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Evaluation of Metabolite Alteration under Flooding Stress in Soybeans

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Abstract

Soybean is a crop known to be susceptible to flooding and enhancing flooding tolerance may be a workable strategy to improve soybean production. To elucidate the effects of flooding on soybean metabolism, metabolite alterations in seedlings during flooding treatment were identified using capillary electrophoresis-mass spectrometry (CE/MS). The principal component analysis (PCA) of soybean seedlings revealed that the first component accounted for 62.2% of total variance, and the alteration of metabolites in control and flooding treatments appears to be separated by this component. Furthermore, comparison of the metabolic loading scores in the first component of PCA show that the significant metabolites for the first component were alanine (Ala), gamma-aminobutyric acid (GABA), citrate, fumarate and malate. Quantitative analysis revealed that the total soluble sugar content of seedlings in both control and flooding treatments had declined and was lower in the latter than the former. Phosphoenolpyruvate, pyruvate and lactate, which belong to glycolytic and fermentation pathways, increased transiently, but decreased 3 to 4 days after treatment. Citrate, 2-oxoglutarate, succinate, fumarate, Ala, and GABA, which are related to the TCA cycle and amino acid metabolism, accumulated during flooding treatment. These results suggest that metabolism associated with the TCA cycle, the Ala synthetic pathway, and the GABA shunt may be strongly influenced by flooding during soybean germination.

Discipline: Agricultural environment / Soil fertilizers and plant nutrition
Additional key words: capillary electrophoresis - mass spectrometry (CE/MS), flooding, metabolomic analysis, soybean seedlings

Introduction

Soybean has been cultivated as an alternative crop in rice fields in many areas of Japan. However, there have been many reports of constraints in maintaining soybean yields and flooding injury in particular during the early growth stages has been recognized as a major constraint to soybean establishment and production[17,28,36].

Oxygen deprivation by flooding is one of the factors reducing plant growth under flooding because flooding often interferes with oxygen and carbon dioxide exchange between plants and their aerial aerobic environment[3,13,62]. Accordingly, plants have adapted to hypoxic stress by activating anaerobic metabolism pathways. Under hypoxic conditions, cells relying on external oxygen limit energy-hungry processes and alter their metabolism to increase the anaerobic generation of ATP by glycolysis[10]. This shift is followed by the fermentation of pyruvate into major end products, ethanol and lactate, yielding NAD^+ to sustain anaerobic metabolism. Conversion

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from pyruvate to acetaldehyde by pyruvate decarboxylase and from acetaldehyde to ethanol by alcohol dehydrogenase is essential for ethanol fermentation during flooding and oxygen deprivation[10,15,24].

Besides the major fermentation end products, lactate and pyruvate, oxygen deficiency is associated with the elevation of alanine (Ala), GABA, succinate, and occasionally malate[9,10,15,49,59,61]. Extrapolation suggested that a high rate of fermentation increases the demand for carbohydrates and that the supply of carbohydrates would be important for survival under prolonged hypoxic conditions, because (1) the exogenous supply of sugars improves the survival of flood-sensitive plants[30,41,63], (2) hypoxic stress activates glycolytic enzymes[29,30,50], and (3) inhibition of the synthesis of glycolytic enzymes decreases flooding tolerance[57].

Soybean is generally intolerant of flooding stress. Flooding injury of soybean seeds before radicle protrusion, namely during seed imbibition, is caused by physical disruption of the rapid uptake of water and can be alleviated by using seeds with high moisture content[37]. The causes of flooding injury after radicle protrusion in the soybean, however, have not been well elucidated to date. In our previous report[21],the growth of soybean seedlings under flooding conditions was similar to that under anoxia, and the level of protein related to fermentation was greatly upregulated during flooding. Furthermore, Hashiguchi et al.[16] and Nanjyo et al.[38] demonstrated that flooding of soybean seedlings affected the expression of certain proteins, involved not only in fermentation, but also glycolysis, suggesting that glycolytic and fermentative metabolism were also affected by flooding stress during germination. However, the influence of flooding stress on metabolites related to fermentation and glycolysis remains unclear, although the expression of these proteins was affected by flooding stress. Recently, transcriptome studies revealed that hypoxia and flooding stress affected the expression of genes related to carbon metabolism, nitrogen metabolism, cell wall formation, and signal transduction, as well as transcription factors[1,19,23,39]. These reports suggested that many metabolites are affected by flooding stress, making it necessary to elucidate the changes in numerous metabolites using a metabolomics technique which may enable a comprehensive understanding of plant response to flooding.

Metabolomics analysis has emerged in recent years as a promising technology to identify metabolic networks in living cells[11,40]. Metabolomics studies have progressed, especially using gas chromatography-mass spectrometry (GC/MS), following enormous efforts to develop methodological standards and informing numerous metabolites in plants[11,46]. Other methodological applications have also been introduced, including Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR/MS), liquid chromatography-mass spectrometry (LC/MS), and nuclear magnetic resonance (NMR). Furthermore, capillary electrophoresis-mass spectrometry (CE/MS) has been developed to detect charged compounds such as carboxylic acids, amino acids and nucleotides[48,53,54,55,58]. Metabolites related to anaerobic metabolism, which may be important in flooding conditions, are mainly charged compounds, whichCE/MS can measure. In this study, using CE/MS, metabolites that respond to flooding stress in soybean seedling tissues, except for cotyledons, were identified to evaluate their common metabolism under flooding stress.

Materials and methods

1. Growth conditions and treatment

Soybean seeds (*Glycine max* L. cv. Enrei) were germinated in silica sand and grown until the 6th day after germination in a growth cabinet under illuminated conditions (600 μmol m^{-2} s^{-1}, 12-h light period/day) at 25°C and 70% relative humidity in a growth chamber. Two days after germination, the seedlings were flooded for 4 days and samples were collected daily during the experiment, which was performed in triplicate. Seedlings were separated into hypocotyls and roots after the cotyledons had been removed. To measure the metabolites, hypocotyls and roots were also frozen in liquid nitrogen, lyophilized, and stored at -80°C until analysis.

