Induction of phytic acid synthesis by abscisic acid in suspension-cultured cells of rice.

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Abstract

A pathway of phytic acid (PA) synthesis in plants has been revealed via investigations of low phytic acid mutants. However, the regulation of this pathway is not well understood because it is difficult to control the environments of cells in the seeds, where PA is mainly synthesized. We modified a rice suspension culture system in order to study the regulation of PA synthesis. Rice cells cultured with abscisic acid (ABA) accumulate PA at higher levels than cells cultured without ABA, and PA accumulation levels increase with ABA concentration. On the other hand, higher concentrations of sucrose or inorganic phosphorus do not affect PA accumulation. Mutations in the genes *RINO1*, *OsMIK*, *OsIPK1* and *OsLPA1* have each been reported to confer low phytic acid phenotypes in seeds. Each of these genes is upregulated in cells cultured with ABA. *OsITPK4* and *OsITPK6* are upregulated in cells cultured with ABA and in developing seeds. These results suggest that the regulation of PA synthesis is similar between developing seeds and cells in this suspension culture system. This system will be a powerful tool for elucidating the regulation of PA synthesis.

Abbreviations

ABA, abscisic acid; PA, phytic acid; LPA, low phytic acid; Pi, inorganic phosphate

Keywords

Oryza sativa, ABA, Suspension-cultured cells, Phytic acid synthesis, Developing seeds

1. Introduction

Phytic acid (PA), or *myo*-inositol-1,2,3,4,5,6-hexa*kis*phosphate, chelates inorganic cations (e.g. Ca^{2+}, Fe^{2+}, and Mg^{2+}), producing insoluble salts (phytate). Most of the phosphorus in seeds is stored in the form of phytate, as reviewed by Raboy [1, 2]. Monogastric animals (e.g. swine and poultry) cannot digest and absorb phytate and their waste, containing phytate, causes phosphorus pollution of aquatic environments. In order to decrease the loading of PA into environmental waters, low phytic acid (LPA) crops have been developed, as reviewed by Rasmussen et al. [3].

The biosynthesis pathway of PA in seeds has been elucidated through studies of LPA plants. In the first step, glucose-6-phosphate is converted to *myo*-inositol-3-phosphate by myo-inositol-3-phosphate synthase. Two pathways, a lipid-dependent and a lipid-independent pathway, have been proposed for the subsequent steps in PA synthesis. The lipid-independent pathway may be the main route in plant seeds because mutations in the inositol-1,3,4-trisphosphate-5/6-kinase (*ITPK*) genes result in LPA phenotypes in *Z. mays* [4] and *A. thaliana* [5]. Finally, PA is stored in the vacuole via the ABC transporter [6, 7].

In addition to the development of LPA crops, another solution to the problem of high phosphorus levels in polluted water may be the development of phosphorus-hyperaccumulating plants, which will take up and accumulate phosphorus at high levels. However, an excess of inorganic phosphorus (Pi) is toxic to plants [8]. On the other hand, insoluble phytate is a non-toxic storage form of phosphorus, so high phytate plants could accumulate phosphorus at high levels. The overexpression of a positive regulator of PA synthesis might result in a high phytate phenotype. In a similar approach, the genes (*ZmLEC1* and *ZmWRI1*) encoding transcription factors for oil production in developing seeds were overexpressed in maize plants, resulting in increased oil production in the seeds [9]. However, the transcription factors controlling PA synthesis have not yet been identified.

Phytic acid accumulates mainly in the aleurone layers of rice seeds from 7 to 20 days after flowering [10, 11]. Starch and abscisic acid (ABA) accumulate during the same period, suggesting that ABA regulates the accumulation of starch [12]. In addition, rice cells that are suspension-cultured with ABA and sucrose accumulate large amounts of starch [13]. Furthermore *RINO1*, which encodes a key enzyme in PA synthesis, is upregulated by ABA and sucrose treatments in cultured cells [14]. These results suggest that PA synthesis may be regulated by ABA and/or sucrose.

In this study, we cultured rice cells with various concentrations of ABA, sucrose, and inorganic phosphate (Pi), and demonstrated that PA levels increase with increasing ABA concentrations, but are not affected by changes in the concentrations of sucrose or Pi. We then analyzed the expression patterns of 15 genes related to PA synthesis in the presence of ABA. Six genes, including *RINO1*, were upregulated in the ABA-treated cells. The same genes are also expressed at high levels in developing seeds. The results suggest that this cell culture system provides a useful tool for studying the regulation of PA synthesis.

