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Proteomic Characterization of Tissue Expansion of Rice Scutellum Stimulated by Abscisic Acid

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We found that appropriate treatment with a highly potent and long-lasting abscisic acid analog enhanced the tissue expansion of scutellum during early seedling development of rice, accompanied by increases of protein and starch accumulation in the tissue. A comparative display of the protein expression patterns in the abscisic acid analog-treated and non-treated tissues on two dimensional gel electrophoretogram indicated that approximately 30% of the scutellar proteins were induced by abscisic acid. The abscisic acid-induced proteins included sucrose metabolizing, glycolytic, and ATP-producing enzymes. Most of these enzyme proteins also increased during the seedling growth. In addition, the expression of some isoforms of UDP-glucose pyrophosphorylase, 3-phosphoglycerate kinase, and mitochondrial ATP synthase beta chain was stimulated in the scutellum, with suppressed expression of α-amylase. We concluded that abscisic acid directly and indirectly stimulates the expression of numerous proteins, including carbohydrate metabolic enzymes, in scutellar tissues.

Key words: abscisic acid (ABA); germination; *Oryza sativa*; proteome; scutellum

The seed contains structural and physiological devices that fit it for its role as a dispersal unit and nutrient reserves that sustain the young plant until a self-sufficient, autotrophic organism can be established. Although there are differences among seeds in their content of reserves, starch is a predominant reserve in cereal seeds. The germination process is comprised of numerous biochemical reactions, but the breakdown of reserve starch in the starchy endosperm and its utilization are thought to be a prerequisite step for the later stage of seed germination and subsequent seedling growth to produce energy and provide carbon skeletons for biosynthesis of new cellular components.

The starch is broken down by amylolytic enzymes, particularly α-amylase secreted from the scutellar epithelium and aleurone cells, and the glucose produced is mobilized to the scutellum, where it is reconverted to sucrose and eventually transported to the embryo axis.[1)] Furthermore, the scutellar epithelium synthesizes and releases gibberellins,[2)] which are an important regulatory factor in germinating cereal seeds, to the aleurone layer to induce α-amylase during germination.[3,4)] Hence the scutellar tissues are considered to be the key site for the control of seed germination.

Abscisic acid (ABA) is a small, lipophilic phytohormone that plays a crucial role in plant development, seed dormancy, germination, cell division, and cellular responses to environmental stresses such as drought, cold, salt, pathogen attack, and UV radiation.[5)] Recently, it has been suggested that ABA possesses dual functions: as a growth inhibitor in the presence of stress (associated with high induced ABA levels), and as a growth promoter in the absence of stress (associated with low endogenous ABA levels).[6,7)] In germinating cereal seeds, it has been reported that the endogenous level of ABA decreased rapidly to almost zero at the early stage of germination and increased gradually

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Abbreviations: ABA, abscisic acid; CBB, Coomassie brilliant blue; GA_3, gibberellic acid; IEF, isoelectric focusing; MALDI TOF-MS, matrix assisted laser desorption/ionization time of flight mass spectrometry; PVDF, polyvinylidene difluoride; TCA, trichloroacetic acid; TFA, trifluoroacetic acid; trifluoro-ABA, (+)-8′,8′,8′-trifluoroabscisic acid; 2D-PAGE, two dimensional polyacrylamide gel electrophoresis

during the seedling growth stage.[8,9] ABA is well-known to negatively regulate the gibberellin-induced expression of α-amylase in cereal aleurones.[10] However, the ABA response manner of scutellar tissues concerning the suppression of α-amylase expression was distinguishable from that operating in the aleurone, and ABA stimulated the uptake of glucose and the formation of sucrose in the scutellar tissues in germinating rice seeds.[11] Thus, it is obscure whether or not ABA only acts as negative regulator in the scutellar tissues.

In the present study, we analyzed the proteins in the process of scutellum expansion enhanced by a highly potent and long-lasting ABA analog, (+)-8′,8′,8′-trifluoroabscisic acid (trifluoro-ABA), which is designed to resist 8′-hydroxylation and inactivation by monooxygenase,[12] and we found that ABA stimulates the expression of enzyme proteins involved in the central carbohydrate metabolic pathway in scutellar tissues of rice seedling.

Materials and Methods

Plant materials and chemicals. Rice seeds (*Oryza sativa* L. cv. Nipponbare) used in this study were supplied from the Niigata Prefectural Agricultural Research Institute (Niigata, Japan). The handling and incubation procedures of rice seeds were described previously.[13] The scutellar tissues, free from the endosperm, consisted of scutellum proper and epithelial tissues were freshly dissected from the rice seedlings. The details of incubation are described in each figure legend. (+)-8′,8′,8′-trifluoroabscisic acid was a gift from Dr. Hirai (Kyoto University).

Assays. ABA level was determined following the protocol for the Phytodetek ABA analysis kit (Agdia, Elkhart, IN). Protein assay was carried out as described by Bradford[14] with bovine gamma globulin as a standard. Starch contents were determined as described by Matsukura *et al.*[15]

Two-dimensional gel electrophoresis.

Procedure 1 (IPGphor): Scutellar tissues (100 mg) were homogenized with 1 ml of lysis buffer consisting of 8 M (48%, w/v) urea, 3.25 mM (2%, w/v) CHAPS, 5% (v/v) 2-mercaptoethanol, and 5% (w/v) polyvinylpyrroridone-40 using an ice-chilled mortar and pestle, and centrifuged at 15,000 × *g* for 10 min. The supernatant was mixed with one-tenth volume of 100% TCA, and the resultant precipitate was suspended in 0.2 ml of isoelectric focusing sample buffer consisting of 8 M urea, 3.25 mM CHAPS, 5% (v/v) 2-mercaptoethanol, and 1.25% (v/v) Ampholine (pH 3–10). The suspension was centrifuged at 15,000 × *g* for 10 min and the supernatant was subjected to isoelectric focusing in immobilized pH gradient from 3 to 10 (Immobiline Drystrip, pH 3–10 L, 11 cm) for 16,000 Vh using IPGphor (Amersham Biosystems, Tokyo, Japan). Equilibration of the strips prior to the second dimension (SDS–PAGE) was performed according to the IPGphor manufacturer's recommendation.

