Quantitative Phosphoproteomics Analysis of Nitric Oxide–Responsive Phosphoproteins in Cotton Leaf

Shuli Fan¹, Yanyan Meng²*, Meizhen Song¹, Chaoyou Pang¹, Hengling Wei¹, Ji Liu¹,³, Xianjin Zhan², Jiayang Lan², Changhui Feng², Shengxi Zhang²*, Shuxun Yu²*

¹ State Key Laboratory of Cotton Biology, Institute of Cotton Research, Chinese Academy of Agricultural Sciences, Anyang, Henan Province, China, ² Key Laboratory of Cotton Biology and Breeding in the Middle Reaches of the Changjing River, Institute of Economic Crop, Hubei Academy of Agricultural Science, Wuhan, Hubei Province, China, ³ College of Agronomy, Northwest A & F University, Yangling, Shaanxi Province, China Crop, Hubei Academy of Agricultural Science, Wuhan, Hubei Province, China

Abstract

Knowledge of phosphorylation events and their regulation is crucial to understanding the functional biology of plant proteins, but very little is currently known about nitric oxide–responsive phosphorylation in plants. Here, we report the first large-scale, quantitative phosphoproteome analysis of cotton (Gossypium hirsutum) treated with sodium nitroprusside (nitric oxide donor) by utilizing the isobaric tag for relative and absolute quantitation (iTRAQ) method. A total of 1315 unique phosphopeptides, spanning 1528 non-redundant phosphorylation sites, were detected from 1020 cotton phosphoproteins. Among them, 183 phosphopeptides corresponding to 167 phosphoproteins were found to be differentially phosphorylated in response to sodium nitroprusside. Several of the phosphorylation sites that we identified, including RQxS, DSxE, TxxxxSP and SPxT, have not, to our knowledge, been reported to be protein kinase sites in other species. The phosphoproteins identified are involved in a wide range of cellular processes, including signal transduction, RNA metabolism, intracellular transport and so on. This study reveals unique features of the cotton phosphoproteome and provides new insight into the biochemical pathways that are regulated by nitric oxide.

Introduction

Nitric oxide (NO) is an important growth regulator that modulates diverse developmental processes in plants over the entire lifespan, including cell elongation and germination [1], root development [2], stomatal movement [3], pollen tube growth [4], and plant senescence [5]. NO is also involved in plant responses to abiotic stresses such as heavy metal exposure [6], low temperature [7], salt [8], and drought [9]. Additionally, NO has been demonstrated to play many roles in biotic interactions, such as fighting pathogen infections [10,11], deterring herbivore feeding [12] and building symbiotic interactions [13].

During the last decade, several medium- and large-scale transcriptional analyses have identified hundreds of putative NO-regulated genes [14–19]. Moreover, eight families of transcription factor–binding sites have been identified in the promoter of NO-regulated genes [19]. Recent research has mainly focused on identifying NO target proteins, attempting to reveal NO involvement in plant cell biology. As such, more than 200 NO-responsive proteins have been identified in plants [20,21]. Unlike the genome, however, protein abundance, structure, stability, subcellular localization, and interactions with other biological macromolecules are in continuous flux and these changes are regulated by post-translational modifications such as phosphorylation, acetylation, glycosylation, and methylation.

Phosphorylation is the most common and the most important dynamic adjustment mechanism and is involved in nearly all cellular and extracellular processes including defense responses, signal transduction, cytoskeleton regulation, and apoptosis [22,23]. Given the importance of phosphorylation, there has been much interest in identifying new phosphorylated proteins and phosphorylation sites and in exploring the functional role of these phosphoproteins. Early studies looking at the effect of abscisic acid (ABA) on protein phosphorylation of rice (Oryza sativa) and Arabidopsis thaliana have identified 6 and 50 proteins, respectively, for which phosphorylation was found to be regulated by ABA [24,25]. Furthermore, examination of changes in the phosphoproteome of rape seeds during the filling stage identified 70 phosphoproteins and 16 non-redundant phosphoproteins, which were verified by mapping the phosphorylation sites [26]. Analysis of vacuolar and cell membranes of rice bud and root revealed 230 membrane and membrane-associated proteins, 20% of which are phosphorylated [27]. In addition, soybean (Glycine max) root hairs contain 1625 unique phosphopeptides, including 1639 non-redundant phosphorylation sites, which originate from 1126 phosphoproteins [28]. A recent study identified multiple components of ABA-responsive protein phosphorylation network by integrating genetics with phosphoproteomics [29]. Furthermore, Wang et al. have shown the role of the SnRK2 protein kinases in the ABA signaling pathway by using quantitative phosphoproteomics.
teomics [30]. To date, only a few plant species have been annotated with protein phosphorylation data and these do not include the economically important plant, cotton (*Gossypium hirsutum*) [31]. We previously examined the proteome of cotton leaves in response to NO treatment [21]. To follow-up this research, we performed quantitative time-course measurements of the phosphoproteome of cotton leaves treated with sodium nitroprusside (SNP), a NO donor. This is the first study to show that NO exposure in leaf tissue alters the phosphorylation states of multiple proteins found in cotton. This information should accelerate research on NO metabolic regulation and will lay a novel, theoretical foundation for further related studies in cotton.