2. Metabolite analysis

Quantification of metabolites was performed using the method described by Takahashi et al.[58], Nakamura et al.[35] and Miyagi et al.[32]. Hypocotyls and roots were ground in liquid nitrogen and added to ice-cold 50% methanol (20 μL mg^{-1} fresh weight) containing internal standards (100 μmol L^{-1} each L-methionine sulfone and piperazine-1,4-bis(2-ethanesulfonic acid)). After centrifugation at 15,000 × g for 5 min, the supernatant was filtered through a 5-kDa cutoff filter (Millipore, Bedford, MA, USA) and used for analysis.

The CE/MS system and conditions were as described by Takahashi et al.[58], Nakamura et al.[35] and Miyagi et al.[32]. The metabolites were separated and detected by a CE/MS system using Agilent ChemStation software (Agilent Technologies, Waldbronn, Germany). Anionic compounds were determined by separation at -20 kV on a polyethylene glycol-coated capillary (DBWAX, J&W Scientific, Folsom, CA, USA, 100 cm × 50 μm i.d.) with a running buffer containing 20 mM ammonium acetate (pH 6.8) at 20°C. The sheath liquid composed of 5 mM

ammonium acetate in 50% (v/v) methanol was applied to the capillary at 6 μL min^{-1} using an Agilent 1100 series isocratic HPLC pump equipped with a 1:100 splitter to stabilize MS analysis. To analyze cationic compounds, the samples were injected into an uncoated fused silica capillary (90 cm × 50 μm i.d.) with 1 M formic acid (pH 1.9) as a running buffer and 0.1% formic acid in 50% (v/v) methanol as a sheath liquid. The applied voltage was set to 20 kV. Before injection of each sample, the capillary was equilibrated for 5 min with the running electrolyte. The sample was injected with a pressure injection of 50 mbar for 30 s (~30 nL) to analyze the anionic compounds and for 3 s (~3 nL) to analyze the cationic compounds. MS analysis for anionic compounds was performed in negative ion mode, and for cationic compounds, in positive ion mode. The capillary voltage was ±3500 V and the flow of the drying nitrogen gas (adjusted to 320°C) was 8 μL min^{-1}. The concentration of each compound was determined by measurement of the known concentrations of standard compounds using Agilent ChemStation software.

3. Sugar content

Total soluble sugars in the ethanol soluble fractions were determined using the method of Irigoyen et al.[18]. A portion (0.3 g) of root and hypocotyl was crushed in 5 mL of 95% (v/v) ethanol, whereupon the insoluble fraction of the extract was washed with 5 mL of 70% ethanol. All soluble fractions were centrifuged at 3,500 × g for 10 min. The supernatants were used to determine the soluble sugars. The extract (0.1 mL) was mixed with 3 mL of freshly prepared anthrone reagent (150 mg anthrone in 100 mL of 72% sulfuric acid) and boiled for 10 min. The reaction was terminated on ice, the absorbance at 625 nm measured by a spectrophotometer (DU700; Beckman, Fullerton, CA, USA), and the soluble sugar concentration calculated using glucose as standard.

4. Statistical analysis

PCA was performed as usual using Pirouette software (Infometrix, Woodinville, WA, USA) with mean-center preprocessing. Data were visualized using the principal component score and loading plots. Each point on the scores plot represents an individual sample, and each point on the loading plot represents the contribution of an individual metabolite to the score plot. Biochemical components responsible for the differences between samples detected in the scores plot can accordingly be extracted from the corresponding loadings.

Results and discussion

Root growth of soybean greatly decreases under flooding conditions[16,20,47,52]. This inhibition is one of the most common responses observed in higher plants to flooding stress and is likely related to the energy available from oxygen. The depression of energy-demanding processes including transportation, lipid metabolism and secondary metabolism is a known as a key oxygen-saving strategy, since it decreases oxygen consumption in cells[5,13,14,45]. Furthermore, oxygen concentration has been reported to influence metabolic activity in various tissues such as stems, seeds, tubers, roots and fruits[5]. This involves a global depression in many energy-demanding processes, such as lipid, protein and phenylpropanoid synthesis[13], meaning the inhibition of root growth is associated with oxygen depletion and providing evidence that root growth is linked to oxygen availability. To investigate the response mechanism of soybean to flooding stress, a proteome technique was used in our previous reports[16,20,52]. Shi et al.[52] reported that cytosolic ascorbate peroxidase2 is involved in flooding stress responses in young soybean seedlings using a proteomic technique. Hashiguchi et al.[16] suggested, via proteome analysis, that flooding stress in soybean seedlings included not only hypoxic stress, but also other stresses such as those due to weak light, disease, and water stress. Furthermore, Komatsu et al.[20] suggested that flooding stress directly affects plasma membrane proteins. Recently, in a transcriptome study[39], the expression of many genes related to photosynthesis, glycolysis, amino acid metabolism, hormone metabolism, protein degradation, metabolite transport, and cell wall metabolism was significantly affected by flooding stress, which suggested that the latter affects many areas of metabolism. However, it is unclear which metabolic pathways were strongly affected, although there was an impact on many biological processes. Thus, in this report, we evaluated the metabolic alteration of soybean under flooding conditions to clarify the metabolic pathways significantly affected by this stress.

