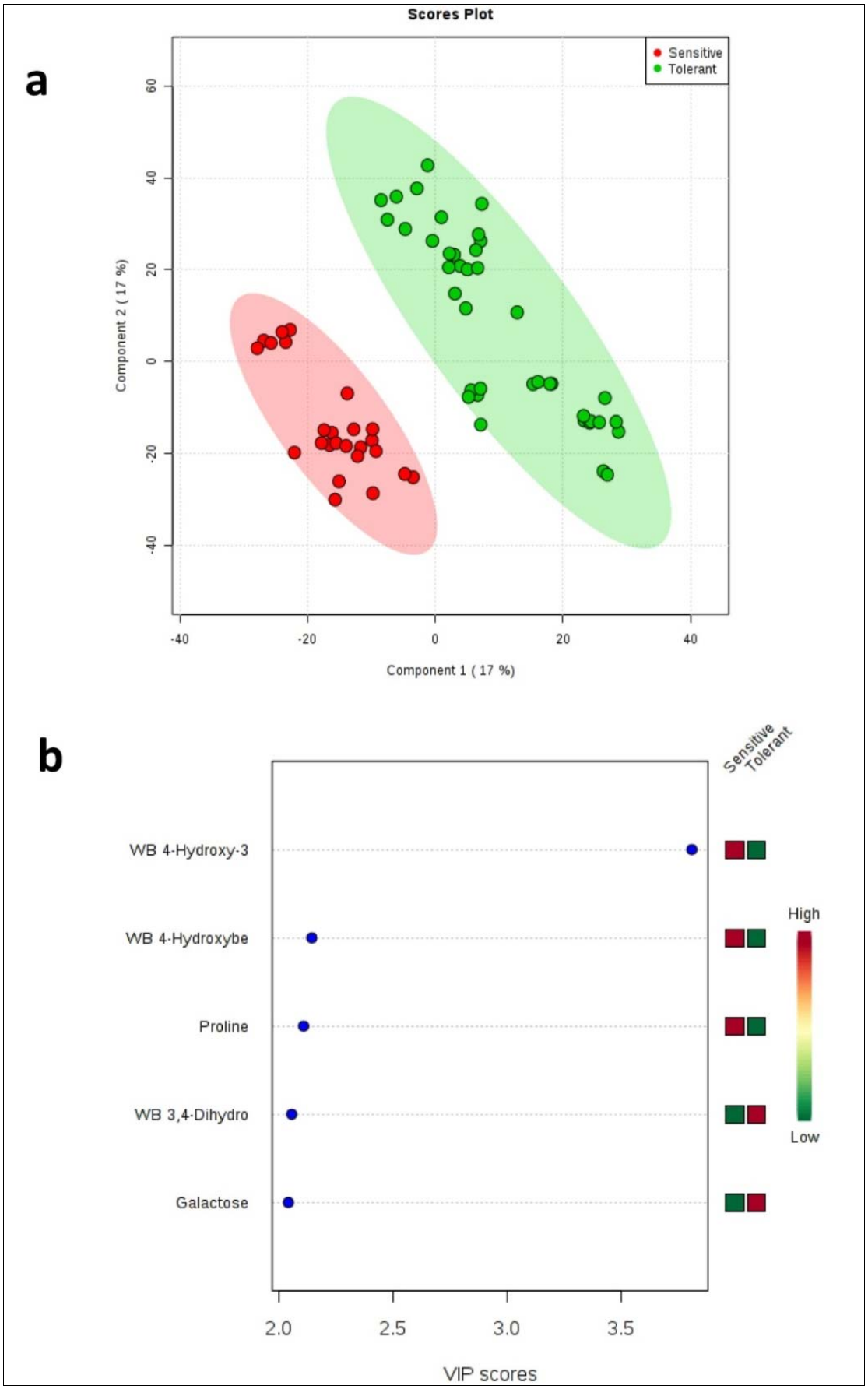

Fig 1: PLS-DA analysis of rice grain metabolites. a: Scores plot for component 1; b: VIP scores. WB 4-Hydroxy-3: wall bound 4-Hydroxy-3-methoxybenzoic acid