2. Materials and methods

2.1. Plant material

Rice (*Oryza sativa* L. ssp. *Japonica* cv. Nipponbare) seeds were sterilized by immersion in 70% ethanol for 1 min and then in sodium hydrochloride (5% available chloride) for 20 min. After 5 rinses in sterile deionized water, the seeds were placed on solid N6 medium [15] supplemented with sucrose (30 g/L), proline (10 mM), casein hydrolysate (300 mg/L), and 2,4-dichlorophenoxyacetic acid (2 mg/L), and incubated at 28°C for 3 weeks. Friable calli were transferred to 15 mL of liquid N6 medium in a 100 mL flask. The calli were cultured in the dark at 28°C on a reciprocal shaker (115 strokes per min), and the resulting suspension-cultured cells were subcultured every 5 days.

To evaluate the effects of ABA, sucrose, and Pi on the accumulation of PA and on genes related to PA biosynthesis, cells were passed through a mesh (opening: 1.5 mm) and cultured in fresh medium for 2 days. Then the cells were passed through another mesh (opening: 1.75 mm) and transferred to

fresh medium containing 1 mg/L 2,4-dichlorophenoxyacetic acid. Twenty four hours after transfer, ABA, sucrose, and/or Pi was added to the medium, and the culture was continued for various time intervals. The cells were then harvested by filtration under reduced pressure. The cells were desiccated at 80°C to measure PA and Pi contents, or frozen in liquid nitrogen and stored at -80°C for gene expression analyses. The medium was stored at -20°C until measurement of its Pi concentration.

2.2. Measurement of PA and Pi

PA was extracted and analysed using a modified ferric chloride precipitation method [16,17]. Ten to twelve mg of dried cells were milled with beads in a 2.0 mL tube, immersed in 1 mL of 0.8 N HCl and then boiled for 10 min to extract the PA and Pi. After centrifugation at 15,000 rpm for 10 min at room temperature, one part (200 μL) of the supernatant was stored at -20°C until measurement of the Pi content in the cells. Another part (500 μL) was transferred to a new 1.5 mL tube, mixed with 300 μL of 1% $FeCl_3$-$6H_2O$/1.0 N HCl, and boiled for 45 min to form an Fe-PA complex. A cooled sample was centrifuged at 15,000 rpm for 10 min at 4°C, and the precipitate was incubated with 400 μL of 0.5 N HCl for 2 h at room temperature to wash the precipitate. After centrifugation as described above, the precipitate was washed twice with 200 μL of 0.5 N HCl for 10 min. After re-centrifugation as described above, the precipitate was dissolved in 1 mL of 0.45 N NaOH and boiled for 15 min. The cooled sample was centrifuged as described above and the supernatant was hydrolyzed in 2 N HCl at 110°C for 24 hours. The Pi concentrations in the hydrolyzed supernatant and in the original cell extract were measured using a modified molybdenum blue method [18]. The PA content in the cells was calculated from the hydrolyzed Pi content: the ratio of PA to Pi is 1 to 6 on a molar basis.

2.3. Quantitative RT-PCR

Cells were cultured for 15 min, 40 min, 90 min, 4 h, 9 h, 16 h, or 24 h after adding ABA to the medium, then harvested. Total RNA was extracted using the ISOGEN® reagent (Nippon Gene), then treated with DNaseI and reverse transcribed using the PrimeScript® RT Master Mix (TaKaRa Bio)

following the manufacturer's instructions. Gene expression was quantified by real-time PCR using the SYBR® *Premix Ex Taq*™ II (TaKaRa Bio) and the Thermal Cycler Dice® Real Time System (TaKaRa Bio). The standard curve method was followed, and primers for the rice actin 1 gene *OsAct1* were used as an internal control.

3. Results

3.1. PA accumulation in suspension-cultured cells was triggered by ABA

Cells were cultured for 24 h with ABA in a range of concentrations from 0 μM to 50 μM. The PA contents of these cells increased in a range from 1.3-fold higher (with 0.05 μM ABA) to 2.5 fold higher (with 50 μM ABA) than the PA contents of cells treated without ABA (Fig. 1). On the other hand, the Pi contents in the same cells were reduced to 96-66% of the Pi contents of cells treated without ABA. Therefore, the ABA treatment affects the PA and Pi contents of the cells in a concentration-dependent manner.