Procedure 2 (Reverse): The cytosolic and solubilized membrane proteins (200 μg) with 2% (v/v) ampholine (pH 3.5–10.0) were applied to the cathode side of first-dimensional disc gel (11.5 cm × ϕ3 mm) composed of 8 M urea, 4% (w/v) acrylamide, 1% IGEPAL CA-630, 2% ampholine (pH 3.5–10), 0.02% ammonium persulfate, and 0.003% *N*,*N*,*N*′,*N*′-tetramethyl ethylenediamine. Electrophoresis was carried out at 300 V for 20 h, followed by 600 V for 30 min at 4 °C. After IEF, the gel was placed in an equilibration solution containing 0.06 M Tris–HCl (pH 6.8), 2.5% (w/v) SDS, 5% (w/v) 2-mercaptoethanol and 10% (w/v) glycerol for 10 min at room temperature, and then subjected to SDS–PAGE.

SDS–PAGE was carried out with 18% separating gel (%C = 0.45) and 5% stacking gel (%C = 2.7) at a constant current of 30 mA, and the proteins separated on 2D-gel were visualized with either Coomassie brilliant blue R250 (CBB) or silver staining. The isoelectric point and relative molecular weight of each protein were determined using 2-D SDS–PAGE standards (Bio-Rad, Hercules, CA). The localization sites and quantification of individual proteins were evaluated by using image analysis software, Image Master 2D Elite (Amersham Biosciences) and PDQuest (Bio-Rad).

Edman sequencing. The N-terminal amino acid sequence was determined by transblotting the proteins separated on 2D-gel onto a polyvinylidene difluoride (PVDF) membrane (Hybond-P, Amersham Biosciences). The protein spots detected by CBB staining were excised from the PVDF membrane and applied to a gas-phase protein sequencer (Shimadzu PPSQ-21, Kyoto, Japan). To determine the internal amino acid sequence, the protein spots detected on the 2D-gel with CBB staining were collected and digested in gel with *Staphylococcus aureus* V8 protease, according to the method described by Cleveland *et al.*[16] After SDS–PAGE, the resultant peptides were transblotted to a PVDF membrane, followed by CBB staining. The peptides detected on the PVDF membrane were excised and applied to the protein sequencer. The amino acid sequences obtained were compared with those proteins compiled in the protein sequence database (DDBJ, http://www.ddbj.nig.ac.jp/; NCBI, http://www.ncbi.nlm.nih.gov/) using the FASTA or the BLAST algorithm.[17,18]

Mass analysis. Protein bands excised from 2D-gel were washed with 25% methanol and 7% acetic acid for 12 h at room temperature, and further destained with 50 mM NH_4HCO_3 in 50% methanol at 40 °C for 1 h. After the destained solution was removed carefully, the gels were rinsed with H_2O twice and dried in a Speed-Vac. The gels were swollen and incubated with 10 mM dithiothreitol, and 100 mM NH_4HCO_3 at 60 °C for 1 h.

The dried gels were swollen again and incubated with 40 mM iodoacetamide, and 100 mM NH_4HCO_3 at room temperature for 30 min in the dark. The gels were rinsed twice with H_2O, crushed, and then dried again in the Speed-Vac. The gel pieces were re-swollen and incubated with 50 µl of 20 nM trypsin (Promega, Madison, WI) and 10 mM Tris–HCl (pH 8.0) at 37 °C for 12 h. Peptide fragments were extracted and collected from the gels with 0.03% (w/v) TFA/33% (v/v) acetonitrile, 0.1% TFA, 0.1% TFA/50% acetonitrile, 0.1% TFA in acetonitrile, sequentially. The obtained peptides were subjected to matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF-MS; AXIMA-CFR Shimadzu, Voyager-DE Applied Biosystems Japan, Tokyo, Autoflex II Bruker Daltonics, Billerica, MA). The matrix solution was prepared by mixing a saturated solution of α-cyano-4-hydroxycinnamic acid in 0.1% TFA and 100% acetonitrile (1:1, v/v). The peptides were further analyzed by post-source decay according to the manufacturer's protocol. Based on the peptide mass fingerprints obtained, the protein homologous to each protein was identified by searching the protein databases (DDBJ and NCBI) using MASCOT (http://www.matrixscience.com).

Results

Tissue expansion of scutellum with or without trifluoro-ABA

When the rice seeds were imbibed with water at 30 °C in the dark, the seed germination occurred at 2 d, and the elongation of shoot and root commenced from that time.[19] Figure 1 shows the change in ABA content in rice seeds during germination and early seedling development. The endogenous level of ABA in germinating seeds decreased rapidly to almost zero within 1 d after imbibition, whereas the ABA level gradually increased from 2 d, and then reached to the same level as dry seeds at 8 d. This result is consistent with observations reported by other groups.[8,9]

The seedling growth under normal condition accompanies a tissue expansion of scutellum, with increasing the protein and starch contents (Table 1). In order to examine the effect of ABA on the expansion of scutellar tissues, rice seeds imbibed for 3 d with H_2O were incubated for a further 3 d with or without ABA. As shown in Fig. 2A, neither 1 µM (+)-ABA nor 1 µM (+)-ABA plus 0.1 µM gibberellic acid (GA_3) affected the seedling growth. Interestingly, trifluoro-ABA (0.5 to 1 µM) was found to enhance the expansion of the scutellar tissues that occurred at this stage (Fig. 2B, Table 1). Furthermore, the protein and starch contents in 1 µM trifluoro-ABA-treated tissues increased markedly to 384 and 155% as compared with those in the wild type, respectively (Table 1).

