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Differentiation of rice varieties using metabolite markers

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Tillering is one of the key yield-related traits for the grass family and affects the quality and quantity of effective panicles produced. For this reason, it is an important characteristic used in breeding rice. It was found that metabolites related to tillering differentiated varieties prior to divergence in tillering phenotype. Selected markers, valine, alanine, leucine, GABA and oxalic acid were quantified in the tiller at different developmental time points. Using principal component analysis it was possible to observe differences between varieties as early as 30 days after planting that correlated to future tillering levels. These results indicate that metabolite markers could be used as an early selection tool in rice breeding studies.

Key words: Metabolomics; multivariate; rice breeding; metabolite marker; tillering

Introduction

The objective of crop breeding is to acquire and combine valuable traits. Tillering is known to be one of the crucial phenotypes that influences the quantity and quality of effective panicles (heads of grain), and therefore yield in rice (Sakamoto and Masuoka, 2004) and exhibits significant variation across varieties (Nuruzzaman *et al*., 2000). Yield is determined by the number of panicles per land area and can be projected by the numbers of tillers per land area. Therefore, understanding the mechanisms that control this plant architecture trait will facilitate the breeding of rice varieties with higher yield potential. Biomarkers associated with phenotypic properties have been advanced in the applied and fundamental medical research fields as a predictive tool (Jacobsen et al., 2008; Kim et al., 2010) and more recently, in agricultural research (Anastasiadi et al., 2009; Stenfath et al., 2010). Combinations of new

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analytical techniques and multivariate statistical analysis tools have made this research possible. Metabolite markers generally fall into two categories: primary or secondary metabolites. Primary metabolites are essential for adequate growth and metabolism, while secondary metabolites are often responsible for interaction with the environment, for example, in response to biotic or abiotic stresses. Although primary metabolites are highly conserved between species, there can be significant quantitative variation between them that can be associated with different growth characteristics (Kim et al., 2009; Ritota *et al*., 2010; Thomas and Pedersen, 1998). Several primary metabolites have been reported as being associated with rice tillering (Tarpley *et al*., 2005) . In particular, amino acids and some organic acids were found to be clearly related to tillering development. A selection of these biomarkers was used in this study to evaluate whether their concentration levels could be used to differentiate rice varieties before differences in tillering phenotypes could be observed.

Materials and methods

Rice varieties

Seeds were taken from the rice germplasm collection of the Malaysian Agricultural Research and Development Institute (MARDI), developed and maintained by Rice Seed Genebank, Penang. The seeds used in this study were the Malaysian varieties MR232, MR219 and MR220, as well as the Indonesian variety Intani-2. Information regarding the varieties was obtained from MARDI records and internal pre-evaluation (unpublished). MR219 and MR220 are siblings while MR232 is a different line that is reported to have comparably high tillering ability. Intani-2 is also reported to be high yielding although no data was obtained specifically regarding its tillering characteristics.

Sample preparation

Experiments were conducted in a closed greenhouse. Forty-eight polystyrene boxes with dimensions 30 x 27 x 24 cm (H x W x L) were filled with soil to a level of 16 cm. Each variety was planted in triplicate for the 4 time points tested. The four varieties; MR232, MR220, MR219 and Intani-2 were evaluated concurrently. Seeds were germinated by soaking then air drying for 24 hours prior to planting. Manual watering and fertilization was conducted evenly and the plot was maintained to prevent weeds. Sampling for metabolite analysis was conducted at 15, 30, 50 and 75 days after planting. At each time point, 20 plants were taken from each of the three replicates of each variety.

Tillers, regardless of the order were separated from roots and leaf blades then washed with purified water and immediately frozen in liquid nitrogen. Fertilizer was applied after each sampling occasion. Metabolite extraction and analysis methods were adapted from those reported by (Tarpley *et al.*,2005).

Extraction protocol

Pulverized tissue (600 mg) was weighed into 15 mL centrifuge tubes. Metabolite extraction was performed by adding milliQ water (1.5 mL) containing ribitol internal standard (60 µL of 20 mg/L in water) and dichloromethane (1.5 mL), followed by vortexing for 1 min. Samples were extracted at 50 °C for 5 h with shaking at 200 rpm then sonicated for 30 s and centrifuged at 2900 ×*g* for 30 min. Aliquots (1.2 mL) were taken from the polar layers, transferred to 2.0 mL HPLC vials, vacuum dried in a centrifugal evaporator then stored at -20 °C until further use.