Sucrose and inorganic phosphorus (Pi) are both substrates of PA synthesis. To investigate whether increased concentrations of these substrates would facilitate PA synthesis, cells were cultured for 24 h under 6 conditions: 0 μM ABA, 3% sucrose, 2.9 mM Pi; 0 μM ABA, 9% sucrose, 2.9 mM Pi; 0 μM ABA, 3% sucrose, 8.8 mM Pi; 17 μM ABA, 3% sucrose, 2.9 mM Pi; 17 μM ABA, 9% sucrose, 2.9 mM Pi; and 17 μM ABA, 3% sucrose, 8.8 mM Pi. After these treatments the PA and Pi contents of the cells were measured (Fig. 2). All cells treated with ABA (treatments C2, S2, and P2 in Fig. 2) showed significantly higher levels of PA and lower levels of Pi than cells treated without ABA (C0, C1, S1, and P1). Cells cultured with 9% sucrose (treatments S1 and S2) exhibited slightly but significantly lower PA and Pi contents than cells cultured with 3% sucrose and the same amount of ABA (treatments C1 and C2, respectively). Cells cultured with 8.8 mM Pi (treatments P1 and P2) exhibited significantly higher Pi contents but similar PA contents, compared with cells treated with 2.9 mM Pi and the same amount of ABA (treatments C1 and C2, respectively). The results indicate that PA synthesis is not facilitated by sucrose or phosphorus.

We also evaluated the accumulation of PA in cells over a period of six days. Cells were subcultured into fresh medium, and ABA was added to the medium 24 h later. Every 24 h after the addition of ABA, the cells were sampled to measure their dry weights, water contents, Pi concentrations, PA contents, and Pi contents (Fig. 3). The dry weights increased over the six days but did not differ between cells cultured with ABA and cells cultured without ABA. On the other hand, the water contents of the ABA-treated cells were lower than those of the untreated cells between 24 h and 120 h after addition of the ABA. In the ABA-treated cells the mean PA content was 4.4 nmol/mg at 0 h, it increased to 22.2 nmol/mg at 72 h, and then deceased to 13.6 nmol/mg at 144 h. The untreated control cells showed only slight increases in PA content between 0 and 72 h. The Pi contents of the ABA-treated cells decreased throughout the sampling period. On the other hand, the Pi contents of the untreated cells remained between 203 and 210 nmol/mg during the first 72 h of culture, and decreased thereafter. At 72 h after adding ABA, the Pi concentrations were 240 and 268 μM in ABA-treated cells and untreated cells, respectively. At 96 h, the concentrations had decreased to 7.57 and 7.94 μM, respectively.

To more closely examine the initiation of PA synthesis after addition of ABA, the PA and Pi contents were measured at shorter intervals (15 min, 40 min, 90 min, 4 h, 9 h, 16 h, and 24 h) after treatment of cells with or without ABA (Fig. 4). At 9 h, the mean PA content of untreated cells was 4.93 nmol/mg, whereas the mean content in ABA-treated cells was significantly higher at 8.68 nmol/mg. During the first 9 h the Pi content in treated cells decreased to 155 nmol/mg, whereas the Pi content in untreated cells remained at the same level as at the beginning of the experiment (about 188 nmol/mg). Previous studies have shown that reduced PA levels in LPA seeds are correlated with increased amounts of phosphorus as Pi and inositol polyphosphate [6, 19-21]. In this suspension culture systems, the amounts of phosphorus as PA and Pi were stoichiometrically consistent between treated and untreated cells at 24 h after adding ABA, but not consistent at 16 h after adding ABA.

3.2. Genes related to the synthesis of PA are up-regulated by ABA

The expression levels of 15 genes related to PA synthesis were analyzed in cells cultured with 17 μM ABA for 15 min, 40 min, 90 min, 4 h, 9 h, 16 h, and 24 h (Fig. 5). The expression levels of nine genes: *RINO1*, *OsMIK*, *OsITPK1*, *OsITPK3*, *OsITPK4*, *OsITPK6*, *OsIPK1*, *OsMRP5*, and *OsLPA1* increased within 15 min after adding ABA and peaked at 90 min to 4 h. These changes were not observed in the untreated cells. Six genes, *RINO1*, *OsMIK*, *OsITPK4*, *OsITPK6*, *OsIPK1*, and *OsLPA1*, showed higher expression levels in treated cells than in untreated cells throughout the experimental period. In contrast, the expression of *OsIPK2* decreased after the addition of ABA.