Germinated soybean seeds were subjected to flooding for 1 to 4 days and sequential changes in metabolites of the seedlings were identified using CE/MS. Under flooding stress, 71 metabolites were identified in a time-dependent manner (Tables 1 and 2). The data for metabolite concentration were subjected to multivariate analysis. PCA converts the complex concentration data into comprehensive matrix data sets. Plots of the first and second PCA scores revealed that distinct clusters clearly corresponded to the differences in flooding treatment (Fig. 1). The first factor accounted for 62.2% of the total variance, and the data of control and flooding treatments

appears to be separated by this factor. Using loading data from the first factor, the scores of Ala, GABA, citrate (CA), isocitrate (ICA), fumarate (FA) and malate (MA) were positive and exceeded the loading score 0.1 (Fig. 2), indicating that these compounds display a high contribution in terms of the response to flooding. Sugar content decreased under both conditions, but the degree of reduction in sugar content was larger in flooding treatment than in control (Fig. 3). A previous report showed that the drop in oxygen concentration most likely caused increased fluxes through sucrose degradation and glycolysis when fermentative processes were induced[60]. Komatsu et al.[21] reported that the expression level of glycolysis and fermentation-associated proteins, UDP pyrophosphorylase, fructose-bisphosphate aldolase, and GA3P dehydrogenase were increased under flooding conditions in soybean. In this study, the amount of phosphoenolpyruvate (PEP), pyruvate and lactate, related to glycolysis and fermentation, transiently increased, but decreased 3 to 4 days after flooding (Fig. 4, Table 2). Therefore, our results also suggested that fermentative processes might be induced in flooded soybean seedlings.

Table 1. Abbreviationlist of metabolites changed under flooding stress

No.	Abbreviation	Metabolite name	No.	Abbreviation	Metabolite name
1	Ala	Alanine	37	6-PG	6-phospho-gluconate
2	Ile+Leu	Leucine+Isoleucine	38	E4P	erythrose 4-phosphate
3	GABA	γ(gamma)-aminobutyric acid	39	R5P	ribose-5-phosphate
4	Val	Valine	40	Ru5P	ribulose-5-phosphate
5	Gly	Glycine	41	RuBP	ribulose-1,5-bisphosphate
6	Ser	Serine	42	GMP	Guanosine 5’-monophosphate
7	Gln	Glutamine	43	GDP	Guanosine 5’-diphosphate
8	Glu	Glutamic acid	44	GTP	Guanosine 5’-triphosphate
9	Arg	Arginine	45	IMP	Inosine 5’-monophosphate
10	Pro	Proline	46	CMP	Cytidine-5’-monophosphate
11	Asn	Asparagine	47	UMP	Uridine 5’-monophosphate
12	Asp	Aspartic acid	48	dTDP	Deoxythymidine 5’-diphosphate
13	Lys	Lysine	49	CDP	Cytidine 5’-diphosphate
14	Met	Methionine	50	UDP	Uridine 5’-diphosphate
15	Thr	Threonine	51	CTP	Cytidine 5’-triphosphate
16	Phe	Phenylalanine	52	UTP	Uridine 5’-triphosphate
17	Tyr	Tyrosine	53	2-OG	2-Oxoglutarate
18	Trp	Tryptophan	54	ACA	cis-aconitate
19	His	Histidine	55	CA	citrate
20	Ornithine	Ornithine	56	ICA	isocitrate
21	Citrulline	Citrulline	57	FA	Fumarate
22	A	Adenine	58	SuA	Succinate
23	G	Guanine	59	MA	Malate
24	Adenosine	Adenosine	60	AcCoA	Acetyl-CoA
25	Guanosine	Guanosine	61	PEP	phosphoenol pyruvate
26	C	Cytosine	62	2,3DPG	2,3-Bisphospho-D-glycerate
27	U	Uracil	63	DHAP	dihydroxyacetone phosphate
28	T	Thymine	64	GA3P	glyceraldehyde-3-phosphate
29	Thymidine	Thymidine	65	G6P	glucose-6-phosphate
30	Cytidine	Cytidine	66	G1P	Glucose-1-phosphate
31	Uridine	Uridine	67	FBP	fructose 1,6-bisphosphate
32	Lac	Lactate	68	Cinnamate	Cinnamate
33	PA	pyruvate	69	Coumarate	p-Coumarate
34	3PGA	3-phosphoglycerate	70	Shikimate	Shikimate
35	Glycolate	Glycolate	71	Glyoxalate	Glyoxalate
36	Glycerate	Glycerate			