Derivatisation method

Samples were derivatised using methyl oxime by dissolving the dried polar extracts in methoxyamine hydrochloride solution (120 µL of 15 g/L in pyridine) at 50 °C, shaking at 200 rpm for 4 h then followed by sonication (<30 s) to dislodge any pellet formed. Samples were then silated by addition of N-methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA) with 1% trimethylchlorosilane (120 µL) followed by shaking at 200 rpm, 50°C for 1 h.

Gas Chromatography Mass Spectral analyses

Samples were analyzed by injecting 1 µL with a split mode injection ratio of 5:1 to Agilent 6890N gas chromatograph via an Agilent 7683 autosampler. The injector temperature was 280 °C. Analyses were performed using a 60 m DB-5MS capillary separation column (30 m × 250 µm i.d., 0.25 µm film thickness; Agilent J&W Scientific, Folsom, CA, USA) using helium as the carrier gas, at a constant flow rate of 1.0 ml/min. Injection and interface temperatures were both set to 280 °C. Separations were achieved using the following temperature gradient: 3 min isothermal heating at 80 °C, followed by a 5 °C/min oven ramp to 315 °C, and final isothermal heating at 315 °C for 14 min. The mass spectra were obtained with electron impact ionization (70 eV) at full scan mode were recorded at 2.48 scans/s with a scanning range of 50 - 650 m/z. All samples were injected in triplicates. Metabolites of interest were matched against reference standards. Reference standards were purchased from

Sigma Aldrich, Malaysia. Retention time of the internal standard, ribitol was locked at 14.80 min.

Standard calibration curve

Serial dilutions of standards were prepared for the purpose of quantification. Standards used were alanine, GABA, uracil, thymine, valine, leucine and oxalic acid. Stock solutions (10 mL) of 4000 ppm were prepared for each standard. Standards were diluted at 500 to 0.5 ppm accordingly. A calibration curve was then plotted for each standard.

Data processing and multivariate analysis

Peaks were identified and annotated according to their retention index (RI) and comparison to the reference mass spectra as well as comparison with the external standard mixture retention times (Schauera *et al.*, 2005). Data were normalized to the internal standard response. Data from different samples were aligned and compiled using an Excel®-based macro developed internally. SIMCA-P 13.0 software was used to perform multivariate analysis.

Results and discussions

Previous work by Tarpley *et.al* 2005 identified a set of primary metabolites that are associated with tillering events. Five of metabolites; oxalic acid, leucine, valine, alanine and GABA were chosen based on their reported principal component loading scores in order to determine whether varietal differences in these putative metabolic markers could be detected before tillering phenotypes are clearly distinguishable. Observations of tillering count and metabolite levels were conducted at 15, 30, 50 and 75 days after planting to sample at times either side of key tillering initiation and development events (Jafuel and Dauzat, 2004). Table 1 shows the concentrations of selected metabolites in rice varieties at the four different time points. It can be seen that in most cases the concentrations of these primary metabolites are at their highest between 30 to 50 days after planting, with oxalic acid being the most abundant and leucine being the least. The concentration maxima observed coincides with the period when primary tillers are known to be actively developing (Jafuel and Dauzat, 2004) supporting their identification as tillering biomarkers(Tarpley *et al.*, 2005). Organic acid metabolism is important at the cellular level in several biochemical pathways such as energy production, formation of precursors for amino acid biosynthesis, and for responses to changing environmental conditions. In this study, oxalic acid was found to be at

the highest level of accumulation in tillers at 30 days after planting, ranging between 3204 ppm to 6684 ppm for the four varieties. During rapid vegetative growth, in this case tillering, the rates of nitrate reduction, carboxylate synthesis and amino acid synthesis are high (López-Bucio et al., 2000; Touraine *et al*., 1988).

