Supporting information for

Indigenous proline is a two dimension safety-relief valve in balancing specific amino acids in rice under hexavalent chromium stress

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2.3 Quantification of amino acids in rice

1. Reagents and standards

Optima LC–MS-grade acetonitrile, methanol, and HPLC grade ammonium acetate and sodium acetate were purchased from Fisher Scientifific. The standard solution of amino acids were purchased from Sigma–Aldrich. Ultrapure water was prepared using a Milli-Q system from Millipore.

2. Preparation of standard solutions and samples

Standard solution series of 0.1, 0.5, 1.0, 2.5, 5.0, 10.0, 25.0, 50.0, 100.0 ng L^{-1} were prepared. The external standards were prepared in ultrapure water with 0.1% hydrochloric acid aqueous solution. The linear equation, correlation coefficient, and LOD conducted by external standard method were presented in **Table S1**. After Cr(VI) exposure, rice tissue samples were respectively collected from treated and controlled seedlings, and then preserved in liquid nitrogen before freeze drying. 50 mg samples were weighed after liquid nitrogen grinding. Each sample was added 1 mL precooling methanol/acetonitrile/water (2:2:1, v/v/v), vortex blending (US, MET VXT-200), low-temperature ultrasound 30 min, incubation with -20℃ for 1 h to deposition of proteins. High-speed low-temperature centrifuge (Eppendorf 5430R) was set to 4℃, and 14000 RCF for 20 min, then take the supernatant, and vacuum drying. For mass spectrometry detection, 100 μL acetonitrile-water solution (1:1, v/v) was added for resolution, and the supernatant was centrifuged at 4℃, 14000 RCF for 20 min. The supernatant was filtered through 0.22 μm membrane and diluted twice before sample analysis.

Table S1 The linear equation, correlation coefficient, and LOD of amino acids.

NO.	Amino acids	Linear equation	Linear range (ng/L)	R^2	LOD (ng/L)
1	proline	y = 0.01343 x + 0.11943	0.1-100	0.9983	0.030
2	glutamate	y = 0.03074 x + -0.08163	0.1-100	0.9980	0.014
3	arginine	y = 0.01895 x + 0.32140	0.1-100	0.9997	0.032
4	ornithine	y = 0.13129 x + 0.59443	0.1-100	0.9998	0.013

3. Instrument conditions

The chromatographic separation and identifification were carried out by Agilent 1290 Infinity Acquity UPLC system. UHPLC separation was performed with Zic-HILIC 3.5μm, 2.1 mm×150 mm chromatographic column. The

gradient mobile phases consisted of acetonitrile (eluent A) and 25 mM ammonium acetate aqueous solution adjusted to pH 6.8 with acetic acid (eluent B). The gradient elution was conducted with the following conditions: 0–2 min, 2% A; 2– 6 min, 2–10% A; 6–10 min, 10% A; 10–16 min, 10–20% A; 16–22 min, 20% A; 22–25 min, 20–30% A; 25–26 min, 30% A; 26–29 min, 30–60% A; 29–33 min, 60% A, followed by washing and reconditioning of the column. The flow rate was 0.25 mL/min, the column temperature was maintained at 40◦C, and the injection volume was 2 μL. A 5500 QTRAP mass spectrometer (AB SCIEX) was used for mass spectrometry in positive ion mode. The collection mode is multi-response monitoring (MRM) mode. 5500 QTRAP ESI Source is as follows: Temperature: 500℃; Ion Source Gas1(Gas1): 40; Ion Source Gas2(Gas2): 40; Curtain gas(CUR): 30; Ion Sapary Voltage Floating (ISVF): 5500 V; MRM mode was used to detect the ion pairs to be measured. The relative molecular weights of the four compounds and the optimized parameters of mass spectrometry were presented in **Table S2**.

Table S2 The relative molecular weights of the four compounds and the optimized parameters of mass spectrometry

NO.	Compund	Retention Time (min)	Precursor Ion (m/z)	Product Ion (m/z)
1	proline	8.94	120.0	74.0
2	glutamate	12.40	148.1	84.1
3	arginine	17.00	175.1	70.0
4	ornithine	17.42	133.1	70.1

4. Recovery rate, precision and detection limit

The standard mixture solution was added to the treated samples, and three samples at the standard level of 0.50, 10, and 100 ng L-1 were prepared. Three parallel samples were set at each concentration and was determined by the above mentioned method. The average recovery rate and relative standard deviation were shown in **Table S3**.

