Comparative phosphoproteomic analysis of blast resistant and susceptible rice cultivars in response to salicylic acid

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Abstract

Background: Salicylic acid (SA) is a significant signaling molecule that induces rice resistance against pathogen invasion. Protein phosphorylation carries out an important regulatory function in plant defense responses, while the global phosphoproteome changes in rice response to SA-mediated defense response has not been reported. In this study, a comparative phosphoproteomic profiling was conducted by two-dimensional gel electrophoresis (2-DE) and mass spectrometry (MS) analysis, with two near-isogenic rice cultivars after SA treatment.

Results: Thirty-seven phosphoprotein spots were differentially expressed after SA treatment, twenty-nine of which were identified by MALDI-TOF/TOF MS, belonging to nine functional categories. Phosphoproteins involved in photosynthesis, antioxidative enzymes, molecular chaperones were similarly expressed in the two cultivars, suggesting SA might alleviate decreases in plant photosynthesis, regulate the antioxidant defense activities, thus improving basal resistance response in both cultivars. Meanwhile, phosphoproteins related to defense, carbohydrate metabolism, protein synthesis and degradation were differentially expressed, suggesting phosphorylation regulation mediated by SA may coordinate complex cellular activities in the two cultivars. Furthermore, the phosphorylation sites of four identified phosphoproteins were verified by NanoLC-MS/MS, and phosphorylated regulation of three enzymes (cinnamoyl-CoA reductase, phosphoglycerate mutase and ascorbate peroxidase) was validated by activity determination.

Conclusions: Our study suggested that phosphorylation regulation mediated by SA may contribute to the different resistance response of the two cultivars. To our knowledge, this is the first report to measure rice phosphoproteomic changes in response to SA, which provides new insights into molecular mechanisms of SA-induced rice defense.

Keywords: Salicylic acid, Rice, Phosphoproteome, Two-dimensional gel electrophoresis, Protein phosphorylation

Background

Rice (Oryza sativa L.) is an economically important cereal crop throughout the world, providing food for over 50% of global population [1]. The ascomycetous fungus Magnaporthe oryzae causes the rice blast, one of the most devastating fungal diseases in rice production and thus poses a great threat to the world’s food security [2]. Thus far, the disease control is mainly based on using fungicides and breeding resistant cultivars. However, fungicide could fail to satisfy the requirement of environment and human health regulations, and resistant cultivars could be overcome by the quick arising/evolving of new races of M. oryzae [3, 4]. As an important signaling molecule, salicylic acid (SA) can induce plant resistance against multiple fungal, viral and bacterial pathogens [5]. SA-mediated plant defense responses are actively involved in both PTI (PAMP-triggered immunity) and ETI (effector-triggered immunity) [6].

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the previous study, we confirmed that SA can protect rice against infection by *M. oryzae* race ZC13 in CO39 (susceptible cultivar) and in a near isogenic line C101LAC, which carries the resistance gene *Pi-1* against *M. oryzae* race ZC13 and thus represents a resistant cultivar [7]. Proteomic analysis further showed that SA coordinates multiple cellular activities to facilitate defense response and recovery in both rice cultivars. However, it awaits further elucidation about the detailed molecular mechanisms of SA-induced rice defense response against *M. oryzae*.

Phosphorylation is one of the most important post-translational protein modifications (PTMs), regulating a wide range of cellular functions in various organisms, including cell signaling, metabolism, stress responses and defense responses [8]. Phosphoproteomics can capture the dynamics and specificity of protein phosphorylation, and therefore enhance our understanding of fundamentals and complex biological processes [9]. In recent years, a large number of emerging evidences suggested that protein phosphorylation can regulate plant stress responses triggered by exogenous hormone and biotic stress [10–13]. It has been also shown that protein phosphorylation was involved in the activation of SA-induced plant resistance using traditional biochemical methodologies [14, 15]. To our knowledge, the general scope of such connections between protein phosphorylation events and SA-induced rice resistance has not been studied.

In the present study, we performed a comparative phosphoproteome to reveal the detail SA-induced mechanism in rice using two-dimensional gel electrophoresis (2DE), Pro-Q diamond phosphoprotein stain and MALDI-TOF/TOF mass spectrometer. Thirty-seven SA-responsive phosphoprotein spots were found and twenty-nine of them were identified. Phosphoproteins involved in similar or different function and expression patterns in resistant and susceptible rice cultivars were discussed. The results provided new insights about the dynamic phosphoproteomes at different time points in rice upon SA treatment, and broadened the understanding of SA-mediated rice resistance against *M. oryzae* infection via regulation on protein phosphorylation.