**Materials and Methods**

**Plant materials**

Seeds of *G. hirsutum* ecotype CCR110 were cultured in a mix of sand and nutritional soil in a culture room under white fluorescent light (14 h light/10 h dark) with day/night temperatures of 30/22°C. Previously we evaluated the effects of nitric oxide on cotton seeds treated with 0, 0.05, 0.1 or 0.5 mM SNP for 24 h. The results showed that the effect of treatment with 0.05 mM SNP on plant growth was not severe, 0.5 mM SNP was lethal, and 0.1 mM SNP was significant but not toxic (Figure S1). Additionally, we also found that the effect of treatment at 0.1 mM SNP for 6 h on cotton seedlings effected protein levels significantly [21]. Based on these results, proteins were extracted from the leaves treated with 0.1 mM SNP. Plants that were 30 days old after sowing were irrigated with 0.1 mM SNP (Sigma, USA) in distilled water for 0 h (control), 1 h, 3 h and 6 h, respectively during the light period. The experiment was performed in triplicate with 30 plants in each group. Fresh, fully expanded leaves were harvested after treatment and immediately frozen in liquid nitrogen and stored at −80°C.

**Protein extraction**

Leaf samples were ground in liquid nitrogen and lysed in buffer containing 4% SDS, 100 mM Tris-HCl, and 1 mM diithiothreitol (pH 7.6) at room temperature and briefly sonicated to reduce the viscosity of the lysate. The ratio of buffer to tissue was 5:1 (v/v). After 3 min incubation in boiling water, the suspensions were ultrasonicated (80 W, 10 s ultrasonic at a time, every 15 s, and 10 times) then incubated at 100°C for 3 min. The crude extract was clarified by centrifugation at 13,000 × g for 3 min. The supernatant was used for the following steps.

**Protein digestion**

Protein digestion was processed by using the method of filter-aided sample preparation (FASP) [32]. The protocol was as follows: For each sample, 300 μg of protein was diluted with 200 μL UA buffer (8 M urea with 0.1 M dithiothreitol in 0.15 M Tris-HCl pH 8.0) and centrifuged at 14,000×g for 20 min. Another 200 μL of UA buffer was added and the samples were centrifuged again at 14,000×g for 20 min. Then 100 μL of 0.05 M iodoacetamide in UA buffer was added, and the samples were incubated at room temperature for 20 min in darkness. After 10 min centrifugation at 14,000×g, 100 μL UA buffer was added and centrifuged at 14,000×g for 15 min. This step was repeated twice. Subsequently, 100 μL ABC buffer (0.05 M NH₄HCO₃ in water) was added to the filters and the samples were centrifuged for 10 min at 14,000×g. This step was also repeated twice as reported previously [32]. Finally, 2 μg of trypsin (Promega, USA) in 40 μL ABC buffer was added to each filter, and incubated overnight at 37°C. The resulting peptides were collected by centrifugation at 14,000×g for 10 min and then the filters were rinsed with 40 μL 10× DS buffer (50 mM triethylammonium bicarbonate at pH 8.5) and centrifuged again [32]. The peptide content was estimated by absorbance at 280 nm using an extinction coefficient of 1.1 for a 0.1% (g/100 mL) solution that was calculated based on the frequency of tryptophan and tyrosine in vertebrate proteins [33].

**Isobaric tag for relative and absolute quantitation (iTRAQ) labeling and phosphopeptide enrichment**

Equal amounts of the trypsin-digested samples from the four groups were labeled as CK-114, 1 h-115, 3 h-116 and 6 h-117 utilizing the iTRAQ Multiplex (4-plex) kit (Applied Biosystems, USA); three biological replicates were performed. For the process of enriching phosphopeptides, each labeled peptide mixture was concentrated in a vacuum concentrator and resuspended in 500 μL buffer containing 2% glutamic acid, 65% acetonitrile, and 2% trifluoroacetic acid. TiO₂ beads (500 μg, GL Sciences, Japan) were added and the mixture was agitated for 40 min then centrifuged for 1 min at 5000×g [34]. The supernatant from the first centrifugation was mixed with another 500 μg of TiO₂ beads and agitated and centrifuged as before. The two bead collections were combined and washed three times with 50 μL of 30% acetonitrile, 3% TFA and then three times with 50 μL of 80% acetonitrile, 0.3% trifluoroacetic acid to remove the remaining non-adsorbed material. Finally, the phosphopeptides were eluted with 50 μL of elution buffer (40% acetonitrile, 15% NH₄OH) followed by lyophilization and mass spectrometry (MS) [35].