4. Discussion

We cultured rice cells with 0 to 50 μM ABA and measured the PA contents in the cells. The PA contents increased with increasing concentrations of ABA (Fig. 1). This effect was not observed when cells were treated with high concentrations of sucrose or Pi (Fig. 2). The PA contents gradually increased and the Pi contents decreased during 72 h after ABA (17 μM) was added to the cells (Fig. 3 and Fig. 4). Six genes related to PA synthesis, *RINO1*, *OsMIK*, *OsITPK4*, *OsITPK6*, *OsIPK1*, and *OsLPA1*, showed sustained upregulation after ABA treatment (Fig. 5).

Previous reports have shown that PA accumulates in the aleurone layers of rice seeds between 7 and 20 days after flowering [10, 11]. In addition, the ABA concentration in developing seeds increases after flowering and reaches a maximum between 14 and 21 days after flowering [12, 22]. These findings suggest that ABA may be a regulator of PA synthesis in developing seeds. We show a clear, concentration-dependent relationship between ABA treatment and PA accumulation in suspension-cultured cells (Fig. 1), and thus confirm that PA synthesis in suspension-cultured cells is regulated by ABA. On the other hand, our results show that Pi and sucrose are not key regulators of PA synthesis.

Fifteen genes [7, 23-26] have been reported or predicted as enzymes or transporters related to PA synthesis and storage, and these genes were used in an expression analysis in this study. Gene disruption experiments showed that the downregulation of five of these genes: *RINO1*, *OsIPK1*, *OsMIK*, *OsMRP5*, and *OsLPA1* resulted in LPA phenotypes [24-29]. The results suggest that these

five genes may play important roles in PA synthesis in developing rice seeds. In addition, knockout mutations in three *ITPK* homologs in *Zea mays* and *Arabidopsis thaliana* resulted in LPA seeds [4, 5]. Six *IPTK* homologs (*OsITPK1-6*) have been annotated in rice [23], however there have been no reports of mutations in these genes causing LPA phenotypes. Expression analyses using qPCR and microarrays have shown that *OsITPK4* and *OsITPK6* are upregulated during the early stages of rice seed development [23, 30]. Furthermore, the ITPK protein(s) have been shown to function as inositol polyphosphate kinases [31, 32] Taken together, these observations suggest that *OsITPK4* and *OsITPK6* may also contribute to PA synthesis in developing rice seeds.

Six genes: *RINO1, OsMIK, OsITPK4, OsITPK6 OsIPK1*, and *OsLPA1* were upregulated in the present study (Fig. 5), suggesting that these genes contribute to PA synthesis in suspension-cultured cells. Interestingly, the same six genes have been implicated to play roles in PA synthesis in developing seeds as described above. Therefore, it is possible that these ABA-responsive genes function in PA synthesis in both cultured cells and developing seeds.

In our experiments the PA contents of cultured cells were increased after ABA treatment, whereas the Pi contents decreased, and the total phosphorus contents in the PA plus the Pi were the same between ABA-treated and untreated cells (Fig. 3, Fig. 4). Similarly, the total phosphorus content was conserved in LPA seeds [6, 19-21]. That is, while the PA content was decreased by mutations or RNAi suppression of genes related PA synthesis, the contents of other inositol polyphosphates and Pi were increased. Consequently, the total amount of phosphorus in the seeds was unchanged. The present study also implies that changes in PA synthesis do not affect the influx of phosphorus.

Suspension-cultured cells of bromegrass showed reduced water contents (on a dry-weight basis) in response to ABA treatment (75 μM). In that case the water content per cell was not changed but the dry weight per cell increased [33]. In this study the dry weight was not affected, but the water content per mg of dry weight was decreased in response to ABA treatment. It is most likely that the dry weight per cell was also increased by ABA treatment in this study, but the cell division rate was decreased, resulting in unchanged total dry weights.

In conclusion, we have developed a rice suspension culture system in which PA accumulation can be manipulated. This system will be a useful tool for elucidating the regulatory networks controlling PA synthesis. We have uncovered many parallels in the regulation of PA synthesis between developing seeds and suspension-cultured cells. Thus, further investigations on the regulation of PA synthesis in suspension-cultured cells may provide critical insights regarding the regulation of PA synthesis in developing seeds.

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Figure 1. The effects of ABA on the PA and Pi contents of cultured cells.

Rice cells were cultured with various concentrations of ABA (0–50 μM) for 24 h. The cells were then desiccated and used for measurements of Pi content (A) and PA content (B), each expressed as nmol/mg dried cells. The "Pi+PA content" is the total phosphorus content in the Pi and PA (PI + 6 × PA) expressed as nmol P/mg dried cells, and is shown for each time point in (C). Values shown are means and error bars represent standard deviations (n=4).