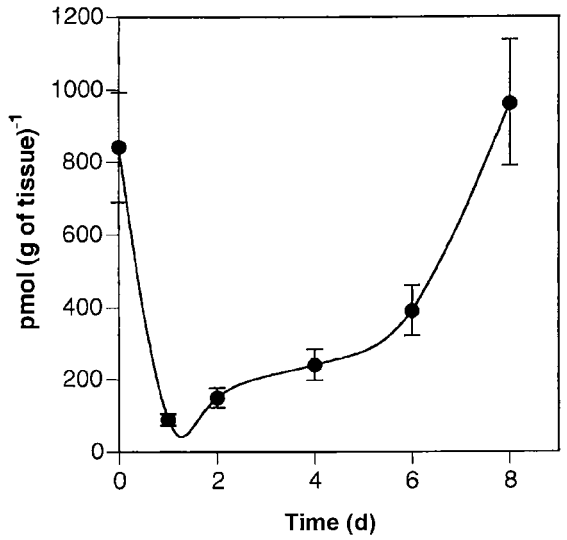

Fig. 1. Changes in Endogenous ABA Contents in Rice Seeds during Germination and Early Seedling Development.

At the selected imbibitional time intervals, the seed parts dissected from rice seedlings were extracted and subjected to the ABA assay. The data represent the average of duplicate experiments.

2D-PAGE database of proteins from scutellar tissues

Proteins extracted from the scutellar tissues of rice seeds imbibed for 4 d with lysis buffer containing 8 M urea and 3.25 mM CHAPS were subjected to IPGphor, followed by SDS–PAGE. Protein spots (428) were detected on 2D-gels by CBB staining (Fig. 3). The separation patterns of scutellar proteins by 2D-PAGE were standardized using an image analyzer with refer-

Table 1. Effect of Trifluoro-ABA on Expansion of Scutellar Tissue of Rice Seedlings

Measurements	Control 1	Control 2	Trifluoro-ABA	
			0.5 µM	1 µM
Tissue size				
Y: longest diameter (mm)	1.97 ± 0.06 (90.4)[a]	2.18 ± 0.04 (100)[a]	2.25 ± 0.08 (103)[a]	2.36 ± 0.14 (108)[a]
X: shortest diameter (mm)	1.28 ± 0.05 (85.3)[b]	1.50 ± 0.01 (100)[b]	1.78 ± 0.08 (119)[b]	1.72 ± 0.10 (117)[b]
Dry weight (mg/scutellum)	0.42 ± 0.03 (65.6)[c]	0.64 ± 0.05 (100)[c]	0.74 ± 0.09 (116)[c]	0.78 ± 0.07 (122)[c]
Protein content (µg/scutellum)	6.85 ± 0.09 (76.5)[d]	8.95 ± 0.08 (100)[d]	28.1 ± 0.09 (314)[d]	34.4 ± 0.11 (384)[d]
Starch content (µg/scutellum)	7.40 ± 0.08 (89.2)[e]	8.30 ± 0.07 (100)[e]	10.2 ± 0.08 (123)[e]	12.9 ± 0.08 (155)[e]

Rice seeds were imbibed for 3 d at 30 °C with water (control 1), and further incubated in 10 mM acetate buffer (pH 5.3) without (control 2) or with trifluoro-ABA (0.5 or 1 µM) for 3 d at 30 °C in darkness, and then the scutellar tissues were carefully dissected. A set of 20 scutella was subjected to each assay. The longest (Y) and shortest (X) diameters in tissue size are defined in Fig. 2. [a–e]The values in parentheses indicate the percentage against those in the control 2 experiments. Data are represented as the mean ± SD obtained from five independent experiments.

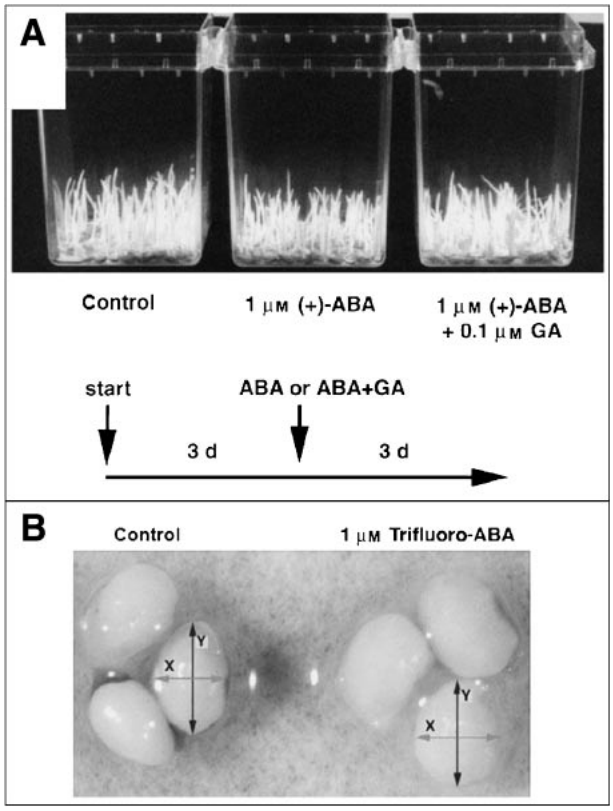

Fig. 2. Effect of ABA on the Seedling Growth and Expansion of Scutellar Tissue.

A, Seedling growth: Rice seeds were imbibed for 3 d at 30 °C with water, and further incubated in 10 mM acetate buffer (pH 5.3) with H_2O, 1 μM (+)-ABA, or 1 μM (+)-ABA plus 0.1 μM GA for 3 d at 30 °C in the darkness. B, Scutellum expansion: Rice seeds were imbibed for 3 d at 30 °C with water, and further incubated in 10 mM acetate buffer (pH 5.3) with or without 1 μM trifluoro-ABA for 3 d at 30 °C in darkness, then the scutellar tissues were carefully dissected. The longest (Y) and shortest (X) diameters of scutellar tissues were defined as indicated.