**Nano-liquid chromatography**

Each iTRAQ sample (5 μL) was mixed with 15 μL 0.1% trifluoroacetic acid (v/v), and subsequently a 5 μL of the mixture was loaded onto a C18-reversed phase column (15 cm long, 75 μm inner diameter, RP-C18 3 μm, packed in house) in buffer A (0.1% formic acid) and separated with a linear gradient of buffer B (80% acetonitrile and 0.1% formic acid) at a flow rate of 250 nL/min (controlled by IntelliFlow technology; Applied Flow Technology, USA) over 240 min on a Q Exactive MS (Thermo Finnigan, USA) equipped with Easy nLC (Thermo Fisher Scientific, USA). The peptides were eluted with a gradient of 0–55% buffer B from 0 to 220 min, 55–100% buffer B from 220 to 228 min, and 100% buffer B from 228 to 240 min.

**Tandem MS analysis**

We analyzed the peptides in positive ion mode and acquired the MS spectra using a data-dependent, top10 method, dynamically choosing the most abundant precursor ions from the survey scan (300–1800 m/z) for subsequent high-energy collisional dissociation (HCD) fragmentation [36]. Determination of the target value was based on predictable automatic gain control (pAGC). The dynamic exclusion duration was 25 s. Survey scans were acquired at a resolution of 70,000 at m/z 200 and resolution for HCD spectra was set to 17,500 at m/z 200. Normalized collision energy was 29 eV and the under fill ratio, which specifies the minimum percentage of the target value likely to be reached at maximum fill time, was defined as 0.1%. The instrument was operated with the peptide recognition mode enabled. Each iTRAQ experiment was analyzed at least three times.
Data analysis

Mascot 2.2 (Matrix Science) and Proteome Discoverer 1.3 software (Thermo Scientific, USA) were used for identification and quantitative analysis [37]. The reference sequences in the Cotton Genome Project (CGP) database derived from the Gossypium raimondii genome include 40,976 non-redundant protein-coding genes sequences and 39316 non-redundant peptide sequences (ftp://public.genomics.org.cn/BGI/cotton/Annotation/G.raimondii. pep.fasta.gz, updated to 07-23-2012, downloaded on 12-05-2012) [30]. A q-value is the minimal false discovery rate at which the identification is considered correct. The dataset was filtered to give a < 1% false discovery rate (q-Value ≤ 0.009, Table S1) at peptide level using the target decoy method [39]. The phosphorylated sites on the identified peptides were assigned again using the PhosphoRS algorithm, which calculated the possibility of the phosphorylated site from the spectra matched to the identified peptides. According to the instruction of the software, for each phosphorylation site(s) on all phosphopeptides, PhosphoRS probabilities were set above 75%, indicating that the site is truly phosphorylated, and PhosphoRS scores were set above 50, indicating a good peptide spectral match [40,41]. Proteome Discoverer 1.3 software was used to extract the peak intensity within 20 ppm of each expected iTRAQ reporter ion from each analyzed fragmentation spectrum. Only spectra in which all the expected iTRAQ reporter ions (four for HCD in this work) were detected were used for quantification.

The intensity of the reporter ions was used for phosphopeptide quantification. We normalized the phosphopeptide ratios by dividing by the median ratio of all peptides identified. As for the quantitative analysis, the log2 fold-change values (Treatment/ Control) for each time point were calculated for each phospho-regulated. We also included the cutoff for the log2 value highly significant if the log2 value of the treated sample was lower than the control by 1.5 fold in phosphorylation activity (Table S2B). The distribution of phosphor-Ser (pS) was found to be consistent with that of rice (95.9%) [22] and Glycine max (89.3%) [28]. Moreover, the distribution of phosphor-Thr (pT) was similar to Arabidopsis (9.9%) [22]. However, the abundance of tyrosine phosphorylation in cotton (0.8%, this study) was slightly lower than in Arabidopsis (2.4%) [22], Medicago (1.3%) [42] and rice (1.6%) [22]. On the other hand, Laurent et al. reported a proportion of pY in soybean of only 0.48% [28]. Additionally, within the same species (e.g. Arabidopsis), the distribution of the phospho-amino acids was not always consistent [22,43]. These differences may be attributed to differences in methodology (e.g. phosphopeptide enrichment and/or LC-MS) or in the biological system, where each cell type, tissue and organism has a unique phosphoproteome profile.