Figure 2. Effects of sucrose and phosphorus on PA and Pi contents.

Rice cells were cultured for 24 h under 6 conditions: C1: 0 μM ABA, 3% sucrose, 2.9 mM Pi; S1: 0 μM ABA, 9% sucrose, 2.9 mM Pi; P1: 0 μM ABA, 3% sucrose, 8.8 mM Pi; C2: 17 μM ABA, 3% sucrose, 2.9 mM Pi; S2: 17 μM ABA, 9% sucrose, 2.9 mM Pi; and P2: 17 μM ABA, 3% sucrose, 8.8 mM Pi. Control cells (C0) were harvested without treatment. The cells were desiccated and used for measurements of Pi content (A) and PA content (B). Values shown are means and error bars represent standard deviations (n=4).

Figure 3. The effects of ABA on cultured cells.

Rice cells were cultured with or without 17 μM ABA and harvested every 24 h for 6 days (144 h). The dry weights (A) and water contents [mg water/mg dried cell] (B) were obtained by weighing the cells before and after desiccation. The Pi concentrations in the media (C) and the PA (D) and Pi (E) contents in the dried cells were measured as described in the Materials and Methods. The "Pi+PA content" is the total phosphorus content in the Pi and PA (PI + 6 × PA) expressed as nmol P/mg dried cells, and is shown for each time point in (F). The diamonds represent controls (0 μM ABA) and the triangles represent ABA-treated cells. Values shown are means and error bars represent standard deviations (n=4).

Figure 4. Initiation of PA accumulation by ABA treatment.

Rice cells were cultured with or without 17 μM ABA and harvested at 15 min, 40 min, 90 min, 4 h, 9 h, 16 h and 24 h after ABA treatment. The PA content (A) and Pi content (B) were measured as described in the Materials and Methods. The "Pi+PA content" (C) is as described for Fig. 1. The diamonds and dotted line indicate controls (0 μM ABA) and triangles and solid line indicate ABA-treated cells. Values shown are means and error bars represent standard deviations (n=3).

Figure 5. Relative expression levels of genes involved in PA synthesis in suspension-cultured cells. Quantitative RT-PCR was used to examine the expression levels of 15 genes in cells cultured with 17 μM ABA (represented by triangles and solid lines) or without ABA (represented by diamonds and dotted lines). *OsAct1* was used as an internal control and the 0 h point was used as the reference value. Values shown are means and error bars represent standard deviations (n=3). Cultivation and sampling of cells was conducted in duplicate.

Supplementary Table S1. Primers used for qRT-PCR

Supplementary Table S1

		Primer (5' → 3')	
Gene	Accession number	F	R
RINO1	AK103501	GCGAATGGGATGGAGCAAGAAAG	AGTCTCACACAAAGCACAGAAGGT
RINO2	AK100872	ACAAGAGGCCAAGTGGGGAAG	AGTGTCAAACTGTCAATAGATGCCC
OsIMP1	AK071149	CTTCGGGAAGCTGGTGGTTTTG	TGTTTGATCCTGCCATTCTTCGTG
OsIMP2	AK103039	CCGTCTTTGATCGTTCTGTCCTTG	AAGTCGGTAGGGTATTTGTCGGG
OsMIK	AK243444	ATCTGTGTAAACACATGACCCAAC	TTCTGCACTCAGCGATTCCCA
OsITPK1	AK106544	GGTCAAGGAGATGTTAGCTTTGAG	TTACACAGGTTGGGCACGGA
OsITPK2	AK100971	CGGCTGAAGTGCAAAGAGTCC	GCCACCATTTTCTGGGAAGACAC
OsITPK3	AK067068	TCATGCGAGGGGTGAAACGA	TGATGCCCAACTTTACCACGGA
OsITPK4	AK071209	CCGACCCAGCGTTTGAACA	GGTTTAGCTTGCACTTGTAGCATTT
OsITPK5	AK059148	TGATGAGGGGAATAGCGAGCAAG	AGGATTTACACCGCAGGTAGGG
OsITPK6	AK102571	GCATACAGGCGACAACATTCATAC	TCATCGCAAGCAGTTCCACAAATC
OsIPK1	AK102842	GTTCAGCTCCAACATTGACGACC	TGGACATGCACACTTGTAGAAACAC
OsIPK2	AK072296	CGGTAACGTAAATGCGAAGCGA	AGTAAGATCCAATGCACCCCGAA
OsMRP5	AK121451	CCCAGCCAAAATTCACTCGCTC	CTCCTCTCACCAACTCCCTCAAC
OsLPA1	AK100921	AAAACAAATCCAAAAGCGAAGAC	TCTCGGCGTCCTCTTCTACC
OsAct1	AK100267	CCCAAGGCCAATCGTGAGAAG	ACCATCACCAGAGTCCAACACAA