Table 2. Concentrationsat which metabolites changed under flooding stress

Days after germination		2	3	4	5	6
Days after flooding		0	1	2	3	4
		(μmol g^{-1}DW ± SE)				
Ala	Control	8.85 ±1.44	9.27 ±2.47	8.60 ±1.75	10.96 ±1.53	16.65 ±1.29
	Submerge		44.10 ±3.78	63.34 ±4.15	67.73 ±4.40	68.65 ±7.40
Ile+Leu	Control	7.58 ±1.43	6.93 ±1.71	6.04 ±1.23	6.99 ±1.02	12.77 ±0.21
	Submerge		14.47 ±1.28	15.25 ±1.18	11.75 ±0.69	12.79 ±1.03
GABA	Control	5.34 ±0.81	6.25 ±0.99	7.84 ±2.22	7.34 ±0.97	8.17 ±0.19
	Submerge		21.46 ±3.80	55.22 ±7.71	34.98 ±3.00	53.51 ±9.50
Val	Control	9.26 ±3.40	12.51 ±4.43	9.83 ±1.57	11.89 ±2.27	21.38 ±3.47
	Submerge		10.85 ±1.39	17.97 ±3.94	12.09 ±1.49	16.65 ±1.89
Lac	Control	0.68 ±0.28	0.76 ±0.15	1.26 ±0.16	0.48 ±0.20	0.30 ±0.10
	Submerge		5.62 ±1.53	5.29 ±0.97	0.63 ±0.27	0.94 ±0.50
PA	Control	0.94 ±0.24	0.77 ±0.14	1.11 ±0.29	0.97 ±0.19	0.65 ±0.26
	Submerge		1.76 ±0.46	2.36 ±0.38	1.19 ±0.24	1.30 ±0.15
Gly	Control	0.84 ±0.14	1.12 ±0.23	1.28 ±0.05	1.32 ±0.26	2.25 ±0.29
	Submerge		6.28 ±2.56	8.06 ±2.61	3.40 ±0.32	4.74 ±0.49
Ser	Control	8.06 ±0.77	9.38 ±1.68	9.75 ±2.05	10.01 ±1.16	14.40 ±0.89
	Submerge		9.85 ±1.34	13.29 ±1.10	15.99 ±1.61	15.81 ±2.00
3PGA	Control	0.33 ±0.16	0.25 ±0.16	0.26 ±0.09	0.15 ±0.07	0.27 ±0.02
	Submerge		0.28 ±0.15	0.51 ±0.17	0.37 ±0.22	0.76 ±0.18
Glycolate	Control	0.50 ±0.15	0.46 ±0.17	2.58 ±0.79	0.77 ±0.61	0.30 ±0.17
	Submerge		1.66 ±0.84	4.08 ±3.18	0.30 ±0.16	0.79 ±0.28
Glycerate	Control	0.18 ±0.04	0.17 ±0.03	0.10 ±0.06	0.16 ±0.03	0.13 ±0.01
	Submerge		0.13 ±0.04	0.20 ±0.04	0.09 ±0.02	0.13 ±0.03
Gln	Control	1.14 ±0.24	1.37 ±0.28	1.42 ±0.18	1.33 ±0.06	1.73 ±0.22
	Submerge		2.77 ±0.68	3.22 ±0.74	2.04 ±0.15	2.16 ±0.27
Glu	Control	5.56 ±0.45	3.50 ±0.33	3.49 ±0.51	3.14 ±0.12	2.59 ±0.09
	Submerge		10.95 ±1.94	9.11 ±1.20	12.49 ±1.54	11.09 ±1.59
Arg	Control	3.38 ±0.86	2.33 ±1.19	2.26 ±0.91	1.26 ±0.14	1.93 ±0.26
	Submerge		8.36 ±3.61	5.89 ±1.43	3.55 ±0.87	3.25 ±1.02
Pro	Control	2.10 ±0.56	8.45 ±6.08	11.70 ±7.04	4.63 ±0.54	5.53 ±1.06
	Submerge		8.43 ±2.13	9.03 ±0.57	6.82 ±1.46	9.06 ±1.27
Asn	Control	0.64 ±0.26	0.92 ±0.45	0.55 ±0.37	0.59 ±0.43	0.77 ±0.45
	Submerge		0.80 ±0.16	1.36 ±0.99	1.05 ±0.56	0.91 ±0.84
Asp	Control	4.34 ±2.64	2.48 ±1.49	2.71 ±1.58	3.84 ±0.51	6.63 ±3.84
	Submerge		0.80 ±0.41	1.93 ±0.49	4.52 ±0.91	4.27 ±1.57
Lys	Control	6.03 ±0.85	5.63 ±0.26	5.39 ±0.37	6.43 ±0.07	8.22 ±1.10
	Submerge		0.57 ±0.09	1.13 ±0.22	4.28 ±0.74	5.92 ±1.83
Met	Control	0.76 ±0.26	0.50 ±0.28	0.33 ±0.05	0.82 ±0.12	1.81 ±0.23
	Submerge		1.02 ±0.14	1.17 ±0.09	1.60 ±0.14	1.71 ±0.54
Thr	Control	3.44 ±0.64	4.44 ±1.10	3.80 ±0.56	3.79 ±0.42	4.64 ±0.27
	Submerge		5.33 ±0.47	5.68 ±0.88	4.64 ±0.18	5.16 ±0.90
Phe	Control	3.57 ±0.64	3.56 ±0.97	3.24 ±0.72	4.21 ±0.28	6.38 ±0.19
	Submerge		7.75 ±0.86	8.13 ±0.23	6.25 ±0.42	7.01 ±0.96
Tyr	Control	1.27 ±0.40	0.38 ±0.18	0.28 ±0.06	0.40 ±0.14	1.46 ±0.73
	Submerge		3.34 ±1.05	2.63 ±0.27	1.59 ±0.61	1.65 ±0.15
Trp	Control	0.50 ±0.15	1.32 ±0.21	1.36 ±0.19	2.26 ±0.40	2.77 ±0.39
	Submerge		0.75 ±0.07	1.07 ±0.14	0.85 ±0.13	0.81 ±0.04
His	Control	7.71 ±1.47	14.03 ±1.82	13.99 ±2.09	15.51 ±2.84	17.54 ±2.12
	Submerge		8.62 ±0.53	11.06 ±0.46	11.39 ±2.57	9.33 ±1.53