Phosphorylation site motifs

To determine the potential consensus sequences for cotton, the motifs of the 1528 non-redundant cotton phosphorylation sites were extracted using the Motif-X program (version v1.2 10.05.06; http://motif-x.med.harvard.edu/motif-x.html) with default parameters [44,45]. Because Gossypium raimondii Protein Fasta data has a size surpassing the upload restrictions to 10 MB, the database included in 20,000 sequences selected randomly from Gossypium raimondii Protein Fasta data was used as the background in Motif-X. Only confidently identified phosphorylation sites with Ascore >19 were used in analysis. All 25 of the phosphorylation site motifs identified in cotton are presented in Table 1. Serine was phosphorylated in 20 of the motifs, threonine in 4, and tyrosine in 1. Table 1 also lists the phosphorylation site motifs that contained at least one fixed position aside from the central phosphorylated residue.

With the purpose of investigating the specificity of the identified motifs to cotton, these motifs were compared with literatures [46,47] as well as in the PhosPhAt 4.0 database of Arabidopsis phosphorylation site motifs (http://phosphat.mpimp-golm.mpg.de/phosphat.html) [48] and Human protein reference database (HPRD) [49]. All the phosphorylation motifs from Arabidopsis and cotton are shown in Table S2A and the information of cotton phosphorylation motif enrichment and/or LC-MS were extracted using the Motif-X program (version v1.2 10.05.06; http://motif-x.med.harvard.edu/motif-x.html) [48] and Human protein reference database (HPRD) [49]. All the phosphorylation motifs from Arabidopsis and cotton are shown in Table S2A and the information of cotton phosphorylation site motifs that contained at least one fixed position aside from the central phosphorylated residue.

Phosphoprotein identification

To identify the corresponding proteins for each phosphopeptide, each phosphopeptide was searched against the CGP peptide database [38]. To overcome the challenge that multiple proteins may share the same peptide, the annotations were confirmed by comparison to the annotation of the top score protein hits from an in-house BLAST search against the CGP peptide database.

Identified phosphoproteins were classified based on: (a) the biological processes of each gene product according to annotations in the CGP database and (b) the gene product subcellular localization predicted using the SherLoc2 web server applying the defaulting settings [http://abi.inf.uni-tuebingen.de/Services/SherLoc2] [21].

**Results and Discussion**

**Phosphopeptide identification**

We profiled the phosphoproteome of the cotton leaf after exposure to SNP in order to gain insight into plant cellular responses to NO. Approximately 2291 phosphopeptides were identified across the cotton database. Subsequently, we removed redundant and invalid peptides and additionally set the PhosphoRS probabilities above 75% and PhosphoRS scores above 50, according to standard practices, to further select for unique phosphopeptides. Following this selection process, we identified 1315 unique phosphopeptides, collectively containing 1528 non-redundant phosphorylation sites (Table S1). Of these non-redundant phosphorylation sites, 1371 (89.7%) were found to be phosphorylated at serine residues, 145 (9.5%) at threonine, and 12 (0.8%) at tyrosine (Figure S2A). Of the 1315 unique phosphopeptides, 1110 were singly phosphorylated, 198 were doubly phosphorylated, 6 were phosphorylated at three sites, and in only one case the phosphopeptide was phosphorylated at four sites (Figure S2B). The distribution of phosphor-Ser (pS) was found to be consistent with that of rice (95.9%) [22] and Glycine max (89.3%) [28]. Moreover, the distribution of phosphor-Thr (pT) was similar to Arabidopsis (9.9%) [22]. However, the abundance of tyrosine phosphorylation in cotton (0.8%, this study) was slightly lower than in Arabidopsis (2.4%) [22], Medicago (1.3%) [42] and rice (1.6%) [22]. On the other hand, Laurent et al. reported a proportion of pY in soybean of only 0.48% [28]. Additionally, within the same species (e.g. Arabidopsis), the distribution of the phospho-amino acids was not always consistent [22,43]. These differences may be attributed to differences in methodology (e.g. phosphopeptide enrichment and/or LC-MS) or in the biological system, where each cell type, tissue and organ has a unique phosphoproteome profile.

**Phosphorylation site motifs**

To determine the potential consensus sequences for cotton, the motifs of the 1528 non-redundant cotton phosphorylation sites were extracted using the Motif-X program (version v1.2 10.05.06; http://motif-x.med.harvard.edu/motif-x.html) with default parameters [44,45]. Because Gossypium raimondii Protein Fasta data has a size surpassing the upload restrictions to 10 MB, the database included in 20,000 sequences selected randomly from Gossypium raimondii Protein Fasta data was used as the background in Motif-X. Only confidently identified phosphorylation sites with Ascore >19 were used in analysis. All 25 of the phosphorylation site motifs identified in cotton are presented in Table 1. Serine was phosphorylated in 20 of the motifs, threonine in 4, and tyrosine in 1. Table 1 also lists the phosphorylation site motifs that contained at least one fixed position aside from the central phosphorylated residue.