Figure1

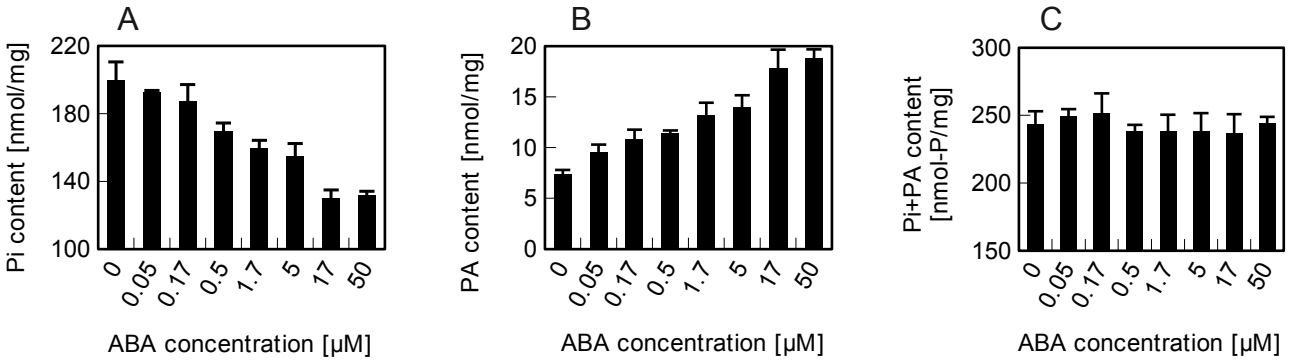

A
Pi content [nmol/mg]
220
180
140
100
0
0.05
0.17
0.5
1.7
5
17
50
ABA concentration [μM]
B
PA content [nmol/mg]
20
15
10
5
0
0
0.05
0.17
0.5
1.7
5
17
50
ABA concentration [μM]
C
Pi+PA content [nmol-P/mg]
300
250
200
150
0
0.05
0.17
0.5
1.7
5
17
50
ABA concentration [μM]

Figure2

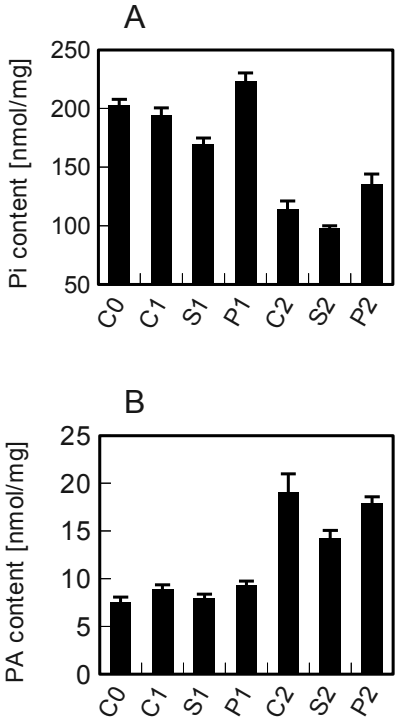

A
Pi content [nmol/mg]
250
200
150
100
50
C0
C1
S1
P1
C2
S2
P2
B
PA content [nmol/mg]
25
20
15
10
5
0
C0
C1
S1
P1
C2
S2
P2