Table 1. Concentration of targeted biomarkers in rice varieties before and during tillering

	DAP	Concentration (ppm)									
		Valine	s.d.	Alanine	s.d.	Oxalic acid	s.d.	Leucine	s.d.	GABA	s.d.
MR219	15	144	9	245	16	1435	484	74	8	124	24
	30	125	2	274	102	3204	35	78	5	111	21
	50	163	1	150	22	2475	329	134	2	129	16
	75	133	1	104	15	139	5	82	13	78	0.1
MR220	15	142	1	230	4	1290	277	83	3	137	31
	30	139	4	455	142	3879	84	70	3	174	19
	50	169	13	146	11	1806	53	135	7	188	30
	75	136	11	114	9	167	16	121	16	133	13
MR232	15	140	0.7	241	21	2839	519	101	9	155	22
	30	145	0.4	314	8	6684	134	100	10	127	21
	50	144	13	102	8	3057	361	145	6	53	2
	75	142	2	108	7	254	35	116	5	82	21
Intani-2	15	134	0.6	162	4	2718	214	98	5	134	32
	30	131	7	174	56	4693	128	86	5	93	17
	50	144	5	105	5	3207	527	132	9	41	7
	75	133	2	90	6	575	103	94	5	79	22

Values are mean of biological replicates (n = 3)

Combined metabolite concentration differences between the varieties at different developmental stages can be efficiently visualized using a principal component analysis (PCA) scatter plot. Figure 1 shows that there is a clear relationship between the metabolites and developmental stages with the varieties tending to cluster together. The varieties cluster the closest together at 15 days after planting while exhibiting the highest variance after 30 days. This is further emphasized in Figure 2 where the individual biological replicate data for day 30 are plotted. The PCA clustering of variety replicates was not observed clearly at other developmental stages. MR219 and MR220 are siblings and are closely clustered along principle component 1 (PC-1), while MR232 and Intani-2 are separated.

Tillering was observed at the same time as samples were collected for metabolite analysis. Tillers can be categorized in the order of the first main culm, followed by primary, secondary and tertiary tillers and the grains produced per tiller will decrease with tiller order (Kariali, 2008; Wang et al., 2007). Generally, tillers emerge from the date of planting until day 60 and panicles initiate from day 55 until day 90 (Kariali, 2008). In this experiment

tillering was calculated irrespective of order and recorded as total number of tillers per plant, Figure 3. The tillering behavior of sibling varieties MR219 and MR220 was nearly identical, increasing until day 30 and decreasing thereafter, while MR232 and Intani-2 both reached a maximum at 50 days after planting.

By day 75, only the main culm and primary tillers remained for grain production in MR219 and MR220, whereas survival of secondary tillers was still observed in MR232 and Intani-2. PCA results showed that MR219, MR220, MR232 and Intani-2 could be differentiated into two groups as early as 30 days after planting (Figure 2), whereas differentiation in tillering is not obvious until day 50 (Figure 3). The PC-1 (x-axis) of Figure 2 appears to distinguish the varieties based on tillering ability. Figure 4 shows the PC-1 loading plot of the biomarkers and indicates that leucine and oxalic acid are most related to the tillering differences observed between the varieties.

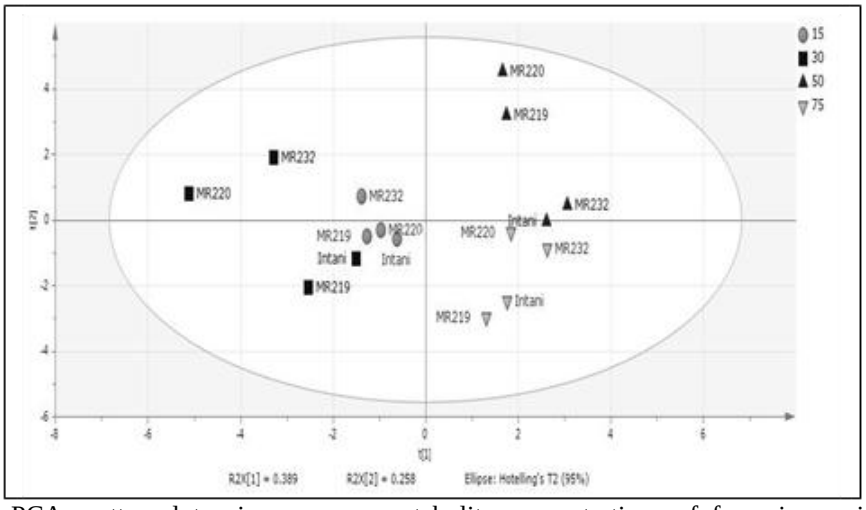


Fig. 1. PCA scatter plot using average metabolite concentrations of four rice varieties at different development stages (days after planting, D15 – D75). Axes of two dimensional plots are derived from PC-1 (x-axis, 38.9% of variance) and PC-2 (y-axis, 25.8% of variance).