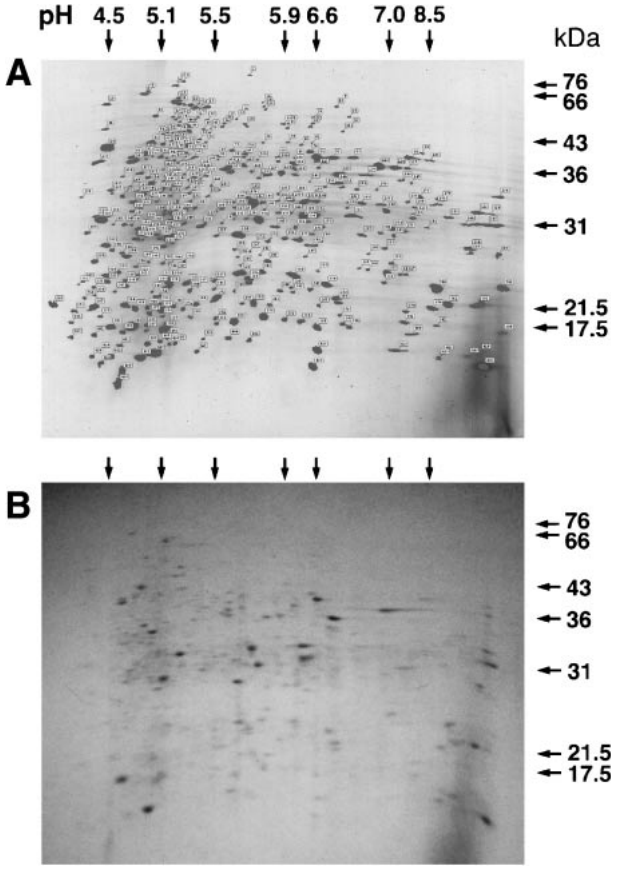

Fig. 3. 2D-PAGE Separation Profile and Numbering of Proteins Expressed in 4-d Scutellar Tissues.

A, Standardization, identification, and numbering (GS-1 to GS-428) of the expressed proteins were done by Image Master 2D Elite. Four different 2D-gels were used in the standardization. B, Typical separation pattern of protein spots by 2D-PAGE (IPGphor).

ence to molecular sizes and isoelectric points of marker proteins, and the standardized picture (Fig. 3A) was used as the base pattern of protein expression in the tissues.

Major protein spots (70) separated on 2D-gels were applied to a gas-phase protein sequencer to determine their N-terminal amino acid sequences. The internal amino acid sequences of GS-17, GS-32, GS-124, GS-128, and GS-129 were also determined by the Cleveland peptide mapping method,[16] followed by Edman sequencing. Furthermore, 41 protein spots were digested in gel with trypsin and then subjected to peptide mass fingerprinting, followed by a data base search MASCOT. The corresponding genes for 53 proteins were identified by Edman sequencing and peptide mass fingerprinting analyses (Table 2). Glycolytic, citric acid cycle, ATP-producing system, and sucrose metabolizing enzymes accounted for almost half of the identified proteins, and in addition to these, ABA- and salt stress-inducible proteins, RAB24 (GS-249), OSR40c1 (GS-128), and OSR40g2 (GS-129),[20,21] were also detected as major proteins in the 4-d scutellar tissues (Fig. 3, Table 2).

Stage-dependent expression of scutellar proteins

To determine the stage-dependent protein expression in scutellar tissues, we analyzed protein extracts prepared from 2-d, 4-d, and 6-d scutellar tissues by 2D-PAGE. As shown in Fig. 4, numerous proteins that include the glycolytic enzymes (GS-17, 32, 91, 92, 96, 100, 104, 108, 248), ATP-producing system enzymes (GS-40, 124), sucrose metabolizing enzymes (GS-47, 363), and ABA-inducible proteins (GS-128, 129) increased in a time-dependent manner. The expression level of some protein spots reached a maximum at 4 d after imbibition, and then they disappeared at 6 d. Fragments of glycolytic (GS-392, 417) and citric acid cycle (GS-298) enzymes were included in this group that was considered to be an intermediate of the degradation process.