**Results**

**Specificity analysis of MOAC-enriched putative phosphoproteins from rice leaves**

A total of 490 ± 15 μg putative phosphoproteins were enriched from 8 mg of total proteins. To test the specificity of MOAC for phosphoproteins, MOAC-enriched putative phosphoproteins were separated by 2DE and sequentially stained for phosphoproteins using Pro-Q Diamond, and for total proteins by silver stain (Fig. 1a, b). 481 ± 9 protein spots could be detected on Pro-Q Diamond-stained gels and 469 ± 12 spots on sequential silver-stained gels; of these spots, 466 were common to the two staining methods (Fig. 1c). To determine the

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**Fig. 1** 2DE analysis of MOAC-enriched putative phosphoproteins from rice leaves. Original-color image of the same 2DE gel was stained by a Pro-Q diamond and b silver nitrate. c Venn diagram analysis of MOAC-enriched putative phosphoproteins in 2DE gels that overlapped between Pro-Q Diamond staining (green) and silver staining (red). False-color images of 2DE gels were visualized with different colors using PDQuest software; d Pro-Q diamond-stained protein spots were colored green and e silver-stained protein spots were colored red. f An overlay of the two images (d and e). The phosphoprotein spots were appeared yellow, and the non-phosphoprotein spots were appeared red (as shown by arrows). Asterisks indicate spots that were very abundant in the silver image but lightly stained in the Pro-Q Diamond image.
investigate further.

changes to these protein spots, which we could in-
treatment resulted in reproducible and significant
(Additional file 1: Fig. S4). We conclude that SA
of SA-responsive phospho proteins were displayed in
these two cultivars. A close-up view of the SA-responsive
phosphoprotein spots were detected in CO39 and
C101LAC, respectively; 21 of which were common in
Figure S1). For each cultivar, 30 and 28 SA-responsive
were obtained from the two cultivars (Additional file 1:
respectively (Additional file 1). Fold changes above 1.5 in
all three replicates were used as thresholds to determine
the SA-responsive phosphoproteins. Additionally, differ-
tential expression pattern was shown to be similar in all
three replicates, and we manually checked all the spots
to ensure confidence in differentially regulated phospho-
proteins. A total of 37 SA-responsive phosphoproteins
were obtained from the two cultivars (Additional file 1:
Figure S1). For each cultivar, 30 and 28 SA-responsive
phosphoprotein spots were detected in CO39 and
C101LAC, respectively; 21 of which were common in
these two cultivars. A close-up view of the SA-responsive
phosphoproteins on the 2DE gels was shown in
(Additional file 1: Figure S3). The relative intensities
of SA-responsive phosphoproteins were displayed in
(Additional file 1: Fig. S4). We conclude that SA
treatment resulted in reproducible and significant
changes to these protein spots, which we could in-
vestigate further.

MALDI-TOF/TOF identification of SA-responsive
phosphoproteins

The 37 SA-responsive phosphoprotein spots were ex-
cised from 2DE gels and further identified by MALDI-
TOF/TOF MS. Of these, 29 phosphoproteins were
identified with high confidence (Table 1), while the
remaining 8 phosphoprotein spots (2, 10, 19, 22, 23, 26,
32 and 35) did not show a good match in the database.

The results of protein spot 11 were provided as an ex-
ample of analysis (Additional file 1: Figure S5). Gener-
ally, one protein spot in 2DE gel represented one unique
protein. However, we noted that three phosphoproteins
were identified in more than one spot in the same gel
(Table 1, Additional file 1: Figure S1). For example, three
phosphoprotein spots (11, 12 and 13) were identified as
probable glutamyl endopeptidase, two (17 and 18) as
glyceraldehyde-3-phosphate dehydrogenase, two (24
and 25) as alpha 1,4-glucan phosphorylase. Consistent with
our results, it has been reported that protein isoforms
migrated as a chain of spots, most likely due to post-
translational modifications [16]. Proteins with multiple
phosphorylation states could also lead to electrophoresis
patterns that multiple spots are with similar molecular
weight but different pI [17].

Based on biological annotations from UniProtKB data-
base (www.uniprot.org), the 29 identified SA-responsive
phosphoproteins were functionally classified into 9
groups: photosynthesis, defense, antioxidative enzymes,
protein synthesis and degradation, molecular chaper-
one, amino acid metabolism, carbohydrate metabolism,
energy metabolism and other metabolism (Additional
file 1: Figure S6). Among them, phosphoproteins in-
volved in carbohydrate metabolism, protein synthesis
and degradation were the most abundant, both repre-
senting 24.14% (7/29) of the phosphoproteins identified,
respectively.

Phosphorylation site identification

Next, we tried to determine the phosphorylation patterns
of total proteins by using NanoLC-MS/MS analysis, follow-
ing the workflow as depicted in Fig. 2a. Totally, 1815
phosphosites were identified, which come from 1537 phos-
phopeptides (Additional file 2: Table S1) on 839 phospho-
proteins (Additional file 3: Table S2). Among these 1815
phosphorylated residues, there were 1539 phosphoserine
(pS), 246 phosphothreonine (pT), and 30 phosphotyrosine
(pY), corresponding to 84.79, 13.55, and 1.65% respectively
of all phosphorylated residues (Fig. 2b). We noticed that
257 phosphopeptides were multiply phosphorylated, and
1279 phosphopeptides were singly phosphorylated (Fig. 2c).
Then, the 29 identified SA-responsive phosphoproteins
were scanned in the NanoLC-MS/MS analysis data. Phos-
phorylation sites within 4 identified SA-responsive phos-
phoproteins were verified (Additional file 4: Table S3). The
MS/MS spectrum of a representative phosphorylated pep-
tide (ELLS*YEYGDENVAGSALK), Elongation factor
Tu (spot 30) was shown in (Additional file 1: Figure S7)
as an example.