Table S3 Recovery rate and relative standard deviation test

Compounds	Original（ng L^{-1}）	Spiked level（ng L^{-1}）	Found（ng L^{-1}）	Recovery（%）	RSD（%）
proline	125.2	0.5	0.48	96.0	8.7
		10	11.6	116.0	5.6
		100	118.2	118.2	7.1
glutamate	441.8	0.5	0.42	84.0	5.8
		10	8.7	87.0	7.1
		100	117.3	117.3	4.6
arginine	390.3	0.5	0.56	112.0	7.1
		10	11.4	114.0	5.2
		100	109.8	109.8	5.3
ornithine	80.4	0.5	0.56	112.0	6.8
		10	11.3	113.0	4.5
		100	108,4	108.4	5.3

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2.4 RNA extraction and RT-qPCR analysis

The RT-qPCR cycling conditions are as follows: 1) denaturation at 95°C for 10 s; 2) annealing at 58°C for 30 s; and 3) extension at 72°C for 32s. This cycle was repeated 40 times. Use of 7500 Fast Real-Time PCR system (Applied Biosystems) and SYBR green chemistry was performed for RT-qPCR analysis. *OsGAPDH1* (rice glyceraldehyde-3-phosphate dehydrogenase, LOC_Os08g03290.1) was selected as the house-keeping gene.

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2.5 Enzymatic activity assays

2.5.1 Measurement of P5CS activity

Rice tissues (0.25 g) were used for crude enzyme extraction. The amount of γ-glutamyl hydroxamate complex produced was estimated from the molar extinction coefficient 250 mol^{-1} cm^{-1} reported for Fe^{3+} hydroxamate complex of the compound. The activity was expressed in U mg^{-1} protein, which represents the amount of enzyme required to

produce 1 μM γ-glutamyl hydroxamate min^{-1}.

2.5.2 Measurement of P5CR activity

Rice tissues (0.25 g) were used for crude enzyme extraction. The activity was determined with the assumption of an extinction coefficient for NADH of 6,220 M^{-1} cm^{-1}. P5C was synthesized by the periodate oxidation of δ-allo-hydroxylysine and purified by cation-exchange chromatography.

2.5.3 Measurement of P5CDH activity

Rice tissues (0.25 g) were homogenized into powder in liquid N2 and mixed with 0.5 mL extraction buffer (50 mM potassium phosphate buffer [pH 8.0] containing 10 mM $MgCl_2$, 1 mM PMSF, 10 mM β-mercaptoethanol, and 1 mM pyridoxal phosphate). After centrifugation at 12,000 3 g for 10 min at 4□C, the supernatant was used to measure P5CDH activity. Protein content in crude enzyme was adjusted to 0.2 mg/mL using a modified bradford protein assay kit (Sangon Biotech). The reaction was started by adding 0.6 mM NAD^+ to a mixture containing 80 mM potassium phosphate buffer (pH 8.0), 0.8 mM P5C, 0.8% Triton X-100 (v/v), and 80 mL crude enzyme in a final volume of 200 mL. A unit of P5CDH is equal to 1 nM NADH formation min^{-1} as calculated from the extinction coefficient for 1 mg NADH, 6.2 M^{-1} cm^{-1}, and its activity was expressed as U mg^{-1} protein. P5C was synthesized as in the P5CR activity assay.

2.5.4 Measurement of ProDH activity

Rice tissues (0.25 g) were homogenized into powder in liquid N2 and mixed with 0.5 mL extraction buffer (100 mM sodium phosphate, 1 mM cysteine, and 0.1 mM EDTA [pH 8.0]). After centrifugation at 12,000 3 g for 10 min at 4□℃, the supernatant was used to measure PDH activity. Protein content in crude enzyme was adjusted to 0.2 mg/mL using a modified bradford protein assay kit (Sangon Biotech). 80 mL crude enzyme was incubated in the reaction buffer (100 mM Na_2CO_3-$NaHCO_3$, 10 mM NAD, and 20 mM L-proline [pH 10.3]) in a 200-mL reaction, the reaction was started at 32□℃ by adding 0.6 mM NAD^+ to the mixture, and then PDH-dependent NAD reduction

was monitored at 340 nm for 4 min at 1-min intervals. A unit of PDH activity was defined as an absorbance change of 0.001 min^{-1}, and its activity was expressed as U mg^{-1} protein.