Influence of trifluoro-ABA on expression of scutellar proteins

To examine the effect of ABA on the protein

Table 2. Rice Scutellar Protein List

GS[a]	pI[b]	MM (kDa)[b]	Identity	Accession number[c]	Identification[d]	Score[e] (% Covered)[f]	Edman sequencing analysis (N)N-terminal sequence (I)internal sequence	ABA response[g]
1	5.70	80.04	N.I.[h]		Ed, MS		N-blocked	Inducible
5	4.83	71.13	Chloroplast heat shock protein 70 (cucumber)	T10248	MS	108 (20.8%)		
6	4.79	70.01	Chloroplast heat shock protein 70 (Narcissus)	AAB47207	Ed		(N)EKVVGIDLGT	
12	4.47	65.05	N.I.		Ed, MS		N-blocked	Inducible
17	5.33	63.82	Phosphoglycerate mutase	AP003411	Ed		(I)YVEIPSDIGI	Inducible
22	4.89	60.01	Protein disulfite isomerase	BAA92322	MS	184 (68.5%)		Inducible
32	5.34	58.18	Enolase	Q42971	Ed		(I)XDNFMVQQLDG	Inducible
40	5.15	55.82	Putative ATP synthase beta chain, mitochondrial	BAB92708	MS	173 (57.3%)		Inducible
41	5.46	55.64	Probable methylmalonate-semialdehyde dehydrogenase	T02721	MS	66 (19.0%)		
47	5,30	54.59	UDP-glucose pyrophosphorylase	BAB69069	MS	278 (53.5%)		Inducible
62	4.85	49.29	α-Amylase I-1	S10013	Ed, MS	125 (36.7%)	N-blocked	Reducible
76	6.22	46.54	UDP-glucose pyrophosphorylase	BAB69069	MS	144 (33.9%)		Inducible
91	6.28	44.65	Glyceraldehyde-3-phosphate dehydrogenase	U31676	Ed		(N)GKIKIGINGFGR	Inducible
92	6.41	44.65	Glyceraldehyde-3-phosphate dehydrogenase	U31676	Ed		(N)GKIKIGINGF	Inducible
96	7.09	44.51	Fructose bisphosphate aldolase C-1, cytosolic	S65073	MS	238 (46.6%)		Inducible
98	5.42	43.94	Putative glutamine synthetase	AAN05339	MS	79 (19.8%)		
100	5.76	43.94	Phosphoglycerate kinase, cytosolic (wheat)	TVWTGY	MS	52 (18.0%)		Inducible
104	5.95	43.52	Glyceraldehyde-3-phosphate dehydrogenase (Maize)	T02722	Ed		(N)AKIKIGINGF	Inducible
105	4.40	43.25	Unknown protein	AAN04952	Ed, MS	85 (37.1%)	N-blocked	Inducible
108	6.08	42.83	Glyceraldehyde-3-phosphate dehydrogenase (Maize)	T02722	Ed		(N)AKIKIGINGF	Inducible
109	5.32	42.56	Probable phosphopyruvate hydratase	T03267	MS	127 (40.4%)	N-blocked	
110	5.65	42.29	N.I.		Ed		N-blocked	
111	5.53	42.02	Cytoplasmic malate dehydrogenase	AAG13573	Ed, MS	78 (40.7%)	N-blocked	Inducible
116	6.08	41.36	N.I.		Ed, MS		N-blocked	Inducible
118	5.49	41.23	N.I.		Ed, MS		(N)NRAALIXYAL	Inducible
124	5.97	40.06	Putative malate dehydrogenase	AP003340	Ed		(I)VNVPVVGGHAGITIL	Inducible
125	4.76	39.93	N.I.		MS			
127	5.85	39.68	UDP-glucose pyrophosphorylase (fragment)	BAB69069	Ed, MS	125 (35.6%)	(N)NSXNTH	
128	6.09	39.55	OSR40c1 protein	T03911	Ed		(I)GNVVLAPXNPRDE	Inducible
129	6.48	39.55	OSR40g2 protein	Y08987	Ed		(I)DASVLWTESKDVGKGFRXIRMV	Inducible
136	5.78	38.68	Putative ethylene-inducible protein	AAL73561	MS	90 (27.8%)		
143	4.86	37.34	Enolase (fragment)	Q42971	Ed, MS	93 (22.9%)	(N)SVIKKKYGQ	
144	6.45	37.34	OSR40g2 protein	Y08987	Ed		(N)XRANEXYCLT	
153	5.22	35.71	Glyoxalase I	BAA36759	Ed, MS	106 (39.2%)	N-blocked	
154	6.62	35.71	N.I.		Ed		N-blocked	
159	4.93	35.37	Probable phosphopyruvate hydratase	T03267	MS	136 (39.2%)		
165	5.95	34.48	N.I.		Ed, MS		(N)QEXAAAXXPX	
168	6.24	34.26	N.I.		Ed		(N)QGGGGGEXX	
180	6.06	32.98	OSR40g2 protein	Y08987	MS	50 (16.9%)		
183	6.18	32.77	OSR40g2 protein (fragment)	Y08987	Ed, MS	156 (52.8%)	(N)QXHPVXLVP	
185	4.70	32.66	N.I.		MS			Inducible
191	5.73	32.45	Glyceraldehyde-3-phosphate dehydrogenase (fragment)	U31676	Ed		(N)GEKPVTFFGI	
193	6.65	32.35	Voltage-dependent anion channel	CAB82853	MS	144 (40.1%)		
201	6.54	31.34	Putative stress-related protein	BAC15991	MS	186 (59.6%)		Inducible
208	5.11	30.55	L-Ascorbate peroxidase	BAB17666	MS	93 (44.2%)		
220	6.17	29.87	N.I.		Ed, MS		N-blocked	
224	4.38	29.49	Putative nascent polypeptide associated complex alpha chain	BAB89723	Ed, MS	62 (29.2%)	N-blocked	
229	5.32	29.03	Embryo-specific protein	AAD02495	MS	104 (41.2%)		Inducible
231	6.16	29.03	N.I.		MS			
233	4.42	28.84	N.I.		Ed		N-blocked	
234	5.78	28.84	Fructose bisphosphate aldolase	CA757005	MS	92 (20.1%)		
248	5.29	27.67	Triosephosphate isomerase	AAB63603	Ed		(N)GRKFFVGGNW	Inducible
249	5.92	27.41	RAB24 protein	D63917	Ed		(N)PGLTIGDTVP	Inducible
262	6.24	26.04	Alcohol dehydrogenase-1 (fragment)	P20306	Ed		(N)XVAKINPAAP	
264	5.60	25.88	Superoxide dismutase	T04072	Ed		(N)VTTVALPDLP	
269	5.00	25.47	Triosephosphate isomerase	AAB63603	MS	147 (46.6%)		
271	6.00	25.23	N.I.		Ed		(N)XGKSGGGLGAX	
275	6.12	24.98	Alcohol dehydrogenase-1	P20306	MS	83 (21.6%)		
280	4.46	24.05	N.I.		Ed, MS		(N)Lh/aXEh/aEh/a	Inducible
281	6.23	24.05	Alcohol dehydrogenase-1 (fragment)	P20306	Ed		(N)ATINVAKPK	
282	4.96	23.89	N.I.		Ed		(N)QAPAYAGEFD	
286	4.51	23.22	IgE-dependent histamine-releasing factor homolog	A38958	MS	62 (28.6%)		Inducible
294	4.64	22.27	Mitochondrial putative ATPase delta chain	AP003856	Ed		(N)STAEVPAEAA	Inducible
298	5.85	21.51	Malate dehydrogenase (fragment, soybean)	AF068687	Ed		(N)VTTLDVVYAK	
302	4.95	21.23	N.I.		Ed		N-blocked	
304	5.02	21.17	N.I.		Ed		N-blocked	
305	5.63	20.96	N.I.		Ed		(N)QQVGQGQEGX	
309	6.29	20.05	N.I.		Ed		N-blocked	
312	5.80	19.92	Alcohol dehydrogenase-1 (fragment)	P20306	Ed		(N)AVGLAAAEGA	
314	5.62	19.73	N.I.		Ed		(N)EIPNAKQDLL	
320	6.08	19.42	N.I.		Ed, MS			Inducible
329	5.69	18.45	N.I.		Ed		N-blocked	
334	6.36	18.22	N.I.		Ed		N-blocked	
340	7.31	17.76	N.I.		Ed, MS		(N)GGGRXRAXLE	
352	5.41	16.56	N.I.		Ed		(N)QQQGYNFYNR	
362	7.67	15.83	N.I.		Ed		(N)WPGKLFIXDE	
363	5.87	15.78	Nucleoside-diphosphate kinase	S43330	MS	110 (41.6%)		Inducible
379	5.82	14.53	N.I.		Ed		N-blocked	
383	5.57	14.03	N.I.		Ed		(N)GLLXHDVVYI	
392	4.68	13.54	Triosephosphate isomerase (fragment)	L04967	Ed		(N)ESNEFM	
396	4.97	13.24	Peroxiredoxin-like protein (fragment)	AP000367	Ed		(N)AVPGAFTPT	
404	5.52	12.50	N.I.		Ed		(N)TVLIVNVWYX	
417	4.88	11.22	Glyceraldehyde-3-phosphate dehydrogenase (fragment, Barley)	M36650	Ed		(N)GMAIRVPGVD	Reducible