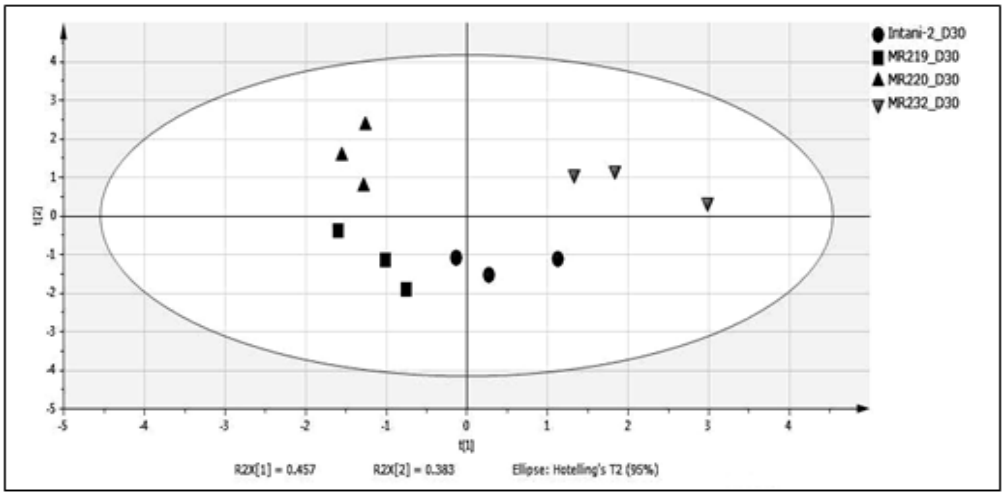


Fig. 2. PCA scatter plot of sample replicates of four rice varieties at 30 days after planting. Axes of two dimensional plots are derived from PC-1 and PC-2. PC-1 (x-axis, 45.7% of variance) distinguishes the two progeny varieties MR219 and MR220 from the other varieties MR232 and Intani-2.

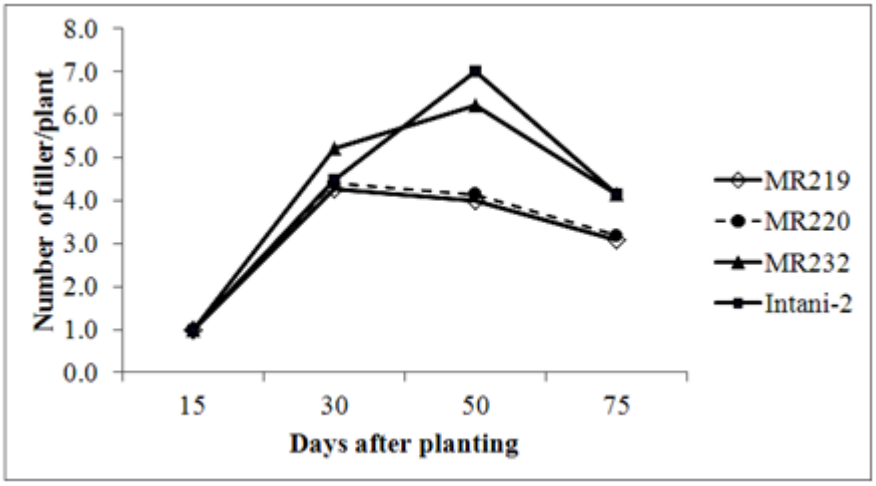


Fig. 3. Number of tillers formed after planting for varieties tested.