Table 2. -continued-

Days after germination		2	3	4	5	6
Days after flooding		0	1	2	3	4
		(μmol g^{-1}DW ± SE)				
6-PG	Control	0.02 ±0.02	0.07 ±0.02	0.07 ±0.01	0.08 ±0.01	0.04 ±0.03
	Submerge		0.11 ±0.10	0.16 ±0.10	0.04 ±0.03	0.02 ±0.01
E4P	Control	0.08 ±0.02	0.05 ±0.02	0.16 ±0.09	0.05 ±0.00	0.02 ±0.00
	Submerge		0.06 ±0.02	0.13 ±0.03	0.09 ±0.03	0.06 ±0.01
R5P	Control	0.12 ±0.03	0.09 ±0.03	0.08 ±0.01	0.16 ±0.04	0.14 ±0.03
	Submerge		0.12 ±0.02	0.19 ±0.05	0.10 ±0.03	0.10 ±0.01
Ru5P	Control	0.19 ±0.02	0.18 ±0.02	0.17 ±0.01	0.19 ±0.02	0.19 ±0.01
	Submerge		0.19 ±0.03	0.28 ±0.03	0.20 ±0.04	0.20 ±0.01
RuBP	Control	0.02 ±0.01	0.04 ±0.01	0.03 ±0.02	0.01 ±0.00	0.05 ±0.03
	Submerge		0.02 ±0.01	0.02 ±0.01	0.04 ±0.01	0.09 ±0.08
Ornithine	Control	47.35 ±3.54	70.58 ±15.45	111.26 ±31.61	110.07 ±31.22	116.20 ±26.86
	Submerge		48.16 ±7.74	67.38 ±9.10	67.17 ±14.29	67.29 ±9.25
Citrulline	Control	0.07 ±0.04	0.12 ±0.05	0.09 ±0.00	0.07 ±0.02	0.09 ±0.04
	Submerge		0.11 ±0.04	0.46 ±0.15	0.10 ±0.06	0.25 ±0.08
A	Control	0.01 ±0.00	0.01 ±0.00	0.01 ±0.01	0.01 ±0.00	0.01 ±0.00
	Submerge		0.00 ±0.00	0.02 ±0.02	0.01 ±0.00	0.01 ±0.02
G	Control	0.01 ±0.01	0.01 ±0.00	0.02 ±0.01	0.01 ±0.00	0.02 ±0.00
	Submerge		0.01 ±0.00	0.01 ±0.00	0.01 ±0.00	0.02 ±0.00
Adenosine	Control	0.11 ±0.01	0.11 ±0.01	0.17 ±0.09	0.12 ±0.01	0.11 ±0.01
	Submerge		0.03 ±0.01	0.03 ±0.01	0.03 ±0.00	0.03 ±0.01
Guanosine	Control	0.04 ±0.01	0.04 ±0.01	0.03 ±0.02	0.04 ±0.00	0.04 ±0.01
	Submerge		0.02 ±0.00	0.03 ±0.01	0.04 ±0.00	0.04 ±0.01
GMP	Control	1.40 ±1.01	2.13 ±1.75	1.17 ±1.17	4.12 ±3.77	0.48 ±0.24
	Submerge		0.19 ±0.09	1.37 ±0.98	5.42 ±5.80	0.52 ±0.43
GDP	Control	0.03 ±0.03	0.05 ±0.05	0.01 ±0.00	0.14 ±0.12	0.00 ±0.00
	Submerge		0.24 ±0.29	0.07 ±0.03	0.02 ±0.01	0.01 ±0.01
GTP	Control	1.34 ±1.43	0.13 ±0.04	0.12 ±0.11	0.03 ±0.03	0.77 ±0.94
	Submerge		0.86 ±0.45	3.78 ±1.64	1.49 ±1.60	0.60 ±0.38
IMP	Control	0.06 ±0.04	0.07 ±0.02	0.05 ±0.03	0.11 ±0.02	0.06 ±0.04
	Submerge		0.06 ±0.05	0.08 ±0.03	0.03 ±0.01	0.04 ±0.00
C	Control	0.03 ±0.00	0.04 ±0.01	0.04 ±0.00	0.04 ±0.01	0.04 ±0.01
	Submerge		0.03 ±0.01	0.03 ±0.02	0.04 ±0.01	0.04 ±0.01
U	Control	0.24 ±0.09	0.32 ±0.12	0.14 ±0.08	0.13 ±0.01	0.12 ±0.02
	Submerge		0.16 ±0.07	0.07 ±0.04	0.14 ±0.04	0.15 ±0.05
T	Control	5.67 ±0.05	6.66 ±1.50	5.58 ±1.18	2.81 ±0.03	2.43 ±0.12
	Submerge		5.43 ±0.21	3.82 ±0.87	6.57 ±0.24	6.37 ±0.43
Thymidine	Control	0.08 ±0.05	0.14 ±0.02	0.09 ±0.01	0.07 ±0.01	0.11 ±0.05
	Submerge		0.11 ±0.04	0.21 ±0.06	0.08 ±0.05	0.11 ±0.02
Cytidine	Control	0.02 ±0.01	0.02 ±0.00	0.02 ±0.00	0.01 ±0.00	0.02 ±0.00
	Submerge		0.01 ±0.00	0.01 ±0.01	0.03 ±0.00	0.03 ±0.01
Uridine	Control	0.08 ±0.07	0.12 ±0.06	0.11 ±0.07	0.03 ±0.02	0.13 ±0.05
	Submerge		0.11 ±0.05	0.10 ±0.05	0.10 ±0.05	0.11 ±0.06
CMP	Control	0.04 ±0.03	0.05 ±0.03	0.08 ±0.08	0.02 ±0.01	0.00 ±0.00
	Submerge		0.02 ±0.01	0.02 ±0.01	0.02 ±0.00	0.02 ±0.01
UMP	Control	0.33 ±0.23	0.44 ±0.13	0.21 ±0.13	0.39 ±0.10	0.25 ±0.19
	Submerge		0.21 ±0.07	0.15 ±0.05	0.23 ±0.03	0.18 ±0.06
dTDP	Control	0.01 ±0.01	0.01 ±0.01	0.00 ±0.00	0.00 ±0.00	0.00 ±0.00
	Submerge		0.01 ±0.01	0.00 ±0.00	0.00 ±0.00	0.00 ±0.00