[a]GS number refers to the spot numbers as given in Fig. 3A. [b]Molecular mass (MM) and pI are from Fig. 3A. [c]Accession number in NCBI database. [d]Methods of protein identification: Ed, Edman sequencing; MS, mass spectrometry. [e]Score is a measure of the statistical significance of a match. [f]Percentage of predicted protein sequence covered by matched peptides. [g]The ABA responses of proteins are from Fig. 6. [h]N.I., not identified.

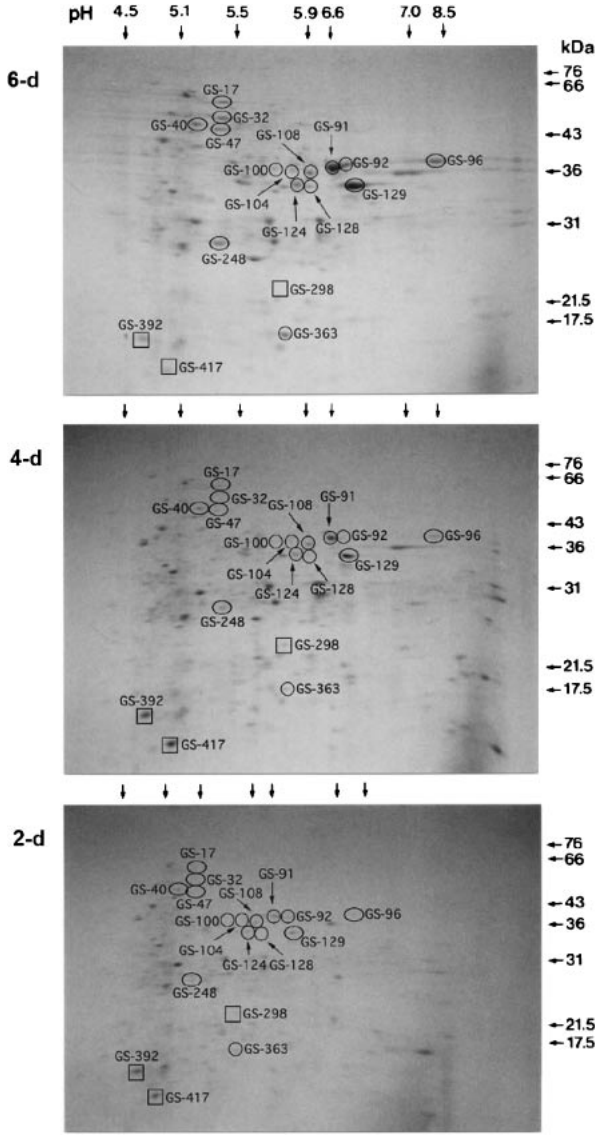

Fig. 4. Comparative Display of Protein Expression Patterns in Scutellar Tissues at Different Germination Stages.

Rice seeds were imbibed for 2 d, 4 d, and 6 d at 30 °C, and the scutellar tissues dissected from rice seedlings at each imbibition period were subjected to 2D-PAGE (IPGphor). Typical protein spots that increased up to 6 d are circled, and proteins that had decreased at 6 d are squared. These increases and decreases were confirmed in four independent experiments.

expression in scutellar tissues, we analyzed proteins extracted from the trifluoro-ABA-treated and -untreated scutellar tissues by 2D-PAGE, followed by scatter plot analysis. We tested the variability of protein quantitation on 2D-gels between individual control experiments, and found that the expressions of most all proteins oscillated within a 4-fold range between the experiments (Fig. 5A). As shown in Fig. 5B and C, 41% of scutellar proteins were induced or reduced over 4-fold by trifluoro-ABA treatment. Furthermore, the number of hormone-induced proteins (30%) was much higher than that of the hormone-reduced proteins (11%). Similar results were obtained by 1 μM (+)-ABA treatment, but this response was relatively weaker by comparison to the above trifluoro-ABA (data not shown). Based on these results, we considered that ABA stimulates protein expression in scutellar tissues rather than inhibiting it.

Figure 6 shows a typical 2D-gel image of protein expression in scutellar tissues in response to trifluoro-ABA. The expression of well-known ABA-inducible proteins, GS-128, 129, and 249, increased in the scutellar tissues treated with trifluoro-ABA as expected. Furthermore, glycolytic enzymes (GS-17, 32, 91, 92, 96, 100, 104, 108, and 248), TCA cycle-ATP producing system (GS-40, 124, and 294) and sucrose metabolizing enzymes (GS-47 and 363) were also induced. Protein disulfite isomerase (GS-22) and an embryo-specific protein (GS-229) were also identified among the trifluoro-ABA-induced proteins. α-Amylase I-1 (GS-62) and a fragment of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (GS-417) behaved as typical ABA-reduced proteins (Table 2).

Changes in protein expression in scutellum with suppressed expression of α-amylase

Scutellar tissues dissected from the germinating seeds of wild-type and transgenic plants (line 15-2)[19] with suppressed expression of α-amylase I-1, which is the predominant α-amylase isoform in rice, were subjected to 2D-PAGE analysis. The α-amylase I-1 expression was totally absent in the 15-2 line, while some proteins including cytosolic 3-phosphoglycerate kinase, UDP-glucose pyrophosphorylase, and ATP synthase beta chain were up-regulated in comparison with those in the wild type (Fig. 7).