Enzyme activities in rice leaves induced by SA treatment

To further validate possible regulation of enzyme activity
by protein phosphorylation, we selected three important
| Protein Spot No. | Protein Name | Uniprot Accession No. | Molecular Mass (kDa) | Isoelectric Point | Pep. Count | Protein Score | Protein Score (C.I.%) | Relative fold changes |
|-----------------|--------------|-----------------------|---------------------|------------------|------------|---------------|----------------------|----------------------|
| 1               | HAD-superfamily hydrolase, subfamily IA, variant 3 containing protein, expressed | Q10I42 | 34.1 | 8.4 | 11 | 560 | 100 | NC | ↓ | 12 h 1.93 ± 0.01 |
| 5               | Rubisco activase, chloroplast precursor, putative, expressed | H2KWJ8 | 39 | 5.4 | 19 | 761 | 100 | ↑ | 12 h 4.06 ± 0.32 | ↑ | 12 h 4.43 ± 0.53 |
| 20              | Rubisco large subunit | POC511 | 26 | 7.0 | 12 | 452 | 100 | ↑ | 12 h 1.83 ± 0.17 | ↑ | 12 h 1.94 ± 0.14 |
| 27              | Putative transketolase 1 | Q5VNW1 | 69.4 | 5.4 | 22 | 521 | 100 | ↑ | 24 h 1.53 ± 0.02 | ↑ | 24 h 1.90 ± 0.02 |
| 34              | Chloroplast 23 kDa polypeptide of photosystem II | B0FFP0 | 20 | 5.6 | 11 | 755 | 100 | ↓ | 24 h 2.89 ± 0.01 | NC |
| 37              | Putative cinnamoyl-CoA reductase | Q69U05 | 36 | 5.7 | 16 | 398 | 100 | NC | ↑ | 24 h 1.63 ± 0.026 |
| 15              | GDP-mannose 3,5-epimerase 1 | A3C4S4 | 43 | 5.8 | 23 | 702 | 100 | ↓ | 12 h 2.36 ± 0.02 | NC |
| 33              | L-ascorbate peroxidase 1 | B7E6Z4 | 273 | 5.4 | 14 | 496 | 100 | ↓ | 24 h 1.93 ± 0.01 | ↑ | 24 h 1.83 ± 0.03 |
| 23              | Putative chaperonin 60 beta | Q9LWT6 | 64 | 5.6 | 23 | 560 | 100 | ↑ | 24 h 3.71 ± 0.17 | ↑ | 24 h 2.20 ± 0.01 |
| 4               | ATP-dependent Clp protease proteolytic subunit | Q6H79 | 32 | 6.7 | 5 | 328 | 100 | ↑ | 12 h 1.59 ± 0.04 | ↑ | 12 h 1.63 ± 0.03 |
| 6               | Alpha/beta hydrolase | Q71OQ1 | 18 | 8.9 | 8 | 415 | 100 | ↓ | 12 h 2.24 ± 0.07 | ↑ | 12 h 3.01 ± 0.47 |
| 11              | Probable glutamyl endopeptidase, chloroplast | Q10MJ1 | 104.48 | 5.7 | 26 | 621 | 100 | NC | ↑ | 12 h 1.75 ± 0.02 |
| 12              | Probable glutamyl endopeptidase, chloroplast | Q10MJ1 | 104.48 | 5.7 | 26 | 621 | 100 | ↑ | 12 h 1.70 ± 0.01 | ↑ | 12 h 2.08 ± 0.28 |
| 28              | Eukaryotic initiation factor 4A-1 | P35683 | 473 | 5.4 | 23 | 719 | 100 | ↓ | 24 h 1.61 ± 0.02 | ↑ | 24 h 1.78 ± 0.03 |
| 30              | Elongation factor Tu | Q6Z53 | 51 | 6.2 | 21 | 608 | 100 | ↓ | 24 h 1.62 ± 0.09 | NC |
| 9               | Phosphoglycerate mutase | Q5OMK7 | 61 | 5.4 | 18 | 386 | 100 | NC | ↓ | 12 h 2.53 ± 0.38 |
| 17              | Glyceraldehyde-3-phosphate dehydrogenase | A2XC18 | 475 | 6.2 | 16 | 652 | 100 | ↑ | 12 h 2.67 ± 0.17 | ↓ | 12 h 2.99 ± 0.02 |
| 18              | Glyceraldehyde-3-phosphate dehydrogenase | A2XC18 | 475 | 6.2 | 16 | 652 | 100 | ↑ | 12 h 2.19 ± 0.05 | ↓ | 12 h 2.89 ± 0.02 |
| 24              | Alpha 1,4-glucan phosphorylase | Q9ATK9 | 105 | 5.4 | 24 | 508 | 100 | ↑ | 24 h 1.58 ± 0.04 | NC |
| 25              | Alpha 1,4-glucan phosphorylase | Q9ATK9 | 105 | 5.4 | 24 | 508 | 100 | ↑ | 24 h 2.47 ± 0.29 | ↑ | 24 h 1.87 ± 0.03 |
| 31              | Phosphoribulokinase | Q6Z5F4 | 449 | 5.7 | 15 | 830 | 100 | ↓ | 24 h 15.98 ± 0.05 | ↑ | 24 h 3.60 ± 0.42 |
| 32              | Phosphoribulokinase | Q8GRU9 | 449 | 5.7 | 18 | 624 | 100 | ↓ | 24 h 2.07 ± 0.01 | ↑ | 24 h 2.25 ± 0.03 |
Table 1  SA-responsive rice phosphoproteins identified by MALDI-TOF/TOF MS (Continued)