Table 2. -continued-

Days after germination		2	3	4	5	6
Days after flooding		0	1	2	3	4
		(μmol g^{-1}DW ± SE)				
CDP	Control	0.07 ±0.01	0.04 ±0.02	0.02 ±0.02	0.02 ±0.00	0.01 ±0.01
	Submerge		0.02 ±0.02	0.03 ±0.00	0.03 ±0.01	0.03 ±0.01
UDP	Control	0.47 ±0.05	0.32 ±0.10	0.11 ±0.08	0.08 ±0.05	0.03 ±0.02
	Submerge		0.24 ±0.06	0.28 ±0.06	0.22 ±0.09	0.28 ±0.12
CTP	Control	0.20 ±0.14	0.04 ±0.04	0.02 ±0.02	0.02 ±0.01	0.03 ±0.03
	Submerge		0.04 ±0.02	0.05 ±0.03	0.09 ±0.07	0.09 ±0.11
UTP	Control	1.03 ±0.71	0.18 ±0.19	0.19 ±0.22	0.06 ±0.03	0.08 ±0.06
	Submerge		0.63 ±0.48	0.63 ±0.39	0.40 ±0.36	0.42 ±0.51
2-OG	Control	0.30 ±0.14	0.31 ±0.10	0.26 ±0.11	0.27 ±0.03	0.33 ±0.03
	Submerge		0.75 ±0.36	1.03 ±0.38	1.97 ±0.09	1.59 ±0.36
ACA	Control	0.17 ±0.04	0.09 ±0.06	0.04 ±0.02	0.05 ±0.02	0.05 ±0.02
	Submerge		0.26 ±0.08	0.24 ±0.04	0.49 ±0.17	0.66 ±0.39
CA	Control	12.12 ±4.02	2.13 ±1.23	2.13 ±0.87	0.69 ±0.34	0.45 ±0.37
	Submerge		10.08 ±2.22	9.78 ±6.12	12.58 ±4.38	21.94 ±7.18
ICA	Control	2.84 ±0.60	1.80 ±0.55	1.47 ±0.32	1.26 ±0.37	0.70 ±0.40
	Submerge		6.83 ±2.20	14.58 ±5.89	7.88 ±1.43	7.58 ±2.24
FA	Control	2.70 ±0.99	3.86 ±2.46	2.73 ±0.72	3.59 ±1.68	5.44 ±3.15
	Submerge		2.35 ±1.27	4.38 ±2.38	19.57 ±8.16	24.60 ±20.69
SuA	Control	1.34 ±0.58	1.07 ±0.34	1.25 ±0.16	1.22 ±0.34	1.49 ±0.79
	Submerge		2.79 ±1.24	3.91 ±0.80	5.18 ±3.50	5.29 ±3.13
MA	Control	44.02 ±13.12	18.89 ±6.48	20.28 ±2.60	26.32 ±7.20	30.11 ±13.46
	Submerge		25.53 ±4.79	23.66 ±13.97	44.53 ±22.27	35.88 ±4.05
AcCoA	Control	0.01 ±0.00	0.01 ±0.00	0.01 ±0.00	0.01 ±0.00	0.00 ±0.00
	Submerge		0.01 ±0.00	0.00 ±0.00	0.00 ±0.00	0.00 ±0.00
PEP	Control	0.16 ±0.03	0.09 ±0.04	0.05 ±0.02	0.03 ±0.01	0.08 ±0.01
	Submerge		0.18 ±0.05	0.24 ±0.03	0.28 ±0.12	0.22 ±0.09
2,3DPG	Control	0.00 ±0.00	0.00 ±0.00	0.00 ±0.00	0.00 ±0.00	0.00 ±0.00
	Submerge		0.00 ±0.00	0.01 ±0.00	0.00 ±0.00	0.00 ±0.00
DHAP	Control	0.24 ±0.02	0.24 ±0.06	0.32 ±0.14	0.24 ±0.02	0.28 ±0.05
	Submerge		0.24 ±0.03	0.43 ±0.22	0.70 ±0.18	0.63 ±0.13
GA3P	Control	0.18 ±0.03	0.16 ±0.03	0.19 ±0.04	0.16 ±0.01	0.18 ±0.02
	Submerge		0.11 ±0.02	0.15 ±0.05	0.25 ±0.06	0.18 ±0.05
G6P	Control	3.30 ±1.50	4.54 ±0.57	3.22 ±0.25	4.57 ±0.64	4.87 ±0.38
	Submerge		4.60 ±0.72	4.91 ±1.95	6.13 ±0.35	5.72 ±0.88
G1P	Control	1.50 ±1.15	0.67 ±0.04	0.60 ±0.04	0.60 ±0.08	0.53 ±0.04
	Submerge		1.26 ±1.09	1.73 ±1.51	0.46 ±0.11	0.35 ±0.02
FBP	Control	0.15 ±0.02	0.16 ±0.01	0.20 ±0.08	0.27 ±0.07	0.22 ±0.04
	Submerge		0.21 ±0.08	0.25 ±0.09	0.26 ±0.16	0.32 ±0.01
Cinnamate	Control	0.20 ±0.08	0.18 ±0.03	0.20 ±0.04	0.21 ±0.02	0.05 ±0.03
	Submerge		0.85 ±0.26	0.51 ±0.15	0.54 ±0.23	0.25 ±0.26
Coumarate	Control	0.12 ±0.12	0.01 ±0.01	0.11 ±0.12	0.09 ±0.06	0.01 ±0.00
	Submerge		0.10 ±0.12	0.06 ±0.05	0.13 ±0.15	0.05 ±0.06
Shikimate	Control	0.17 ±0.09	0.13 ±0.06	0.28 ±0.08	0.11 ±0.01	0.17 ±0.08
	Submerge		0.15 ±0.11	0.18 ±0.04	0.14 ±0.03	0.07 ±0.04
Glyoxalate	Control	0.66 ±0.13	0.55 ±0.08	1.53 ±0.59	0.69 ±0.40	0.33 ±0.18
	Submerge		0.87 ±0.49	1.87 ±1.25	0.64 ±0.28	0.54 ±0.19

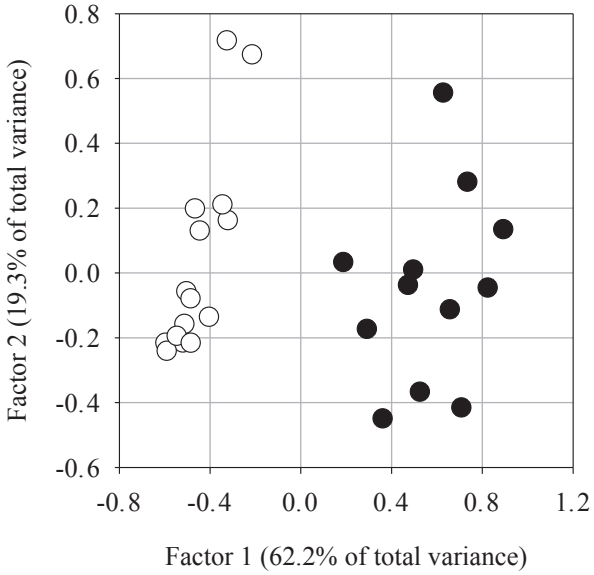

Fig. 1. Sample scores for the first (Factor 1) and second (Factor 2) components provided by PCA analysis for the metabolites identified in soybean seedling extracts

Each plot represents an individual sample. Open and closed circles in the figure represent control and flooding treatment, respectively.
○ : Control, ● : Flooding.