2.5.5 Measurement of OAT activity

Rice tissues (0.25 g) were used for crude enzyme extraction. The OAT activity was assayed following the measurement of P5C amount produced in 30 min using the ninhydrin method. A unit of OAT was defined as the micromolar P5C produced mg^{-1} of protein hr^{-1}.

2.5.6 Measurement of ARG activity

Crude rice tissues proteins were extracted by grinding leaf material in 100 mm Tris-HCl (pH 7.5), 1 mm EDTA and 0.1 mm PMSF. The homogenate was centrifuged at 20,000 g for 10 min at 4 ℃ and the resulting supernatant was used as enzyme source. The reaction mixture of 80 µL contained 17 µL enzyme extract, 5 mm Tris-HCl (pH 7.5), 0.75 mm MnCl2 and 250 mm l-arginine. The reactions were carried out at 37 ℃ for 60 min and stopped by addition of 320 µL acid mix [1:3:7 (v:v:v) H2SO4:H3PO4:H2O]. The produced urea was quantified by addition of 5 µL 9% a-isonitrosopropriophenone (Sigma) in ethanol and heated for 30 min at 95 ℃. After incubation in the dark for 10 min, the OD_{540} was determined. The produced urea was calculated from a standard curve ranging from 0 to 100 mM urea. One unit enzyme activity was defined as the amount of enzyme that catalyses the formation of 1 µM urea per minute. The concentration of proteins was determined following the method of Bradford (1976) using a BSA standard curve from 1 to 10 $\mu L \cdot ml^{-1}$.

Reference:

Bradford M.M. (1976) Rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. Analytical Biochemistry, 72, 248–254

Table S4 Sequence of forward and reverse primers used in gene expression analysis

Enzyme	Gene symbol	MSU ID	Subcellular location	Primer sequences(5'-3')	Amplicon size (bp)
Δ 1-pyrroline-5-carboxylate synthetase（P5CS）	*OsP5CS1*	LOC_Os05g38150.1	Cytoplasm	F-AGAATGCGAATTTGTGGGAATC R-GCTTCTCATATCCAGCAACTTG	216
	OsP5CS2	LOC_Os01g62900.1	Cytoplasm	F-GAACACCGACTCCACCCG R-TGCCTCCCAACACCAACA	198
Arginase(ARG)	*OsARG*	LOC_Os04g01590.1	Mitochondrion	F-GGAAGGTGTGAAGGGAGTGT R-CTGCAACCATAGCCGTCATC	212
Ornithine δ-aminotransferase(OAT)	*OsOAT*	LOC_Os03g44150.1	Mitochondrion	F-CTGGAGCTGAAGGAGTGGAA R-TCCAACCCATCAGTGTCTCC	226
Pyrroline-5-carboxylate reductase(P5CR)	*OsP5CR*	LOC_Os01g71990.1	Chloroplast	F-CATGGCTGATGGTGGAGTTG R-AACGGCATTTATCAGCGTCC	214
Proline dehydrogenase (ProDH)	*OsProDH*	LOC_Os10g40360.1	Mitochondrion	F-ATCGAGTCATGAGTAGAAGTCG R-CGATTGATACACCAATGTCTGT	80
Δ 1-pyrroline-5-carboxylate dehydrogenase（P5CDH）	*OsALDH12B1*	LOC_Os12g40440.1	Cytoplasm	F-TTGAGGCGAATGGAGGAGTT R-ACAACCACATCAACAGCGAG	233
	OsALDH10A5	LOC_Os04g39020.1	Peroxisome	F-GGCTGGGGTCAGTCGTTAGT R-CCCTTTTGAGGTGTTGTGGT	120
	OsALDH2B1	LOC_Os06g15990.1	Mitochondrion	F-TATCACCCCCAGTCCAAG R-CAAGTCAGCAAACCGCAA	248
	OsALDH10A8	LOC_Os08g32870.1	Peroxisome	F-TCGGAAAGAGCCTATCGGTG R-AAGCTCCAAACAAGTCACGG	151
mitochondrial carrier protein	*OsBAC1*	LOC_Os11g23170.2	Chloroplast	F-TACCTTCTGCTGCCTGTAGT R-GACCACAAACCCCTTCACTT	168
Amino acid transporter	*OsProT1*	LOC_Os01g68050.1	Plasma Membrane	F-GTCAACCTCTTCGGCTCCTT R-GTGCCAAATCCTGTTCCATC	114
	OsProT2	LOC_Os03g44230.1	Plasma Membrane	F-GGGGAGGAGGAGAGGGAG R-CAATGCTAATCTGGTGCG	106
	OsProT3	LOC_Os07g01090.1	Plasma Membrane	F-TCCCATTCCTTGGCGACTTC R-GAAGCCAGCAACATTGAGCC	152
NADH dehydrogenase subunit	*OsNDS1*	LOC_Os01g66000.1	Chloroplast	F-GAGACCACCCTCACCAAGAT R-GAGAACCTTGGCTTCCATGA	159
	OsNDS2	LOC_Os04g24410.1	Mitochondrion	F-CTCGCACCGCCTATCTCTAC R-GGGGAGAACTTAGCGAGGTC	186
Pyridine	*OsNDA1*	LOC_Os07g37730.1	Mitochondrion	F-CCGAGAACGCCATCTTCCTC	319