| Protein Spot No. | Protein Name | Uniprot Accession No. | Molecular Mass (kDa) | Isoelectric Point | Pep. Count | Protein Score |
|------------------|--------------|-----------------------|---------------------|------------------|------------|--------------|
| 16               | Aspartate aminotransferase | Q84V24                | 46                  | 5.9              | 25         | 859          | 100          | ↓ 12 h 2.09 ± 0.03 | NC |
| 36               | Cysteine synthase | Q2Q1X5                | 43.8                | 8.8              | 18         | 479          | 100          | ↑ 24 h 2.04 ± 0.05 |
|                  |              |                       |                     |                  |            |              |              |               |
| 21               | ATP synthase epsilon chain, chloroplastic | P0C2Z3                | 15.3                | 5.0              | 9          | 559          | 100          | ↓ 12 h 1.82 ± 0.02 |
|                  | Energy metabolism |                       |                     |                  |            |              |              |               |
| 7                | Probable bifunctional riboflavin biosynthesis protein RIBA 1, chloroplastic | Q6Z233                | 592                 | 5.6              | 18         | 306          | 100          | ↑ 12 h 1.62 ± 0.13 | ↑ 24 h 2.74 ± 0.10 |
| 8                | Probable bifunctional riboflavin biosynthesis protein RIBA 1, chloroplastic | Q6Z233                | 592                 | 5.6              | 18         | 306          | 100          | ↑ 12 h 2.21 ± 0.09 | ↓ 12 h 3.15 ± 0.15 |
| 29               | Glucose-1-phosphate adenylyltransferase large subunit 3, chloroplast, putative, expressed | Q6AVT2                | 55.8                | 7.0              | 31         | 844          | 100          | ↓ 24 h 1.68 ± 0.09 | ↑ 24 h 3.46 ± 0.09 |

aSpot numbers are according to 2DE gels as shown in Fig. 1
bNumber of unique peptides matched to mass peaks
cMS and MS/MS combined score
dC.I. % for the protein score rates the confidence level of the Protein Score
The fold changes listed in the table were derived by comparison of relative protein intensity between SA-treated and control, which were calculated with PDQuest 8.0 software. The detailed information of relative protein intensity was listed in Additional file 1: Fig. S2. Only the proteins with changes ≥1.5-fold were listed. "NC" indicates the proteins with no significant changes (<1.5-fold). Differentially regulated phosphoproteins were marked by arrow, which indicates up-↑) or down-regulated ↓) phosphoproteins in response to SA treatment. The number with no parentheses indicated the fold changes of differentially regulated phosphoproteins in 2DE gels.
enzymes from the identified phosphoproteins, and assessed their activities. A significant increase in cinnamoyl-CoA reductase (CCR) activity was detected in C101LAC at 24 h after SA treatment (Fig. 3a). CCR showed an increased phosphorylation level in C101LAC at 24 h after SA treatment, suggesting that the enzyme activity of CCR was regulated by phosphorylation. Consistent with the phosphoproteomic results, a significant decrease in APX activity was noted in both cultivars 24 h after SA treatment. A significant decrease in PGAM activity was detected only in C101LAC 12 h after SA treatment, but little change was found in CO39 (Fig. 3b, c). Phosphoproteomic analysis also showed that phosphorylated PGAM and phosphorylated APX were down-regulated only in C101LAC at 12 h after SA treatment, but little change was found in CO39 (Table 1). These results above strongly suggesting that the enzyme activity of PGAM and APX was regulated by phosphorylation.

ROS accumulation in rice leaves induced by SA treatment
SA and ROS production in rice defense response to biotic and abiotic stress has been well documented [18, 19]. In this study, the phosphoproteomic analysis revealed several phosphoproteins involved in biogenesis of ROS. In agreement with the phosphoproteomic results, a significant increase in $\text{O}_2^-$, $\text{H}_2\text{O}_2$ and MDA contents was noted in both rice cultivars after SA treatment, compared with the corresponding controls (Fig. 4). Relative to the control, SA resulted in an increase of $\text{H}_2\text{O}_2$ contents by 1.77 and 2.72 times in CO39, 2.47 and 3.15 times in CO101LAC at 12 h and 24 h, respectively (Fig. 4a). An increase of $\text{O}_2^-$ content was also noted by 1.94 and 3.75 times in CO39, 2.59 and 4.46 times in CO101LAC at 12 h and 24 h after SA treatment, respectively (Fig. 4b). MDA contents was significantly increased by 1.83 and 2.61 times in CO39, 2.61 and 2.79 times in CO101LAC 12 h and 24 h post SA treatment, respectively, compared with the controls (Fig. 4c). Taken together, SA treatment significantly increased the contents of $\text{O}_2^-$, $\text{H}_2\text{O}_2$ and MDA in both resistant (C101LAC) and susceptible (CO39) cultivars, but the fold changes in C101LAC were significantly higher than that in CO39.