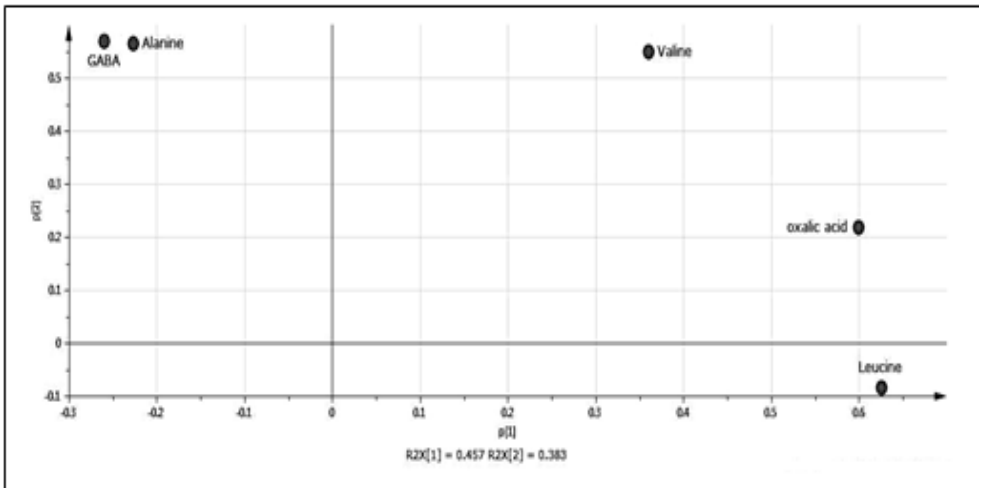
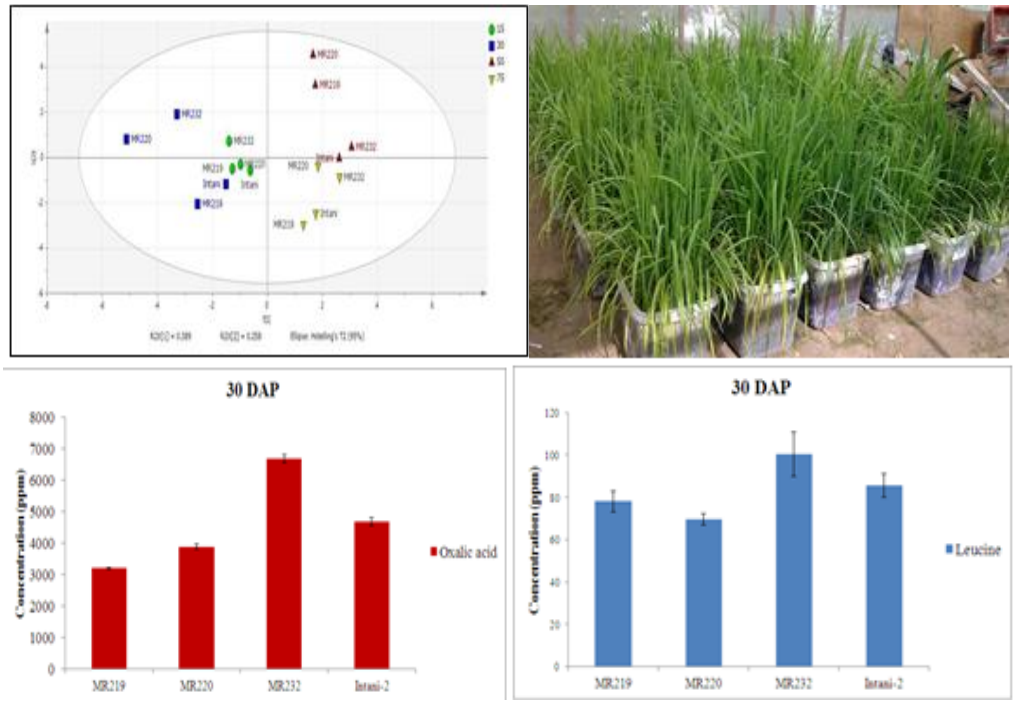


Fig. 4. PC-1 Loading plot of biomarkers at 30 days after planting.



TOC GRAPHIC

Conclusion

In conclusion, it can be seen that a relatively small set of metabolites can be used as a distinguishing parameter between varieties as well as potentially predicting tillering ability earlier than phenotypic divergence. High levels of

leucine and oxalic acid appear to be associated with good tillering ability. This study further highlights the potential for metabolite biomarkers to be used as early predictors of useful traits in crop breeding programmes.