Enzyme	Gene symbol	MSU ID	Subcellular location	Primer sequences(5'-3')	Amplicon size (bp)
nucleotide-disulphide oxidoreductase				R-TACTTGGAGAGGTGGTCCGT	
NDH-dependent cyclic electron flow	*OsNDF1*	LOC_Os08g17390.1	Chloroplast	F-GAAGCACAGGGAAGAGGTTG R-AGCCCTTCTTCTCCTCCAAG	227
	OsNDF6	LOC_Os02g51020.1	Chloroplast	F-GCTGCATTCTGTAATACCGTAC R-CTTTCCTTTTGAGTCTGATGCC	99
FAD dependent oxidoreductase domain containing protein	*OsNPB*	LOC_Os06g11140.1	Chloroplast/Mitochondrion	F-AAAGGCCATCCTCGAACTTT R-CGCAGCATCACTTCTACCAA	210
	OsETFQO	LOC_Os10g37210.1	Mitochondrion	F-CCTGACCATGAAGCAACAGA R-GATCCCTCAAACGGAGATGA	154
Electron transfer flavoprotein subunit beta	*Osb-ETF*	LOC_Os04g10400.1	Cytoplasm	F-CAACCAAACAGGACAAATGCTA R-CAAATCCAAGCTAATGGTCTCG	139

Table S5 The correlation coefficients between Pro and Cr(VI) accumulation under different N source conditions.

Exposure duration	**Cr(VI)-N**		**Cr(VI)+NO_3^-**		**Cr(VI)+NH_4^+**	
	shoots	**roots**	**shoots**	**roots**	**shoots**	**roots**
R^2 of 2-d exposure	0.004	0.269	0.926	0.572	0.572	0.654
R^2 of 4-d exposure	0.001	0.534	0.947	0.980	0.980	0.444

Table S6 The ratios of Pro to Glu in rice seedlings under Cr(VI) stress in the presence of different N sources

Rice tissues	Cr(VI) conc.	2-d exposure			4-d exposure		
		Cr(VI)-N	Cr(VI)+NO_3^-	Cr(VI)+NH_4^+	Cr(VI)-N	Cr(VI)+NO_3^-	Cr(VI)+NH_4^+
shoot	0 mg/L	0.908	0.161	0.723	0.942	0.188	1.091
	2 mg/L	1.376	0.171	0.467	1.009	0.318	0.558
	8 mg/L	1.315	0.239	0.518	1.059	0.387	0.738
	16 mg/L	1.136	0.277	0.591	0.857	0.439	0.549
root	0 mg/L	0.224	0.227	0.736	0.261	0.209	0.609
	2 mg/L	0.217	0.227	0.209	0.170	0.230	0.189
	8 mg/L	0.195	0.210	0.207	0.133	0.218	0.187
	16 mg/L	0.172	0.186	0.196	0.140	0.212	0.178