Transcriptional expression analysis of SA-responsive phosphoproteins
Six genes encoding the identified phosphoproteins (Additional file 4: Table S4) were selected for expression analysis via qRT-PCR. The gene encoding putative chaperonin 60 beta was significantly down-regulated in CO39 after SA treatment, but up-regulated in C101LAC (Fig. 5a). Elongation factor Tu significantly decreased in CO39 at mRNA levels, did not significantly vary in C101LAC (Fig. 5b). The expression changes of the above
Fig. 3 Quantitative analysis of a CCR, b PGAM, and c APX activities in the leaves of rice seedlings after SA treatment. Bars indicate ± standard error of the mean. Different small letters in each group indicate significant differences at $P \leq 0.05$. 

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Fig. 4 ROS production in rice leaves induced by SA. Water was used as a control (CK). a $\text{O}_2^-$ production. b $\text{H}_2\text{O}_2$ content. c MDA contents. Bars indicate ± standard error of the mean. Different capital letters in each group indicate significant differences at $P \leq 0.01$. 

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two phosphoproteins at mRNA levels were consistent with the 2DE results both in CO39 and C101LAC. Two phosphoproteins (eukaryotic initiation factor 4A-1 and phosphoribulokinase) showed a significant decrease in mRNA levels but a significant increase in phosphorylation levels in C101LAC; however, they decreased both in mRNA levels and phosphorylation levels in CO39 (Fig. 5c, d). Interestingly, glyceraldehyde-3-phosphate dehydrogenase significantly decreased in mRNA levels, but increased in phosphorylation levels in CO39; however, the expression decreased both in mRNA levels and phosphorylation levels in C101LAC (Fig. 5e). L-ascorbate peroxidase 1 significantly increased in mRNA levels both in CO39 and C101LAC; while its phosphorylation level showed a typical decrease in C101LAC and no significant variation in CO39 (Fig. 5f). In total, these data showed a lack of correlation between transcriptional regulation and post-translational regulation (by protein phosphorylation) in rice plants treated with SA. Consistent with this results, several reported phosphoproteomics studies had also revealed different changes in the mRNA levels and their corresponding proteins levels [20, 21]. The results highlighted the importance of SA-induced resistance mechanism at multiple molecular levels in rice plant.
Discussion

SA is an important signaling molecule that plays key roles in the regulation of plant defense against pathogens. In our previous work, we explored the molecular mechanisms of SA-mediated protection of rice against *M. oryzae* infection by proteomic profiling analysis with blast-resistant vs. -susceptible rice cultivars after SA treatment [7]. Protein phosphorylation, as one of the most common and best characterized post-translational modifications, is a key process that regulates a large number of biological processes in plants, including cell signaling, metabolism, hormone and stress responses [22]. However, it is very difficult to detect phosphoproteins by the "proteomic" approach, due to the low abundance of phosphoproteins [23]. For further understanding the molecular mechanisms, we performed a comparative phosphoproteome analysis of two near rice isogenic lines after SA treatment. In total, we identified 29 phosphoproteins in response to SA treatment, belonging to 9 functional categories. Among the 29 phosphoproteins, 17 protein spots were common in both resistant (C101LAC) and susceptible (CO39) cultivars, suggesting that some physiological processes could be commonly influenced by SA. However, the other 12 phosphoproteins showed different changes between CO39 and C101LAC. The expression patterns of these phosphoproteins could help to understand the different molecular mechanism of SA-induced resistance against *M. oryzae* in the two rice cultivars.

Photosynthesis has been well-known to be highly sensitive to various exogenous stimulus, including SA, JA and some plant hormone [24, 25]. In this study, total 5 phosphoproteins related to photosynthesis were differentially regulated after SA treatment, in which chloroplast 23 kDa polypeptide of photosystem II (PSII, spots 34) was firstly reported to be phosphorylated which was validated by NanoLC-MS/MS. It has been previously demonstrated that photosynthesis could be regulated through phosphorylation of photosynthesis-related proteins. For example, phosphorylation of rubisco activase (RCA), known as an ancillary photosynthetic protein essential for Rubisco activity, was reduced following abiotic stress, resulting in the malfunctioning of photosynthesis [26]. Transketolase (TKL), a key enzyme linking the non-oxidative pentose phosphate pathway and the Calvin cycle, is presumed to participate in the functional regulation of numerous metabolic pathways by phosphorylation [27]. Previous studies have shown that dephosphorylation leads to a dramatic decrease in the TKL activity, which declines the rates of photosynthesis [28]. In this study, most of the phosphoproteins, including RCA (spots 5), Rubisco large subunit (Spots 20) and TKL (spots 27) were up-regulated in both rice cultivars at 12 h and 24 h post SA treatment. Consistent with our results, it has previously been reported that SA could alleviate decreases in plant photosynthesis against pathogen infection to produce various metabolites and energy, thus improving plant defense response [7]. However, we also noted that HAD-superfamily hydrolase (subfamily IA, variant 3 containing protein, spot 1) were down-regulated in C101LAC 12 h post SA treatment. It is suggested that SA regulates plant photosynthesis via phosphorylation of photosynthesis-related proteins and the effect of SA on the photosynthetic machinery is complex in rice plants.