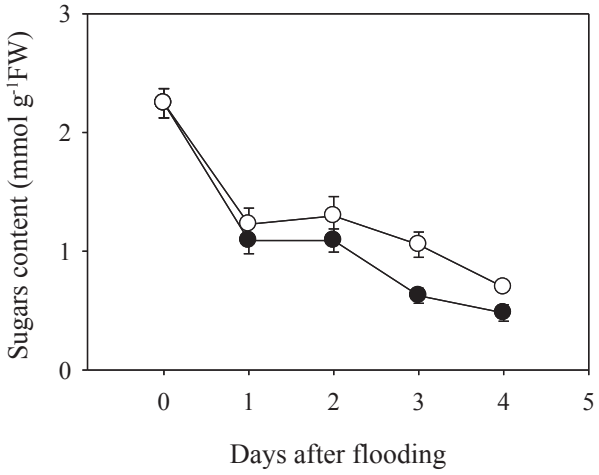

Fig. 3. Total soluble sugar content in soybean seedlings

Error bars indicate the SE. Open circles and closed circles in the figure represent control and flooding treatments, respectively. FW, fresh weight.
—○— : Control, —●— : Flooding.

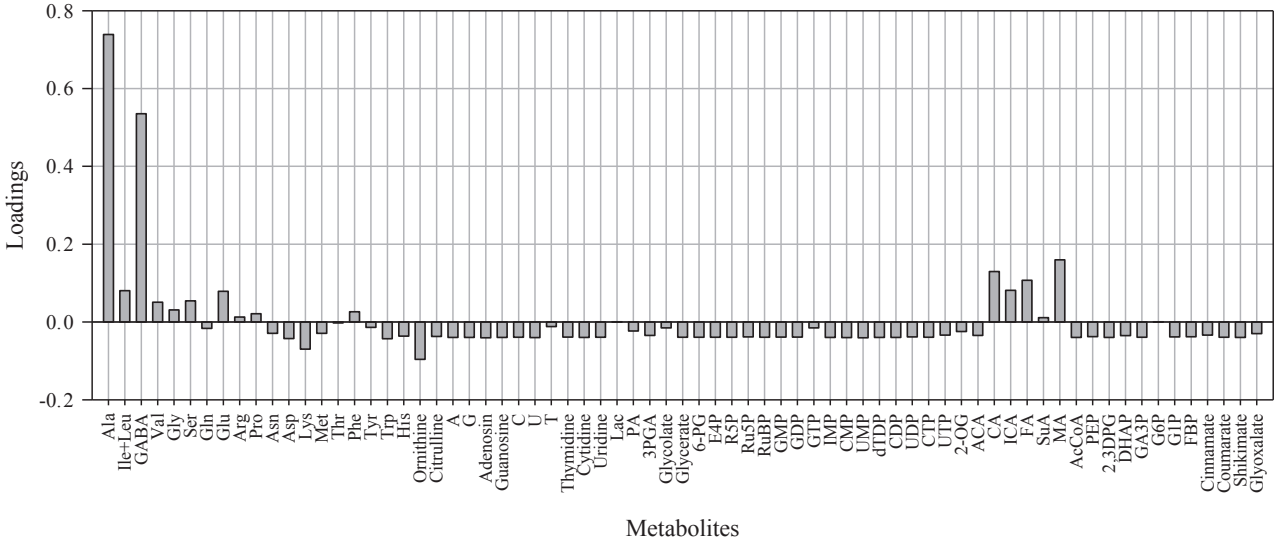

Fig. 2. PCA loadings of metabolites with the first principal component (Factor 1) in soybean seedlings

CA, 2-oxoglutarate, FA, Ala and GABA, related to the TCA cycle, amino acid metabolism, and the GABA shunt, accumulated in response to flooding treatment (Fig. 4, Table 2). In our previous report[22], the expression of the TCA cycle-related proteins malate dehydrogenase, aconitase, isocitrate dehydrogenase, and 2-oxoglutarate dehydrogenase were upregulated by flooding stress in soybean. In our study, organic acids related to the TCA cycle accumulated and the TCA cycle could not be suppressed in soybeans exposed to flooding conditions. Under flooded conditions, Ala and GABA were reportedly the two major amino acids synthesized, while organic acids were also accumulated[3]. It has been suggested that the accumulation of these amino acids is a stress response