With the purpose of investigating the specificity of the identified motifs to cotton, these motifs were compared with literatures [46,47] as well as in the PhosPhAt 4.0 database of Arabidopsis phosphorylation site motifs (http://phosphat.mpimp-golm.mpg.de/phosphat.html) [48] and Human protein reference database (HPRD) [49]. All the phosphorylation motifs from Arabidopsis and cotton are shown in Table S2A and the information of cotton phosphorylation site motifs that contained at least one fixed position aside from the central phosphorylated residue.

**Phosphoprotein identification**

To identify the corresponding proteins for each phosphopeptide, each phosphopeptide was searched against the CGP peptide database [38]. To overcome the challenge that multiple proteins may share the same peptide, the annotations were confirmed by comparison to the annotation of the top score protein hits from an in-house BLAST search against the CGP peptide database.

Identified phosphoproteins were classified based on: (a) the biological processes of each gene product according to annotations in the CGP database and (b) the gene product subcellular localization predicted using the SherLoc2 web server applying the defaulting settings [http://abi.inf.uni-tuebingen.de/Services/SherLoc2] [21].
tides were confirmed by comparing them to the annotation of the top score protein hits in CGP peptide database. From 1528 unique phosphopeptides, 1020 unique phosphoproteins were identified and all of sequence information could be found in Table S3. Passing through several steps of protein digestion, phosphopeptide enrichment, fractionation and mass spectrometry analysis, it is possible that for a single protein, two or more phosphopeptide is retained and can be detected in the LC/MS analysis. As much, our results indicated that, among the 1020 phosphoproteins, 81% were represented by a single phosphopeptide, 13.2% by two phosphopeptides, 3.7% by three phosphopeptides, and 2.1% by more than three phosphopeptides (Figure S3).

To obtain a general overview of the roles of phosphorylation in cotton, we next analyzed the biological functions/pathways and cellular localization of the 1020 phosphoproteins. The proteins were classified into 30 different categories based on their predicted functions, covering a wide range of pathways. The largest functional groups were ion/protein transport–related proteins, various enzymes (including kinases, transferases, and hydrolases), RNA splicing/processing, transcription, and sensory and signal transduction, accounting for 9.3, 7.7, 7.1, 6.4 and 5.3% of the 1020 proteins, respectively (Figure 1A). No function/pathway could be assigned to 32.0% of the phosphoproteins identified. For the subcellular localization analysis, these 1020 phosphoproteins were located in 12 different cellular compartments, including primarily the nucleus (22.5%), membranes (15.0%), cytoplasm (13.7%) and chloroplasts (4.4%). Exact subcellular localization information was not available for 35.0% of the proteins (Figure 1B).

### Quantitative analysis of protein phosphorylation changes in response to NO

The fold change (treatment/control) for every phosphopeptide at each time point was calculated, and the amounts of 183 unique phosphopeptides derived from 167 phosphoproteins were found to change significantly (≥1.5 fold change and P-value ≤ 0.05) after SNP exposure (Table S4). The total number of differentially expressed phosphopeptides was 95, 89, and 85 at 1, 3, and 6 h, respectively (Figure 2). A decreasing trend was observed for the number of upregulated phosphopeptides, which decreased from 53.7% in the 1 h group to 40.0% in the 3 h group and 52.8% in the 6 h group. Conversely, the number of downregulated phosphopeptides increased gradually from 42.1% in the 1 h group to 52.8% in the 3 h group and 58.8% in the 6 h group. Quantitative analysis revealed that most of the phosphopeptide changes occurred rapidly, i.e., within an hour of first exposure to SNP, which is consistent with the initiation of signal transduction and is similar to other phospho-proteomics studies [28]. As such, we hypothesized that, for phosphoproteins regulated by NO, we should see a shift from the

| No. | Cotton* | Arabidopsis | PhosphoMotif Finder Database |
|-----|---------|-------------|------------------------------|
| 1   | ….SPR   |             |                              |
| 2   | ….RS    |             |                              |
| 3   | .SP.S... |             |                              |
| 4   | ….S...SP.|             |                              |
| 5   | ….SP.R..|             |                              |
| 6   | ….RS...  |             |                              |
| 7   | ….SPK... |             |                              |
| 8   | ….S...SP.|             |                              |
| 9   | R.S,S...  |             |                              |
| 10  | ….S...SP.|             |                              |
| 11  | ….GSP... |             |                              |
| 12  | ….RO...S |             | N                            |
| 13  | ….SDE... |             | N                            |
| 14  | …LR.S... |             | N                            |
| 15  | ….SPS... |             | N                            |
| 16  | ….SDE... |             | N                            |
| 17  | ….SP...S |             | N                            |
| 18  | ….SRS... |             | N                            |
| 19  | ….SD.D...|             | N                            |
| 20  | ….DS.E...|             | N                            |
| 21  | ….RT...S.|             | N                            |
| 22  | ….T...SP.|             | N                            |
| 23  | ….SPT... |             | N                            |
| 24  | ….SP...T.|             | N                            |
| 25  | ….PY...  |             | N                            |

*The phosphorylated residues are indicated in bold and underlined, and (.) indicates any amino acid. N indicates the motif was not found.

doi:10.1371/journal.pone.0094261.t001
Figure 1. Functional classifications (A) and protein subcellular locations (B) of all 1020 phosphoproteins.
doi:10.1371/journal.pone.0094261.g001
phosphorylated to the dephosphorylated forms as the treatment continued.