We identified a putative cinnamoyl-CoA reductase (CCR, spot 37) as SA-responsive phosphoprotein. CCR catalyzes the first step of the monolignol pathway for lignin biosynthesis and therefore plays an essential role in defense-related processes in rice [29]. In our previous study, CCR was firstly reported to be dephosphorylated in susceptible rice plants after *M. oryzae* infection, which resulted in a decrease in enzyme activities [30]. In this study, CCR was notably up-regulated only in C101LAC at 24 h after SA treatment (Table 1). Correspondingly, CCR activity was significantly increased in the resistant cultivar at 24 h after SA treatment, but not in the susceptible cultivar (Fig. 3a). CCR, as an effector of small GTPase Rac in rice defense signaling, is considered to be activated by OsRac1, which further leads to efficient production of monolignols, deposition of lignin, increased ROS accumulation [31]. Taken together, we speculate that phosphorylation of CCR may increase enzyme activities, accelerate lignin synthesis and ROS accumulation, thus further activate rice defense responses against infection of pathogens.

Phosphorylation of antioxidant enzymes has been reported to be involved in antioxidant defense [32]. In this study, two phosphoproteins were identified as GDP-mannose 3,5-epimerase 1 (OsGME 1, spot 15) and L-ascorbate peroxidase 1 (OsAPx01, spot 33), which are known as antioxidant enzymes. Phosphorylated OsGME 1 was down-regulated only in the susceptible cultivar at 12 h after SA treatment, and phosphorylated OsAPx01 was down-regulated in both cultivars. Previous studies have showed that dephosphorylation decreases the activities for APX and OsGME 1 upon biotic or abiotic stress in several plants [26, 33]. To validate phosphorylated regulation of enzyme activity, APX activity was performed (Fig. 3b). The results showed that APX activity was significantly decreased in both cultivars at 24 h after SA treatment, which were consistent with the phosphoproteomic analysis. It has been suggested that down-regulation of scavenging/antioxidant systems and/or activities may contribute to increase ROS accumulation [34]. Earlier study reported that plant cells presumably regulated ROS levels by coordinating activities of ROS-generating enzymes such as SOD and ROS-degrading enzymes such as APX, POX, and CAT [35]. A detailed comparison of the influence of SA on H2O2 production,
SOD activities and H₂O₂-degrading enzymes showed that tobacco leaves treated with SA may have enhanced H₂O₂ largely by activating enzymes capable of generating H₂O₂, and by inactivating enzymes that are capable of degrading H₂O₂ [36]. In this study, the findings that phosphorylation of APX and OsGME 1 was down-regulated in rice suggests a significant decrease of the activities of ROS-scavenging antioxidant enzymes, further accelerates oxidative burst in both cultivars. Consistent with this notion, significant ROS accumulation was observed in both rice cultivars after SA treatment (Fig. 4), which indicated that SA could regulate antioxidant enzymes’ activity by phosphorylation, then enhance ROS accumulation in rice plants as a defense response. However, SA and ROS interactions are complicated and awaits further investigation in future to fully elucidate.

One phosphoprotein was identified as 60 kDa chaperonin beta (Cpn60β, spot 23), which is a molecular chaperone involved in protein destination and assembly. Molecular chaperones play regulatory roles in prevention of stress injury, immune response, and cell death in plants, which might help plant enhance defense system against pathogen infection [37]. Phosphorylation is a common regulatory mode for the function of different classes of molecular chaperones, which markedly improves the capacity to enhance its binding to unfolded proteins, facilitate rapid degradation of certain abnormal proteins, and protect against oxidative stress injury [38, 39]. In this study, phosphorylated Cpn60β was up-regulated at 24 h after SA treatment in both cultivars (Table 1). A previously proteomic study showed that 60 kDa chaperonin was significantly up-regulated in resistant tomato cultivar compared with susceptible tomato cultivar after bacterial infection, which might provide enhanced defense system against Pseudomonas solanacearum [40]. Taken together, we speculate that SA-induced phosphorylation of Cpn60β may improve the activities of Cpn60β, further contributing towards rice defense against pathogen attack.

Seven phosphoproteins were involved in carbohydrate metabolism, including phosphoglycerate mutase (PGAM, spot 9), glyceraldehyde-3-phosphate dehydrogenase (GAPDH, spot 17 and 18), alpha 1,4-glucan phosphorylase L isozyme (α-GP, spots 24 and 25), phosphoribulokinase (PRK, spot 31 and 32). PGAM catalyzes the conversion of 3-phosphoglycerate to 2-phosphoglycerate, which is a crucial step in glycolysis. Previous phosphoproteomics studies showed that PGAM can be phosphorylated at multiple tyrosine sites, which enhances PGAM activity and upregulates glycolysis [41, 42]. In this study, phosphorylated PGAM was down-regulated only in C101LAC 12 h after SA treatment. To validate regulation of enzyme activity by SA-induced protein phosphorylation, PGAM activity was assessed (Fig. 3b). Agreeable with the phosphoproteomic analysis, the results showed that a significant decrease in PGAM activity was noted in C101LAC 12 h after SA treatment, but little change was found in CO39. α-GP catalyzes the reversible phosphorolysis of glucan chains and releases 1-glucose-1-phosphate for starch synthesis. Phosphorylated α-GP is active, and dephosphorylated α-GP is inactive [43]. GAPDH catalyzes a critical step in glycolysis; GAPDH activity was significantly decreased if phosphorylated [44]. In this study, phosphorylated α-GP was up-regulated both in CO39 and C101LAC 12 h after SA treatment, suggesting that SA might increase α-GP activity. However, phosphorylated GAPDH was down-regulated in C101LAC, but up-regulated in CO39 12 h after SA treatment. The results above suggest that accumulation of carbohydrates was differently regulated in two different rice cultivars by SA induction, indicating that carbohydrate metabolism pathways may be regulated by reverse phosphorylation of important enzymes. However, little is known about the potential role for phosphorylated regulation in these enzymes of carbohydrate metabolism and further investigation is needed to decipher their role in rice.