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References

Anastasiadi, M., Zira, A., Magiatis, P., Haroutounian, S., Skaltsounis, A. L. and Mikros, E. (2009). H-1 NMR based metabolomics for the classification of greek wines according to variety, region and vintage. Comparison with HPLC data. . Journal of Agricultural and Food Chemistry 57:11607-11618.

Jacobsen, M., Mattow, J., Repsilber, D. and Kaufmann, S.H. (2008). Novel strategies to identify biomarkers in tuberculosis. Biological Chemistry 89:487-495.

Jafuel, S. and Dauzat, J. (2004). Synchronism of leaf and tiller emergence relative to position and to main stem development stage in a rice cultivar. Annals of Botany 95: pp.12.

Kariali, P.K.M.a.E. (2008). Time of emergence determines the pattern of dominance of rice tillers. Australian Journal of Crop Science 1:53-62.

Kim, J.Y. K., Park, J. Y., Kim, O.Y., Ham, B.M., Kim, H.J., Kwon, D.Y., Jang, Y., and Lee, J. H. (2010). Metabolite profiling of plasma in overweight/obese and lean men using ultra performance liquid chromatography and Q-TOF mass spectrometry (UPLC-Q-TOF MS). Journal of Proteome Research 9, 4368-4375.

Kim, Y.K., Xu, H., Park, N.I., Boo, H.O., Lee, S.Y. and Park, S. U. (2009). Amino acid and GABA contetnt in different cultivars of Momordica charantia L. J. Journal of Medicinal Plant Research 3, 897-911.

López-Bucio, J., Nieto-Jacobo, M.a.F., Ramírez-Rodríguez, V. and Herrera-Estrella, L. (2000). Organic metabolism in plants: from adaptive physiology to transgenic varieties for cultivation in extreme soils. . Plant Science 160:1-13.

Nuruzzaman, M. N., Yamamoto, Y., Nitta, Y., Yoshida, T. and Miyazaki, A. (2000). Varietal differences in tillering ability of fourteen Japonica and Indica rice varieties. Soil Science Plant Nutrition 46:381-391.

Ritota, M., Marini, F., Sequi, P. and Valentini, M. (2010). Metabolomic characterization of Italian sweet pepper (Capsicum annum L.) by means of HRMAS-NMR spectroscopy and multivariate analysis. Journal of Agricultural Food Chemistry 8:9675-9684.

Sakamoto, T. and Masuoka, M. (2004). Generating high-yielding varieties by genetic manipulation of plant architecture. Current Opinion in Biotechnology 15:144-147.

Schauera, N.S., Steinhausera, D.S., Strelkovb, S., Schomburg, D., Allisonc, G., Moritzd, T., Lunggrend, K., Tunalie, U. R., Forbese, M. G., Willmitzera, L., Fernie, A.R. and Kopka, J. (2005). GC-MS libraries for the rapid identification of metabolites in complex biological samples. . FEBS Letters 579:1332-1338.

Stenfath, M., Strehmel, N., Peters, R., Schauer, N., Groth, D., Hummel, J., Steup, M., Selbig, J., Kopka, J., Geigenberger, P. and Dongen, J.T.v. (2010). Discovering plant metabolic biomarkers for phenotype prediction using an untargeted approach. . Plant Biotechnology Journal 8:900-910.

Tarpley, L., Duran, A., Kebrom, T. and Sumner, L. (2005). Biomarker metabolites capturing the metabolite variance present in a rice plant developmental period. BMC Plant Biology 5:8-20.

Thomas, M.O. and Pedersen, D. (1998). The Molecular Basis of Quantitative Genetic Variation in Central and Secondary Metabolism in Arabidopsis. Genetics 149:739-747.

Touraine, B., Grignon, N.G. and Grignon, C. (1988). Charge balance in NO3- fed soybean. Estimation of K+ and carboxylate circulation. Plant Physiology 88:605-612.

Wang, F., Cheng, F.m. and Zhang, G.p. (2007). Difference in grain yield and quality among tillers in rice genotypes differing in tillering capacity. Rice science 12:135-140.

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