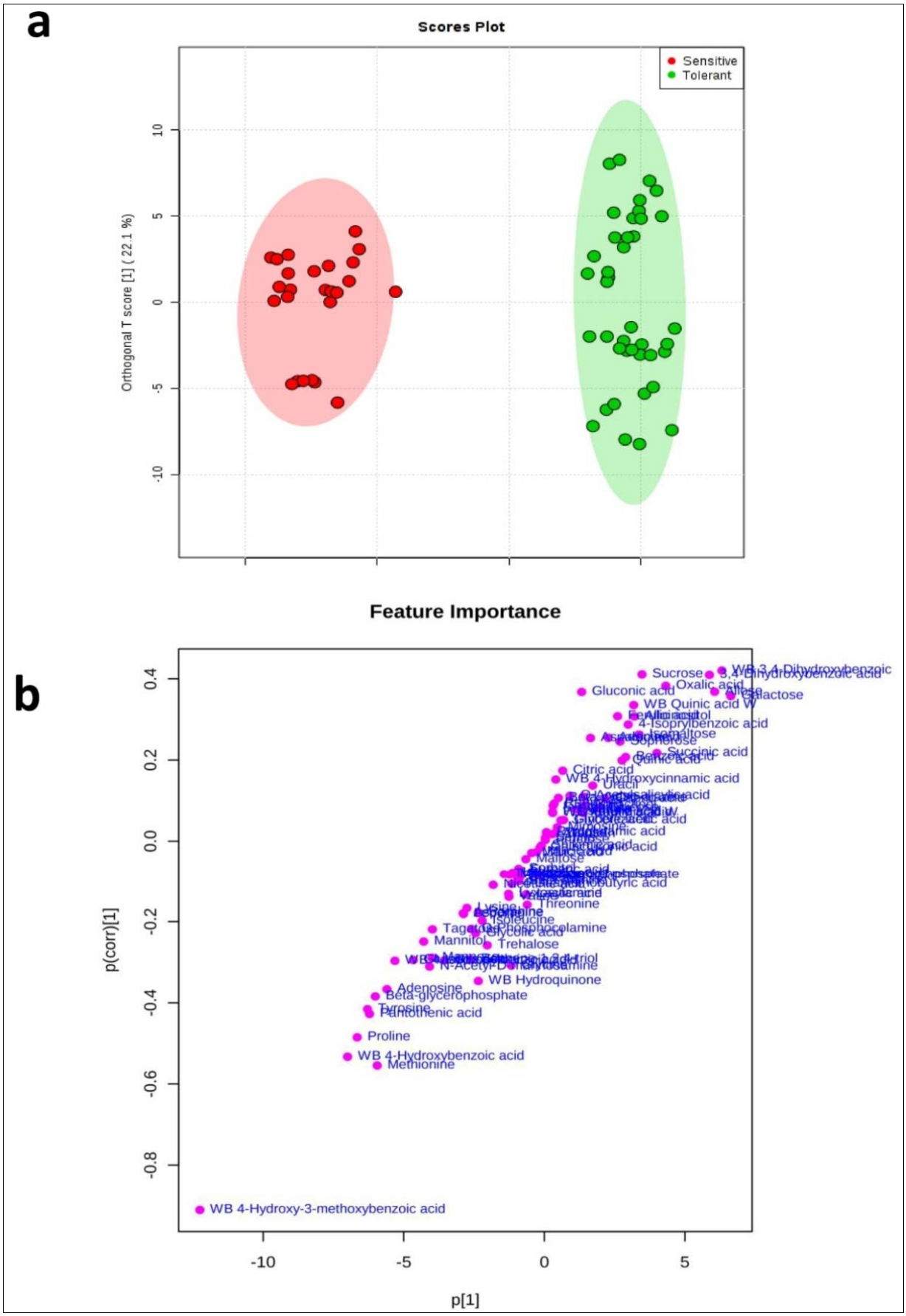

Fig 2: OPLS-DA analysis of rice grain metabolites. a: Score plot; b: Loading plot. WB: wall bound

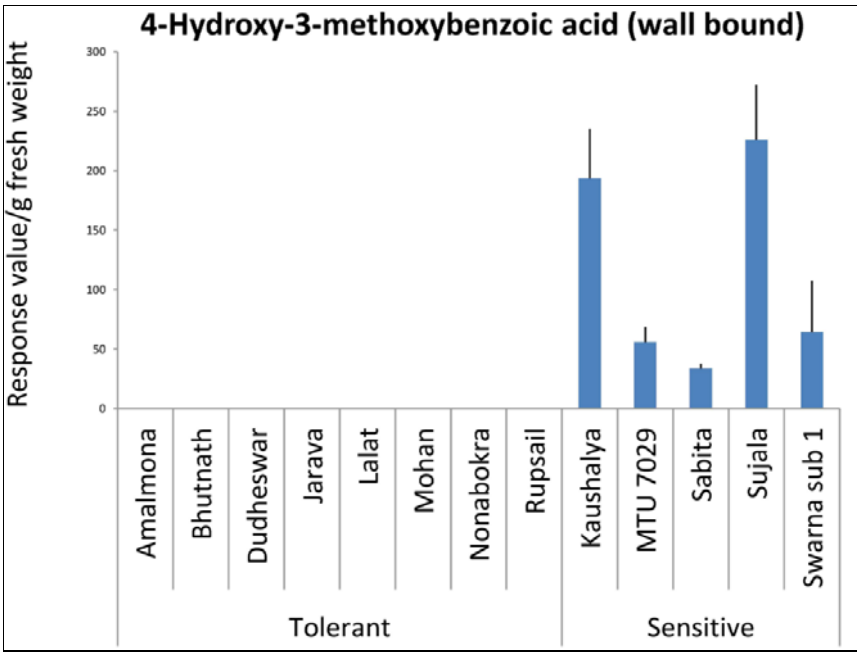

Fig 3: Important metabolite of rice grains distinguishing salinity tolerant and sensitive varieties

Conclusion

Thirteen varieties of rice grains were analysed by GC-MS for aqueous methanol soluble metabolites and cell wall bound phenols. Of these varieties 5 were sensitive to salinity and 8 were tolerant. Chemometric analysis (OPLS-DA and sPLS-DA) of 86 metabolites including 8 wall bound phenols separated the salt sensitive and tolerant varieties. Interestingly, only one wall bound phenol 4-hydroxy-3-methoxybenzoic acid (vanillic acid) was found to be significantly different by t-test. This metabolite was detected only from the sensitive varieties and not from the tolerant varieties. So, this metabolite is considered as a constitutive biomarker compound to identify sensitive / tolerant varieties.

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