Conclusions

In this study, we performed a comparative phosphoproteomic analysis to investigate the molecular mechanisms of SA-induced defense response in different rice cultivars. A total of 29 SA-responsive phosphoproteins were successfully identified by MNAIL-TOF/TOF analysis. Phosphoproteins involved in photosynthesis, antioxidative enzymes, molecular chaperones showed similar changes in the two cultivars, while phosphoproteins related to protein synthesis and degradation, defense, amino acid metabolism, and carbohydrate metabolism were differentially expressed. Furthermore, phosphorylation within four identified phosphoproteins was validated by NanoLC-MS/MS analysis, and phosphorylated regulation of three important enzymes (CCR, PGAM and APX) was verified by activity determination. To best of our knowledge, it is the first report to measure rice phosphoproteomic changes induced by SA, which may broaden our understanding of SA-responsive mechanisms in rice.

Methods

Chemicals

The following chemical reagents were used in this study: SA (Sigma-Aldrich, St. Louis, MO, USA); Immobiline™ DryStrip pH 4–7 NL, 18 cm and IPG buffer pH 4–7 (GE Healthcare, Uppsala, Sweden); Pro-Q Diamond phosphoprotein gel stain (Molecular Probes, Eugene, OR, USA); Glycine, 1.5 mol/L Tris-HCl buffer pH 8.8, 30% acrylamide/bis solution (37.5:1), and overlay agrose (Bio-Rad, Hercules, CA, USA).
Plant materials
Two rice near isogenic lines (Oryza sativa indica) were obtained from the International Rice Research Institute, including C101LAC carrying the resistance gene Pi-1 against M. oryzae, and background line CO39 carrying no known resistance gene. Rice seedlings were sprayed with 0.1 mM SA solution (containing 0.02% v/v Tween 20) at the four-leaf stage [7]. The fourth leaves were harvested at 12 and 24 h after SA treatment. Spraying with sterilized water containing 0.02% v/v Tween 20 served as blank control. The leaves were sampled by freezing in liquid nitrogen, and stored at −80°C before assessment.

Phosphoproteome enrichment, 2DE and gel analysis
Total proteins were extracted essentially from 5 g of rice leaf samples by using a PEG-mediated prefractionation method [45]. Enrichment of phosphorylated proteins follows the well-established Al (OH)₃-MOAC method [30]. The protein content was determined using the coomassie blue dye-binding method, with BSA as the standard [46]. Three replicates with different pools of leaf samples were performed, and all the procedures were carried out at 4°C.

2DE follows the established protocol [47]. Phosphoproteins were visualized with Pro-Q Diamond fluorescent gel stain according to the methods described previously [48], before imaging with a Typhoon Trio Variable Mode Imager (GE Healthcare, Uppsala Sweden). PDQuest software (Version 8.0, Bio-Rad, Hercules, CA, USA) was used for quantitative analysis with gel spots, including spot detection, measurement, matching and calculation. Each phosphoprotein sample was analyzed by 2DE for at least three times. The protein spots showing ≥1.5-fold increase or decrease in all three biological repeats were selected as putative differentially regulated phosphoproteins.

Phosphoprotein identification by MALDI-TOF/TOF MS
The differentially expressed phosphoprotein spots were manually excised from the gels, before in-gel digestion [7]. The peptides were subsequently analyzed using the ABI 4800 Proteomics Analyzer MALDI-TOF/TOF (Applied Biosystems, Foster City, CA). Database search was performed in the Oryza sativa database (Uniprot, v.2016.08.24) using the MASCOT search engine 2.2 (Matrix Science, Ltd.) with GPS-Explorer Software 3.6 (Applied Biosystems). The parameter settings were as following: peptide mass tolerance: 100 ppm; fragment tolerance: ±0.3 Da; protein score C.I.%: ≥95%; total ion score C.I.%: ≥95% and significance threshold: p < 0.05. Besides, to eliminate the redundancy of proteins that appeared in the database under different names and accession numbers, the single-protein member belonging to the species of O. sativa or others with the highest protein score (top rank) was singled out from the multi-protein family.

Identification of phosphorylation sites by NanoLC-MS/MS
Total proteins extraction from rice leaves was performed using a PEG-mediated prefractionation method [30], and protein was digested with trypsin following the FASP method [49]. The trypsin-digested peptide mixture was then loaded onto aliquot of titanium dioxide (TiO₂) beads (5 μm Titansphere, GL Sciences, Japan), which were then collected by centrifugation after washing twice with 30 mg/mL DHB (2,5-dihydroxybenzoic acid) buffer. The beads were further washed for twice with 60% ACN/0.1% TFA and 0.1% TFA respectively, before elution with a 60% ACN/4% ammonium solution. NanoLC-MS/MS was performed with a Q Exactive MS (Thermo Finnigan) equipped with Easy nLC1000 (ThermoFisher, San Jose, CA). The peptide mixture was separated on a C18-reversed phase column with a flow rate of 250 nL/min over 240 min. Peptides were analyzed by MS/MS in positive ion mode, and the MS/MS spectra search was performed against the Uniprot_Oryza database (v.2018.02.27), using Mascot 2.2 engine. Proteome Discoverer 1.3 (Thermo Electron, San Jose, CA) was used for identification of phosphorylation peptides, with the threshold setting as pRS score above 50 indicating a good PSM (Peptide Spectrum Matches) and pRS probabilities above 75% indicating a truly phosphorylated site.