Figure3

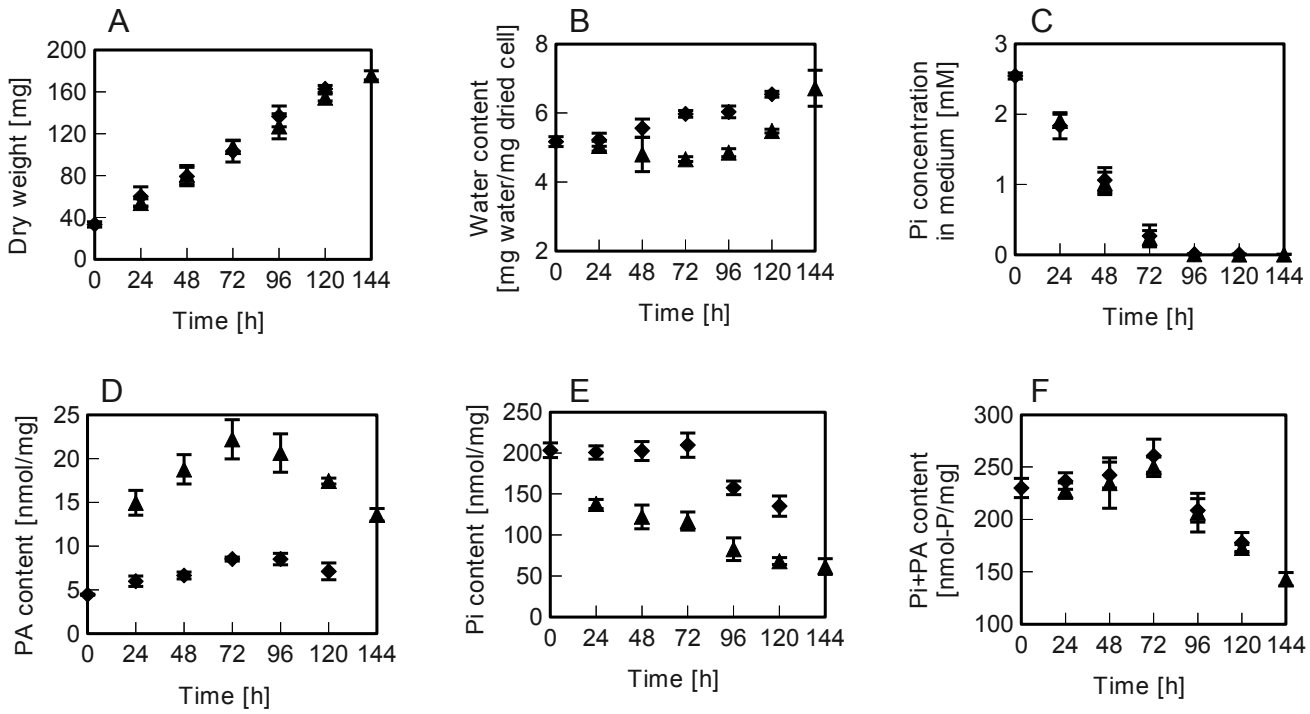

A
Dry weight [mg]
200
160
120
80
40
0
0 24 48 72 96 120 144
Time [h]
B
Water content
[mg water/mg dried cell]
8
6
4
2
0 24 48 72 96 120 144
Time [h]
C
Pi concentration
in medium [mM]
3
2
1
0
0 24 48 72 96 120 144
Time [h]
D
PA content [nmol/mg]
25
20
15
10
5
0
0 24 48 72 96 120 144
Time [h]
E
Pi content [nmol/mg]
250
200
150
100
50
0
0 24 48 72 96 120 144
Time [h]
F
Pi+PA content
[nmol-P/mg]
300
250
200
150
100
0 24 48 72 96 120 144
Time [h]