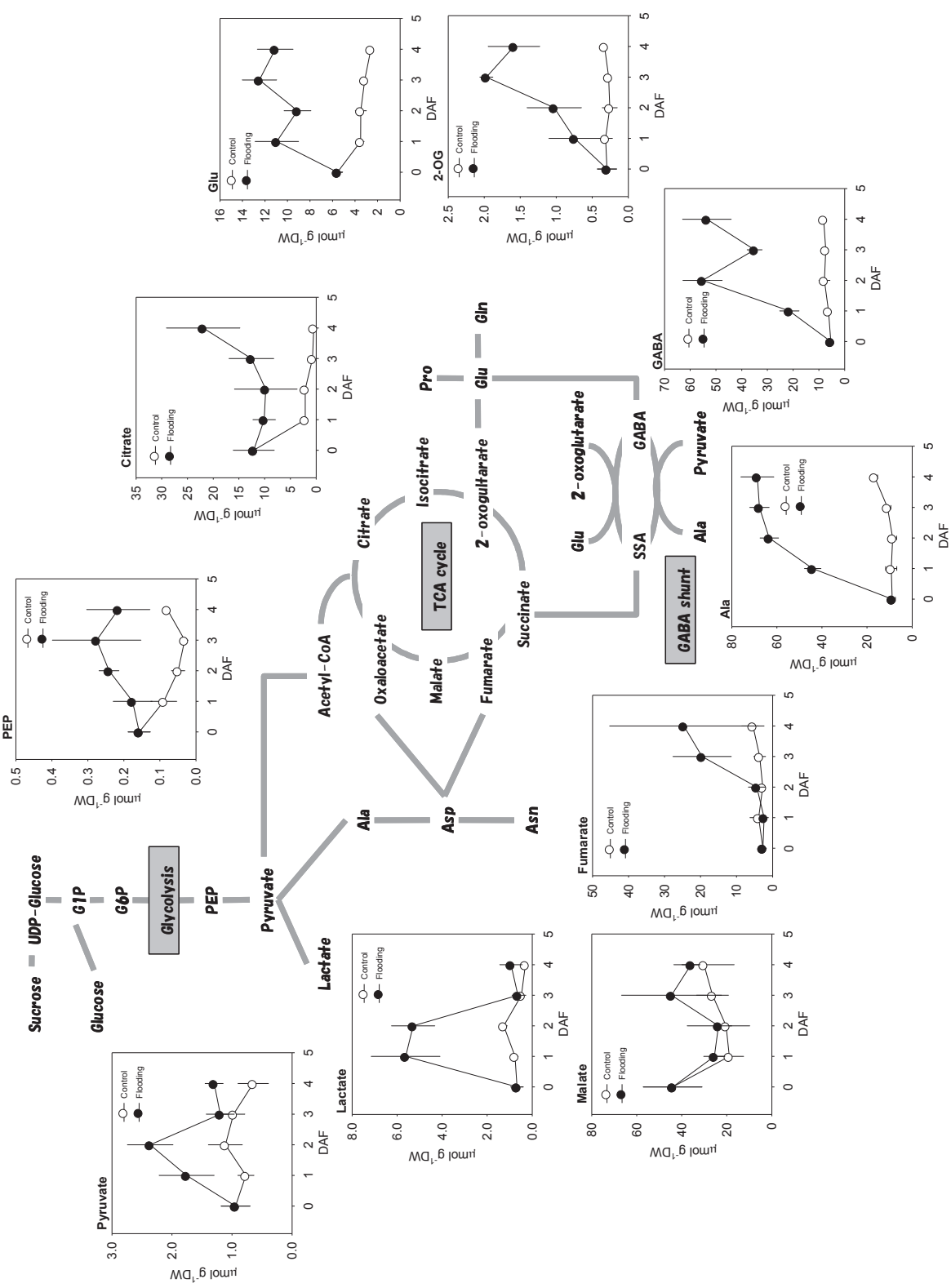

Fig. 4. Mapping of the changes in metabolites on the biosynthetic pathway

DAF means days after flooding. The changes in metabolites that represent a high loading score (>0.1) are shown on the map. Also, the changes in Glutamate (Glu) and 2-oxoglutarate (2-OG), which is a key organic acid related to nitrogen metabolism[12], and of phosphoenolpyruvate (PEP), pyruvate and lactate, which are related to anaerobic metabolism[10], are shown. Error bars indicate the SE. DW, dry weight.

mechanism to flooding stress. Cytoplasmic pH, regulated by glutamate decarboxylase (GAD), has been proposed since GABA synthesis by GAD consumes a proton, and its activity is stimulated under acidic conditions[6,51,56]. Moreover, the production of Ala and GABA was suggested as an important adaptive mechanism to store the carbon and nitrogen that would otherwise be lost under oxygen-deficient conditions[49]. Amino acids such as Ala, Pro, and GABA accumulated in rice coleoptiles under flooding conditions, compensating for a decrease in soluble sugars, to maintain osmotic potential for coleoptile elongation[25,26,31]. Thus, the role of these amino acids in maintaining the osmotic potential in stressed tissues may also be important to counteract the rapid fall in cellular carbohydrate levels[2,4,42,43].

Previous reports showed that a strong increase in mRNA and/or enzymatic activity of alanine aminotransferase (AlaAT), aspartate aminotransferase, and glutamate dehydrogenase (GDH) were consistent with pyruvate conversion to Ala or GABA[19,27,29,30,44,61]. However, the metabolic phenotype of the *Arabidopsis* AlaAT1 mutant (*alaat1-1*) demonstrated that AlaAT activity is not necessary for Ala accumulation under hypoxia[34]. In fact, Ala accumulation proceeded at a faster rate in the roots of *alaat1-1*, despite the virtually complete loss of AlaAT activity in the mutant roots[59]. Characterization of *alaat1-1* indicated that AlaAT1 primarily catalyzes the breakdown of the accumulated Ala during recovery from hypoxic conditions[34]. Most of the increase in AlaAT activity takes place after Ala production ceases, suggesting that the major role of AlaAT occurs during the recovery from low-oxygen stress in soybean[8]. These results suggest that AlaAT activity in soybean under hypoxia would have only a limited role in the accumulation of Ala and indicated the presence of another mechanism responsible for the hypoxia-induced Ala accumulation. However, gamma-hydroxybutyrate (GHB) accumulation in anaerobically stressed plant tissues has been reported[7,49]. GHB was synthesized via succinic semialdehyde from GABA and catalyzed by GABA transaminase (GABA-T), which also synthesizes Ala from GABA in the GABA shunt pathway[7]; Breitkreuz et al.[7] also suggested that GABA-T is operative and that its reaction involves Ala accumulation under hypoxia in flooding stress. Thus, the GABA shunt would be involved in Ala accumulation in response under flooding stress; Miyashita and Good[33] partially proved this using *gad1* and *gaba-t1* mutants of *Arabidopsis*. These findings, together with our results, suggest that the accumulation of Ala and GABA is one of the distinctive responses to flooding stress in the soybean. To determine the mechanism of Ala and GABA accumulation in the soybean during flooding stress, it will be necessary to study the activity and expression of the enzymes related to Ala and GABA metabolism.

Conclusions

Soybean is susceptible to flooding stress during germination and the early vegetative and early reproductive growth stages[17]. To understand the response mechanism of soybean to flooding stress, investigation of the metabolic response is one of the key issues. In this study, we clarified that the accumulation of Ala and GABA was one of the distinctive responses to flooding stress in the soybean. We simply investigated the response to flooding stress of soybean in this study, and in future, must also elucidate the relationship between the results in this study and tolerance against flooding stress. Therefore, it will be necessary to select tolerant varieties or breeding lines and elucidate the differing mechanisms of tolerance to this stress. The results in this study suggested that the metabolism of organic acids in the TCA cycle, Ala synthesis and the GABA shunt are significantly influenced by flooding stress during soybean germination.

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