Determination of enzyme activities and reactive oxygen species
The fourth leaves were sampled at 12 and 24 h after SA treatment. Enzymatic activity of APX (ascorbate peroxidase), CCR (cinnamoyl-CoA reductase) activity, and phosphoglycerate mutase (PGAM) was assayed respectively following the established protocols [50–52]. Hydrogen peroxide (H₂O₂) follows Brennan and Frenkel’s method [53]. The rate of superoxide (O₂⁻) production was measured based on nitroblue tetrazolium (NBT) reduction [54]. Determination of malondialdehyde (MDA) content follows the previously described method [55], using the following formula to calculate MDA content (C): C (μmol/L) = 6.45 (A₅₃₂− A₆₀₀) − 0.56 A₄₅₀.

Quantitative real-time PCR (qRT-PCR) analysis
Total RNA isolation from rice leaves was performed using Eastept® Super Total RNA Extraction Kit (Promega, Shanghai, China). The first-strand cDNA was prepared from 2 μg of normalized total RNA, with FastKing RT Kit (with gDNase) (Tiangen Biotech, Beijing, China). Gene-specific primers used for selected gene transcription assessment were designed using the Primer 5.0 software and were listed in (Additional file 4: Table S4). The tubulin gene (Uniprot Accession No. Q58G87) was used
as reference. qRT-PCR was conducted on a CFX Connec
t™ Real-Time System (Bio-Rad, Hercules, CA, USA) with
the iTaq™ SYBR® Green Supermix (Bio-Rad, Hercules,
CA, USA) according to the manufacturer’s protocol. Three independent biological replicates were
performed for each gene. Relative transcript levels for
each gene were calculated by 2\(^{-\Delta\Delta C_{T}}\) method [56].

Statistical analysis
Means ± standard error (SE) was derived from three bio-
logical replicates. The data were analyzed using the one-
way analyses of variance (ANOVA) and the significant
differences were determined as p ≤ 0.05, by the Duncan’s
test using SPSS software (version 19.0, SPSS Inc., Chi-
icago, IL, USA).

Supplementary information
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Additional file 1: Figure S1. Representative 2DE patterns of
phosphopeptides from rice leaves treated with MQ water (as the control)
and SA. Figure S2. All additional 2DE gels of rice phosphopeptides
were shown as replicate gels. Figure S3. Close-up views of the regions of 2DE
gels showing all SA-responsive phosphoprotein spots in two rice cultivars.
Figure S4. Quantitative analysis of the SA-responsive phosphoproteins in
rice leaves. Figure S5. Identification of spot 11 by MALDI-TOF/TOF MS.
Figure S6. The functional category distribution of the 29 SA-responsive
phosphoproteins. Figure S7. The MS/MS spectra of representative phosphorylated peptides of ELLS*YEYDGEVPIVAGSALK, corresponding
to Elongation factor Tu (Q6ZI53).

Additional file 2: Table S1. Phosphopeptides and their
phosphorylation sites (marked by lowercase letters of the amino acid
residue) identified by NanoLC-MS/MS analysis.

Additional file 3: Table S2. Rice phosphoproteins identified by
NanoLC-MS/MS analysis.

Additional file 4: Table S3. Mapping of SA-responsive phosphoproteins
with the NanoLC-MS/MS data. Table S4. Gene-specific primers designed for
qRT-PCR.

Abbreviations
2DE: Two-dimensional gel electrophoresis; APX: Ascorbate peroxidase;
CCR: Cinnamoyl-CoA reductase; IEF: Isoelectric focusing; IPG: Immobiline pH
gradient; LC: Liquid chromatography; MOAC: Metal oxide affinity
chromatography; MS: Mass spectrometer; MS/MS: Tandem mass
spectrometry; PEG: Polyethylene glycol; PGAM: Phosphoglycerate mutase;
Pro-Q DPS: Pro-Q diamond phosphoprotein stain; PTMs: Post-translational
modifications; qRT-PCR: Quantitative real-time PCR; ROS: Reactive oxygen
species; SA: Salicylic acid

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Authors’ contributions
RS, SQ, TZ, ZW, HL, YL and YN conceived the study, participated in the
design of the study and discussed the results critically. TZ and HL performed
qRT-PCR analysis. RS, SQ, TZ performed the phosphoproteomic analysis and
biochemical analysis. ZW, HL, YL and YN were responsible for NanoLC-MS/MS
analysis. RS, YL and YN wrote and revised the manuscript. All authors read
and approved the final manuscript.

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Availability of data and materials
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Ethics approval and consent to participate
Not applicable.

Consent for publication
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Competing interests
The authors declare that they have no competing interests.

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