Table S7 The ratios of Pro to Orn in rice seedlings under Cr(VI) stress in the presence of different N sources

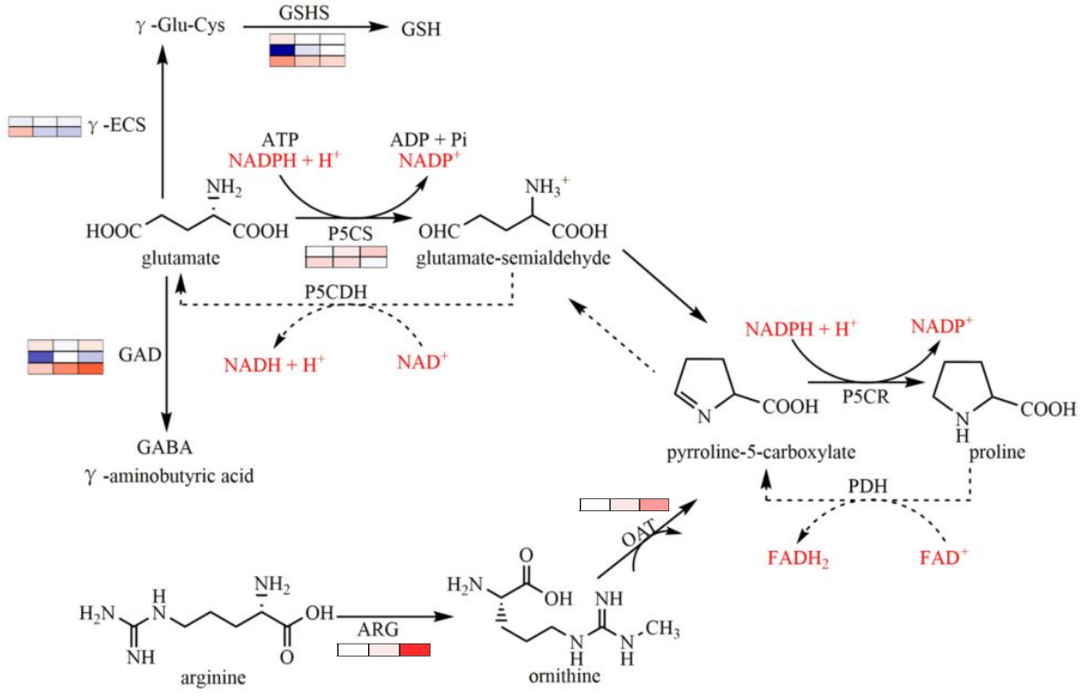
Rice tissues	Cr(VI) conc.	2-d exposure			4-d exposure		
		Cr(VI)-N	Cr(VI)+NO_3^-	Cr(VI)+NH_4^+	Cr(VI)-N	Cr(VI)+NO_3^-	Cr(VI)+NH_4^+
shoot	0 mg/L	127.619	17.191	0.526	298.135	14.589	2.469
	2 mg/L	479.981	26.065	1.480	1379.294	29.966	3.754
	8 mg/L	370.455	28.504	2.729	241.928	36.646	6.098
	16 mg/L	237.472	31.610	3.852	361.792	33.279	7.299
root	0 mg/L	19.512	20.230	14.324	24.678	30.825	11.805
	2 mg/L	28.228	26.153	45.780	48.764	24.037	35.260
	8 mg/L	26.037	27.416	46.082	55.318	30.344	32.647
	16 mg/L	36.584	30.478	49.684	49.922	17.900	29.415

Table S8 The residual of Glu in both roots and shoots of rice seedling under Cr(VI) stress in the presence of different N sources.