Discussion

Function of ABA in early seedling development

ABA is the most critical phytohormone in the promotion of embryo maturation and the prevention of germination. Adding 1 μM (+)-ABA from initial imbibition, indeed, prevented the germination of rice seed completely. However, we observed that the seedling growth normally occurred at least for 3 d when 1 μM (+)-ABA added after 3 d imbibition (Fig. 2A), and that appropriate treatment with trifluoro-ABA enhanced the expansion of scutellar tissues, increasing the protein and starch contents (Fig. 2B, Table 1). In addition to these results, the increase in the endogenous ABA level in the seeds during early seedling development[8,9] (see Fig. 1) strongly suggests that ABA has another physiological function after setting up the germination systems.

Recent studies employing ABA-deficient mutants of *Arabidopsis* (*aba2/gin1*)[6] and tomato (*flc*)[22] strongly suggest that endogenous ABA plays a role in the growth promotion of plants. The mutant plants showed severe growth retardation as compared with the wild-type plants under optimal growing conditions, and treatment with exogenous ABA apparently increased the growth of *flc*, particularly leaf expansion.[22] ABA appears to

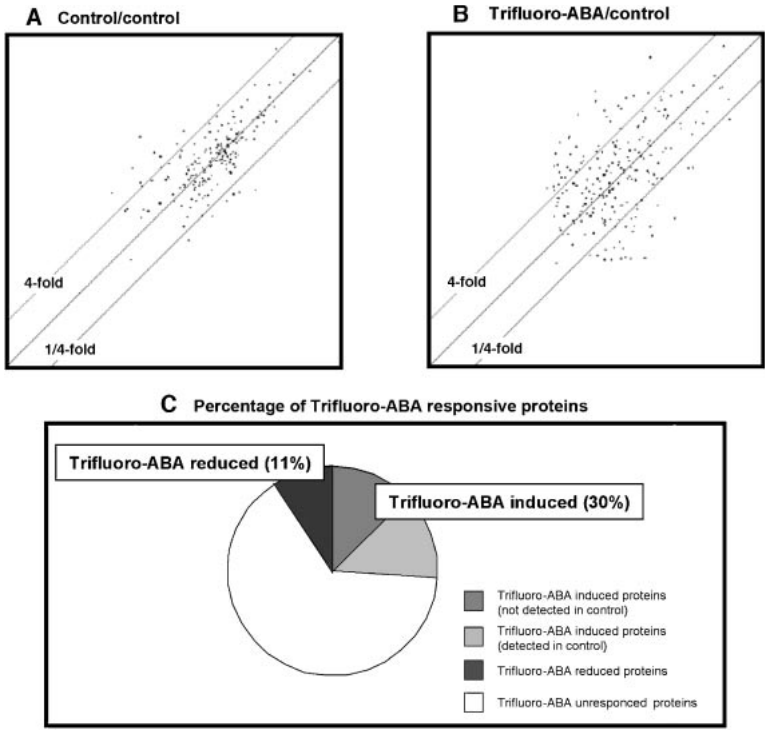

Fig. 5. Effect of Trifluoro-ABA on the Protein Expression in Scutellar Tissues.

Rice seeds imbibed for 3 d were incubated with 10 mM acetate buffer (pH 5.3) containing 1 μM trifluoro-ABA (Trifluoro-ABA) or the buffer alone (control) for an additional 3 d at 30 °C (see Fig. 2). Total protein extracts prepared from the dissected scutellar tissues were subjected to 2D-PAGE (IPGphor). A, Technical repetition: scatter plot comparing the protein spots on 2D-gels prepared in independent control experiments. B, Scatter plot comparing the protein spots on 2D-gels prepared from trifluoro-ABA-treated and control scutellar tissues. The quantitation of protein expression and scatter plot comparison were performed using PDQuest. Upper and lower lines represent 4-fold up and down variations, respectively. C, Ratio of trifluoro-ABA-responsible proteins in scutellar tissues. The ratio of trifluoro-ABA-induced, reduced, and unresponsive proteins were calculated from duplicate experiments.

promote root and shoot growth independent of its effects on plant water balance.[22,23] It is generally accepted that sugars are not only fuel cellular carbon and energy metabolism but also play pivotal roles as signaling molecules.[24] In the scutellar tissues of 15-2 line seeds (α-amylase-deficient) germinated with supplementation of 1.5% sucrose, several sucrose metabolizing and glycolytic enzymes and ATP synthase were markedly induced in comparison with those in the wild type (Fig. 7), strongly suggesting that sugar strength provided by starch degradation coordinates the protein expression in scutellum. Interestingly, trifluoro-ABA was shown to stimulate the sugar uptake and conversion of glucose to sucrose in suspension-cultured cells of rice.[11] The uptake of sugars was also found to be increased in soybean embryo by exogenous ABA.[25] Consequently, we infer that endogenous ABA possesses dual functions: as a negative regulator controlling the breakdown of reserved starch in the endosperm, and as a positive regulator promoting the conversion of glucose to sucrose in the scutellum. The complex interaction of sugar and plant hormones in tissue- and environmental-specific contexts is involved in controlling the growth of plants.[26] The promoting effects of ABA on the scutellar functions might occur in the balance of the signal strengths of ABA-, sugar-, and the other plant hormones.

ABA activates the protein expression involved in central metabolic pathway in scutellum

In the present study, we found that (i) trifluoro-ABA enhances the expression of approximately 30% of the scutellar proteins detected in 2D-PAGE (Fig. 5C), (ii) most of glycolytic and citric acid cycle enzymes, ATP-producing system, and sucrose metabolizing enzymes that increase in a time-dependent manner are included among the trifluoro-ABA-induced proteins (Figs. 4 and 6).