The 167 phosphoproteins whose phosphopeptides levels changed significantly in response to NO were classified into 23 functional categories based on their predicted biological function (Figure 3A). Except for the unknown proteins (25.7%), the largest functional groups were RNA splicing or processing (17.4%), ion or protein transport (7.8%), sensory or signal transduction (7.2%), and transcription (6.6%). Compared with the total dataset of all the non-redundant phosphoproteins identified, our results suggest that these functional categories are the most important in terms of response to NO. Correspondingly, the largest subcellular location groups were the nucleus (35.3%), unknown (26.3%), the cytoplasm (15.6%), and membranes (9.6%) (Figure 3B).

Ion/Protein transport proteins

Many (95) of the cotton phosphoproteins that we identified are involved in ion/protein trafficking processes, such as potassium, ammonium, sucrose, and peptide transport, or are transporter proteins themselves (i.e., aquaporin and ABC transporter). ATPases are post-translationally regulated by complex mechanisms, including phosphorylation [50,51]. In our present study, the abundance of phosphopeptides corresponding to 16 ATPases was found to be altered, including 2 V-type and 9 plasma membrane-type phosphopeptides. These results suggest that NO regulation of transporters by phosphorylation may play a role in quick and reversible activating/inactivating transport channels, leading to rapid changes in water potential and turgor pressure that are necessary for plant growth and stimulus response.

Miscellaneous enzymes

As shown in Figure 1A, a considerable number (78) of the cotton phosphoproteins identified were classified as “miscellaneous enzymes”, including kinases, transferases, and hydrolases. However, only 4 of these proteins, all kinases, appeared in our quantitative phosphoproteomic data (Figure 3A). We hypothesize that changes in the abundance of these proteins might reflect a coordinated activity of one or more kinase cascades involved in the NO-response process. These proteins fit into the context of NO signaling and their relationships with other well-known receptor and protein kinases need to be further explored in future studies.

Proteins involved in DNA/RNA binding and transcription

Among the 72 transcription-related phosphoproteins identified, the content levels of only 11 significantly changed in response to SNP (Figure 3A), including transcription initiation/elongation factors, transcriptional co-repressors, and some sequence-specific transcription factors. DNA/RNA-binding proteins have been identified in several phosphoproteomic studies [28,42]. Similarly, we found 41 phosphoproteins in cotton that appear to be involved in a variety of DNA/RNA processes (Figure 1A). In addition, we found 7 differentially expressed phosphoproteins involved in DNA/RNA binding, including RNA-binding motif protein (Cotton_IGene_10033131) expressed predominantly in spermatocytes [52]. HMG1/2-like protein (Cotton_IGene_10033211) containing 1HMG box DNA-binding domain that belongs to the HMGB family which was regulated by darkness and circadian rhythm [53], uncharacterized RNA-binding protein (Cotton_IGene_10011862) and a number of predicted proteins (Table S1). We speculate that the phosphorylation and the presence of differential phosphorylation of these proteins may be essential for the rapid responses to NO treatment.

Phosphorylation changes of protein in response to plant hormones

The 187 phosphopeptides, corresponding to the 167 identified NO-responsive phosphoproteins, were placed into appropriate signaling pathways to better understand which physiological processes in cotton are regulated by NO. The signaling pathways analysis was carried out using KEGG Automatic Annotation Server (http://www.genome.jp/tools/kaas/) with the default settings.

Ethylene-insensitive protein 2 (EIN2) acts downstream of ethylene receptors and upstream of ethylene-regulated transcription factors and is involved in regulating early leaf senescence caused by NO deficiency [54,55]. We found that the levels of phosphorylated EIN2 (Cotton_IGene_10027821) increased gradually after SNP exposure and were significantly higher at 6 h. Levels of another negative regulator in the ethylene response pathway, the serine/threonine-protein kinase CTR1 (Cotton_IGene_10011591), were also increased in response to NO as early as 1 h after treatment began. In our previous study, we found that NO is involved in regulating ACS [21], a synthase catalyzing the synthesis of a precursor (1-Aminocyclopropane-1-carboxylic acid) in the ethylene biosynthesis pathway. Here, we found that NO appears to regulate the phosphorylation of EIN2 and CTR1, central factors in the signaling pathways regulated by ethylene. Thus, we postulate that NO regulates not only the biosynthesis of ethylene but also the ethylene-signaling pathway by modulating phosphorylation of EIN2 and CTR1.