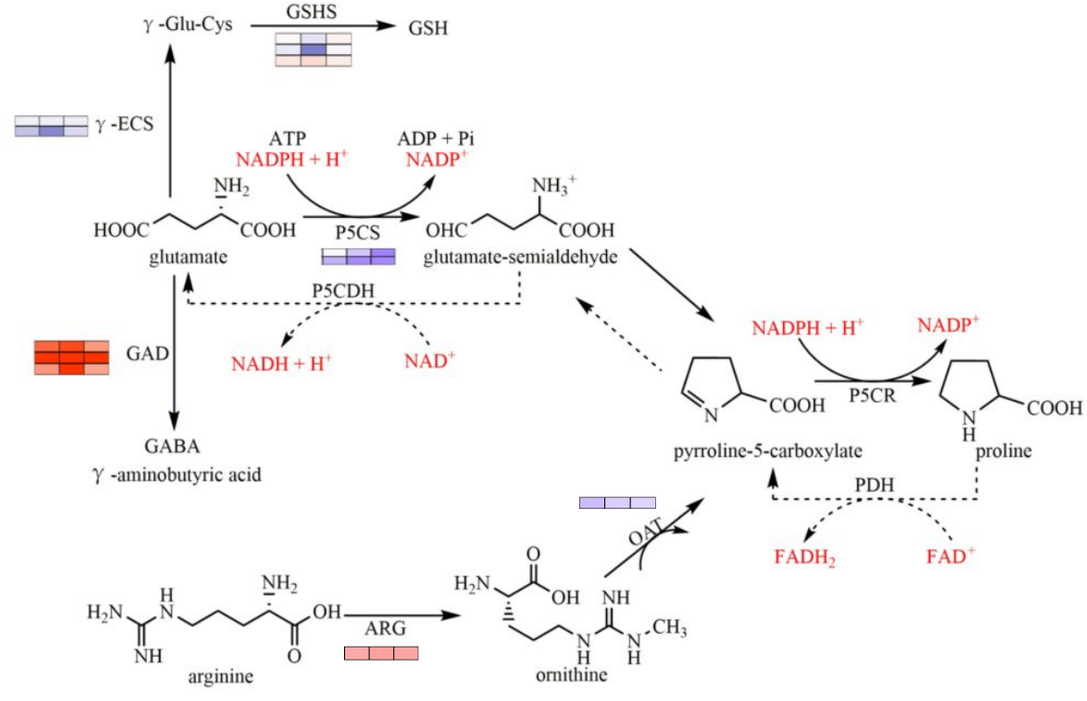
Rice tissues	Cr(VI) conc.	2-d exposure			4-d exposure		
		Cr(VI)-N	Cr(VI)+NO_3^-	Cr(VI)+NH_4^+	Cr(VI)-N	Cr(VI)+NO_3^-	Cr(VI)+NH_4^+
shoot	0 mg/L	29.74	831.99	140.72	16.31	732.52	-27.22
	2 mg/L	-68.14	762.09	431.03	-2.17	380.81	324.56
	8 mg/L	-68.37	604.62	387.03	-14.91	374.4	148.11
	16 mg/L	-36.15	571.45	278.96	43.45	336.37	336.69
root	0 mg/L	545.87	278.71	67.93	426.55	352.81	136.99
	2 mg/L	548.61	210.43	938.77	774.86	237.79	909.01
	8 mg/L	576.81	263.05	856.76	842.69	241.06	859.73
	16 mg/L	742.03	298.13	929.62	710.61	238.19	929.47

Table S9 The genes involving in GABA and GSH synthesis in rice plants

Enzyme	Gene locus	Gene name	Subcellular localization
γ -glutamylcysteine synthetase (γ -ECS)	LOC_Os07g27790	OsSG1	Chloroplast
	LOC_Os05g03820	OsECS	Chloroplast
Glutathione synthase (GSHS)	LOC_Os11g42350	OsGS1	Chloroplast
	LOC_Os12g16200	OsGS2	Chloroplast
	LOC_Os12g34380	OsGS3	Cytoplasm
Glutamate decarboxylase (GAD)	LOC_Os08g36320	OsGAD1	Cytoplasm
	LOC_Os04g37500	OsGAD2	Cytoplasm
	LOC_Os03g13300	OsGAD3	Cytoplasm



（a）



（b）

Fig. S1 Response of these genes involving in GABA and GSH synthesis in rice seedlings under Cr(VI) stress in the presence of NO_3^-. (a) expression of GABA and GSH synthesis related genes in rice shoots; (b) expression of GABA and GSH synthesis related genes in rice roots.