Several components have been identified in the ABA signal transduction pathways in plants, particularly the role of the *cis*- and *trans*-acting promoter elements in the response to ABA.[27] The promoter regions have revealed conserved DNA elements that are ABA-responsive, named ABREs, G-boxes, and ACGT-boxes.[28] Typical ABA-induced proteins in the scutellar tissues, GS-129 and 249 were identified to be OSR40g2 and RAB24 respectively (Table 2 and Fig. 6). A PLACE database search (http://www.dna.affrc.go.jp/htdocs/PLACE/)[29] indicated that the 5′ flanking region of the *OSR40g2* gene contains ACGT-box with a coupling element,[30] and that the *RAB24* promoter has (C/T)ACGTGGC consensus.[31] Like OSR40g2 and RAB24, many glycolytic enzymes, citric acid cycle-ATP producing system, and sucrose metabolizing enzymes were induced by

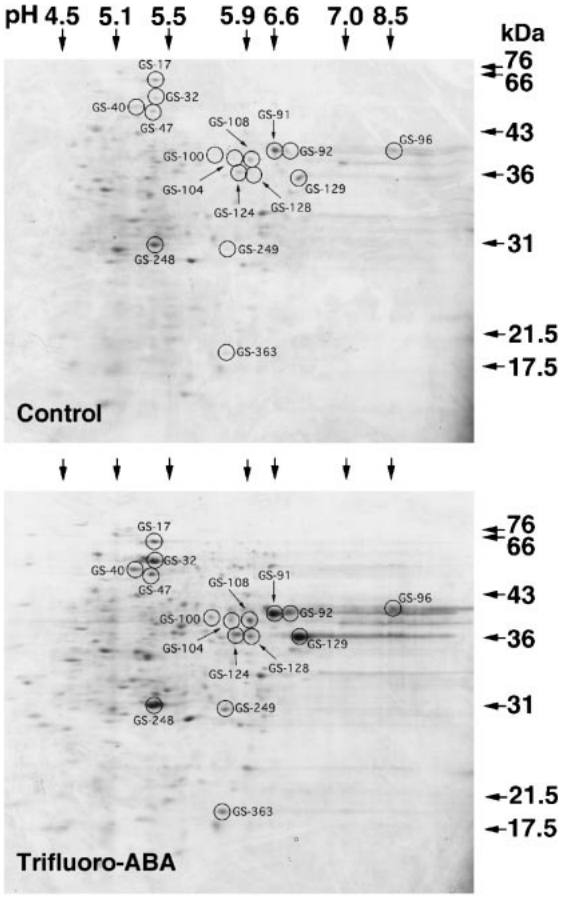

Fig. 6. Comparative Display of Protein Expression Patterns in Scutellar Tissues with and without Trifluoro-ABA Treatment.

The trifluoro-ABA-treated and control scutellar tissues were subjected to 2D-PAGE (IPGphor), as described in Fig. 5. Typical protein spots induced by ABA are circled. These inductions were confirmed in four independent experiments.

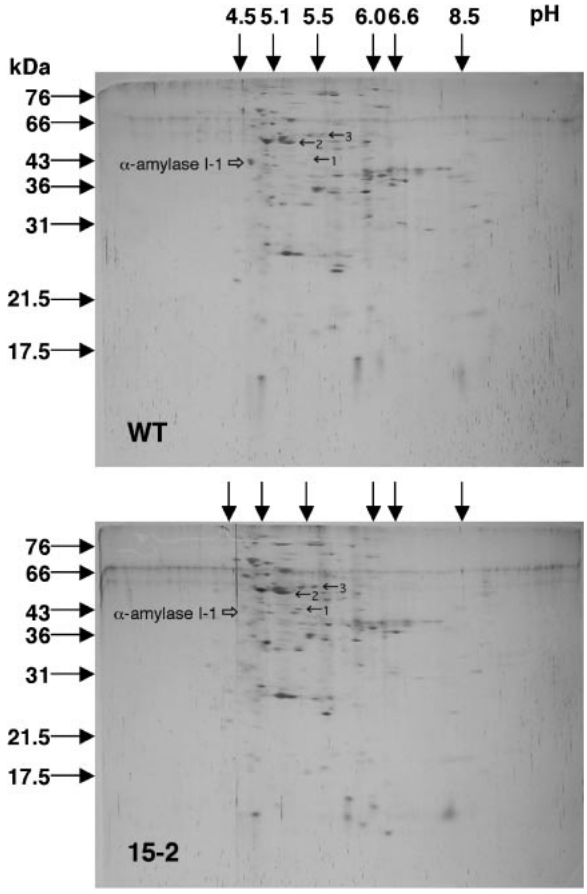

Fig. 7. Comparative Display of Protein Expression Patterns in Scutellar Tissues of 4-d-Germinating Seeds of the 15-2 Line and the Wild Type with Supplementation of 1.5% (w/v) Sucrose.

The protein extract from scutella dissected from each germinating seed was subjected to 2D-PAGE (reverse), followed by silver staining. Arrows indicate α-amylase and typical proteins induced in the 15-2 line. 1, cytosolic 3-phosphoglycerate kinase (ABI74567); 2, UDP-glucose pyrophosphorylase (BAB69069); 3, mitochondrial ATPase beta chain (NP_916979).

ABA treatment (Fig. 6), although we did not find typical ABA-responsive *cis*-acting elements in their promoter regions. The mRNA levels of GAPDH in the desiccation-tolerant plant *Craterostingma plantagineum*[32] and enolase in the common ice plant *Mesembryanthemum crystallium* L.[33] have been reported to be increased by ABA treatment, but the ABA-responsive DNA elements in glycolytic enzyme genes have not yet been identified. In addition to the transcriptional and translational regulation of genes encoding glycolytic enzymes, the proteolytic turnover of enzyme molecules is regulated possibly.[34] As presented in Fig. 6, the appearance of GS-417, that is thought to be an intermediate of GAPDH degradation, is reduced by trifluoro-ABA. Thus, the glycolytic systems in scutellar tissues might be regulated by ABA transcriptionally, translationally, and posttranslationally, though the precise mechanism of regulation is totally obscure.

Acknowledgments

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