In our study, NO also appeared to affect the signal transduction pathways of other plant hormones, such as cytokinine and ABA (Figure S4). Inhibitors of NO-synthase have been shown to inhibit expression from the cytokinine-responsive ARR5 promoter [56]. The two-component response regulator ARR2 is also a transcriptional activator involved in cytokinine signaling and is phosphorylated in response to cytokinine [57,58]. In the current paper, we found that levels of phospho-ARR2 (Cotton_IGene_10029097) were markedly reduced in the 1 and 3 h treatment groups. This decrease is likely related to the NO-regulated cytokinine signaling pathway. Protein phosphatase 2C (PP2C) 37 is a major negative regulator of ABA responses during seed germination and cold acclimation [59,60]. We also found that the levels of phospho-

Figure 2. Analysis of the 183 phosphopeptides whose levels changed after exposure to SNP. N/a, not applicable, indicates that the phosphopeptides were not detected in this group and were only expressed in other treatment groups.
doi:10.1371/journal.pone.0094261.g002
ylated PP2C 37 (Cotton_D_gene_10021530) were increased at both the 1 and 3 h time points. Moreover, NO mediates the induction of ABA biosynthesis involved in oxidative stress tolerance in maize (Zea mays) [61,62]. Additionally, PP2C inhibits SnRK2, a vital kinase in the regulation of ABA-induced gene expression, thus inhibiting activation of the ABA pathway [25]. Given this extensive cross-talk between NO and ABA, it is plausible that the apparent dephosphorylation of PP2C 37 that we observed may play a role in ABA signal transduction regulated by NO.

Many components of the auxin efflux (but not influx) system are activated by phosphorylation [28,63]. In this study, the phosphopeptide levels of one auxin-related protein were significantly altered in response to NO. Furthermore, we found that a phosphoprotein required for auxin influx-facilitator (AUX1) polar trafficking (Cotton_D_gene_10018241) was significantly increased 1 h after SNP treatment. Thus, it appears that increases in phosphorylation of both efflux and influx carriers facilitate auxin transport in cotton.

Phosphoproteins involved in RNA metabolism

Our study also identified numerous phosphoproteins involved in RNA metabolism, including components of the spliceosome, mRNA surveillance, and RNA transport proteins. Specifically, 21 phosphoproteins, corresponding to 13 KEGG orthology entries involved in spliceosome activity were detected (Figure S5), 8 of which were SR proteins (serine/arginine-rich splicing factors; Cotton_D_gene_10019600, Cotton_D_gene_10022365, Cotton_D_gene_10019600, Cotton_D_gene_10039654, Cotton_D_gene_10032493, Cotton_D_gene_10026606, Cotton_D_gene_10024595, and Cotton_D_gene_10030715). SR proteins belong to a highly conserved family of splicing regulators involved in RNA splicing.

Figure 3. Functional classifications (A) and protein subcellular locations (B) of the 167 phosphoproteins. doi:10.1371/journal.pone.0094261.g003
in constitutive and alternative splicing in response to stress and hormones [64] and have been shown to be phosphorylated in both animals and plants [65–67]. Moreover, kinase-protein interactions related to pre-mRNA splicing are modulated by the phosphorylation of SR proteins [69]. The 9 SR proteins we detected were extensively phosphorylated at serine residues in their serine/arginine-rich domains (Table S1), similar to the phosphorylation of SR proteins in Arabidopsis [69].

The phosphorylation of some helicases in humans and Arabidopsis has been shown to regulate pre-mRNA splicing [69–71]. Our analysis identified two ATP-dependent RNA helicases (Cotton_D_gene_10003086, Prp2; Cotton_D_gene_10004269, Prp2), suggesting that phosphorylation of the cotton homologs may also function in splicing. Small nuclear RNA conformation and the phosphorylation of related proteins strongly affect 3′-splice site recognition and catalytic activation of the spliceosome [72,73]. We found that the levels of phosphorylated forms of two small nuclear ribonucleoproteins (Cotton_D_gene_10032473, Prp3; and Cotton_D_gene_10038196, Prp4) and one small nuclear ribonucleoprotein helicase (Cotton_D_gene_10020278, Bn2) increased in response to NO within 3 h of exposure to SNP. In addition, the phosphorylated form of a pre-mRNA processing factor (Cotton_D_gene_10025929, FBP11), the nuclear cap-binding protein (Cotton_D_gene_10014749, CBP80/20), and a nuclear apoptotic chromatin-condensation inducer (Cotton_D_gene_10036545, ACINUS) also increased 1 h and 3 h after SNP treatment began. The levels of phosphorylated U2-associated protein (Cotton_D_gene_10039568, SR110) and RNA-binding protein (Cotton_D_gene_10022997, HnRNPs) also decreased to varying degrees. We also found three splicing factors in this pathway (Cotton_D_gene_10040894, SF36; Cotton_D_gene_10001970, U2AF; Cotton_D_gene_10027627, U2AF) that are involved in phosphorylation in response to NO, indicating a direct link between NO signaling and spliceosome regulation through phosphorylation and dephosphorylation of related proteins. Except for a special pattern that sharp increase followed by sharp decrease (Cotton_D_gene_10019970), the changes in the other 20 spliceosome-related phosphoproteins could be categorized as two distinct patterns—a gradual decline in phosphorylation followed by a sharp increase or a stable decline from 1 to 6 h (Figure 4).