Figure4

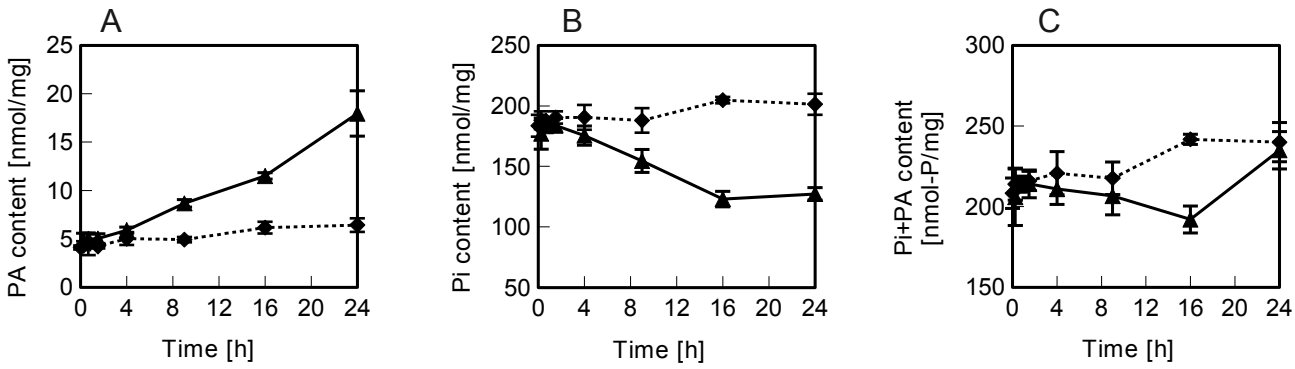
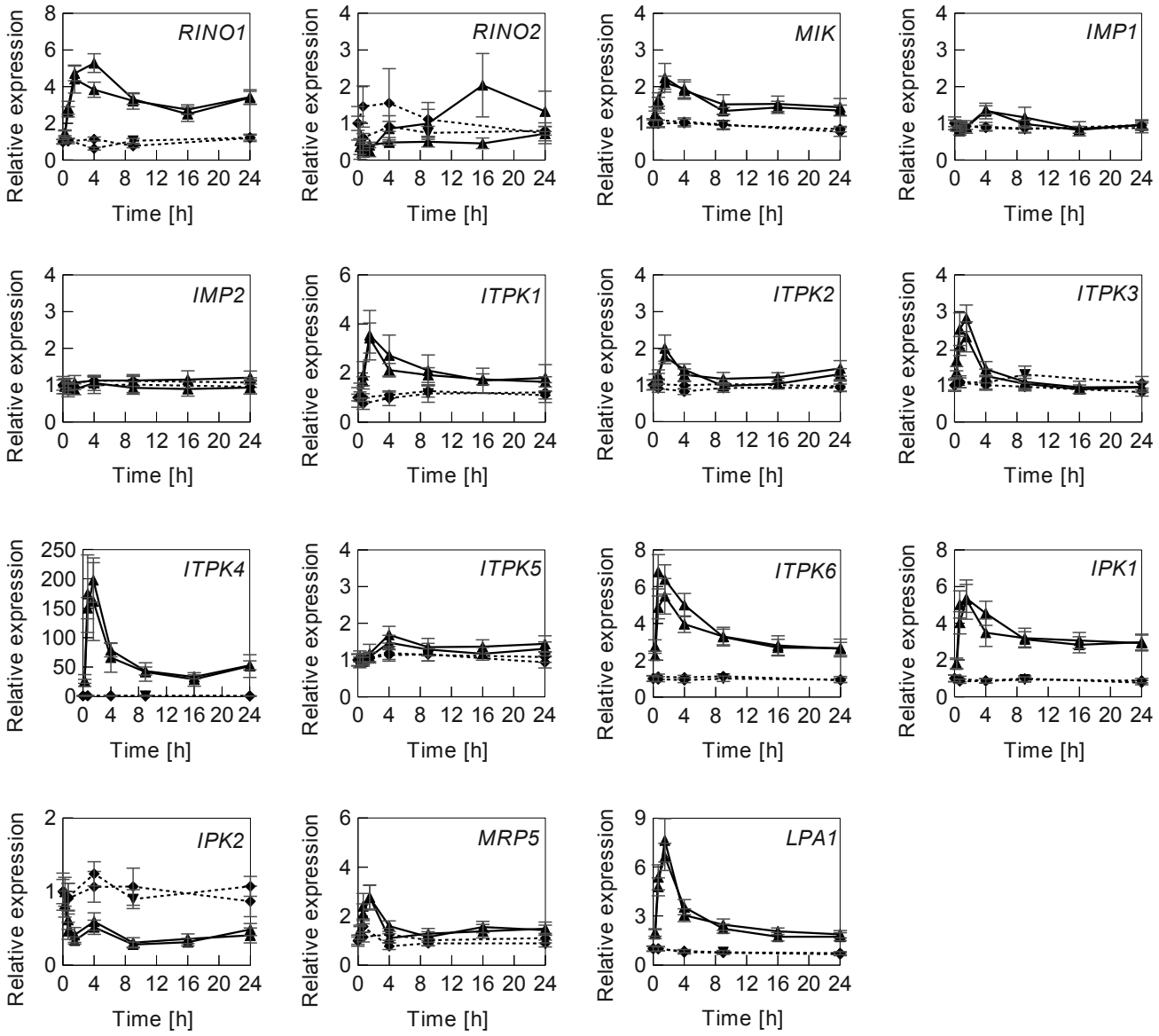

A
PA content [nmol/mg]
25
20
15
10
5
0
0 4 8 12 16 20 24
Time [h]
B
Pi content [nmol/mg]
250
200
150
100
50
0 4 8 12 16 20 24
Time [h]
C
Pi+PA content [nmol-P/mg]
300
250
200
150
0 4 8 12 16 20 24
Time [h]

Figure5


RINO1
RINO2
MIK
IMP1
IMP2
ITPK1
ITPK2
ITPK3
ITPK4
ITPK5
ITPK6
IPK1
IPK2
MRP5
LPA1
Relative expression
Time [h]