The mRNA surveillance pathway is a quality-control mechanism that detects and degrades abnormal mRNAs. Upf3, together with Upf1 and Upf2, detects the presence of premature termination codon and instigates decapping and rapid exonucleolytic digestion of the mRNA [74]. The dephosphorylation of hUpf1 by PP2A, contribute to the remodeling of the mRNA surveillance complex [75]. We found that Upf3 (Cotton_D_gene_10004090, Upf3) levels were higher at 3 h in the treated samples than at 1 or 6 h, and the levels of the phosphorylated forms of three PP2A subunits (Cotton_D_gene_10025447, PP2A; Cotton_D_gene_10028017, PP2A; and Cotton_D_gene_10028691, PP2A) also changed over time. In addition, levels of the phosphorylated RNA-binding motif protein (Cotton_D_gene_10035131, CPSF6/7), heterogeneous nuclear ribonucleoprotein (Cotton_D_gene_10035148, Musashi), cleavage and polyadenylation specificity factor (Cotton_D_gene_10004099, CPSF4) and serine/threonine-protein kinase (Cotton_D_gene_10001769, SMG1) also changed significantly in response to NO (Figure 5, Figure S6). The changes in these proteins are consistent with the known critical role of the mRNA surveillance pathway in cellular homeostasis.

In addition to the apoptotic chromatin condensation inducer, nuclear cap-binding protein, and Upf3, all mentioned above, we identified another six phosphoproteins involved in RNA transport (Figure S7), including four translation initiation factors (Cotton_D_gene_10020409, eIF3; Cotton_D_gene_10004109, eIF3; Cotton_D_gene_10012222, eIF4G; and Cotton_D_gene_10010902, eIF4A), one ribonuclease (Cotton_D_gene_1001365, RNaseZ), and one unknown protein (Cotton_D_gene_10022069, Nup133). Eukaryotic initiation factor 3 (eIF3) is an essential, highly conserved multiprotein complex that is a key component in the recruitment and assembly of the translation initiation machinery [76], eIF4A is a protein complex that mediates recruitment of ribosomes to mRNA [77,78]. The activity of both proteins is regulated by phosphorylation and in turn influences translation, apoptosis, and growth [78,79]. Analysis of the cotton eIFs revealed that the phosphorylated peptides of all four proteins increased to varying degrees in response to SNP. The pattern for all the RNA transport phosphopeptides showed a rapid increase followed by a steady downward trend (Figure 6).

RNA splicing/processing was the largest group of proteins whose phosphorylation level was significantly affected by NO (Figure 3A). Correspondingly, we found the numerous proteins in the spliceosome, mRNA surveillance and RNA transport pathways that were altered noticeably in regards to their phosphorylation status as regulated by NO. These results suggest that the plant mRNA splicing machinery is a major target of phosphorylation or dephosphorylation induced by NO.

Other phosphorylated proteins

Other phosphoproteins, whose peptide levels changed noticeably in response to NO in the current paper, were found to be involved in protein processing and/or to be related to plant-pathogen interactions [21,80]. In protein processing, the binding protein, Bip, in the endoplasmic reticulum can recognize improperly folded or assembled and facilitates their refold and reassembly [81,82]. It has been reported that the transcription of the gene encoding Bip is induced by SNP [21]. Similarly, in the current paper we found that phosphopeptides of Bip (Cotton_D_gene_10017746) were increased significantly after treatment with SNP in the 6 h group. In terms of plant-pathogen interaction proteins, we found that the phosphorylated forms of three heat shock proteins were also dramatically increased by SNP (Cotton_D_gene_10018942, Cotton_D_gene_10030775 and Cotton_D_gene_10025013). A mitogen-activated protein kinase (Cotton_D_gene_10039089, MKK1/2), a calcium-dependent protein kinase (Cotton_D_gene_10038462, CDPK), and a respiratory burst oxidase (Cotton_D_gene_10034071, Rboh) also showed significant upregulation in phosphorylation in varying degrees. On the other hand, an unknown protein (Cotton_D_gene_10020033, CaMCM1) showed significant decreases in the phosho-forms (Figure 7, Figure S8). These results indicate that NO regulates plant disease resistance by modulating phosphorylation of the relevant proteins.

It is noteworthy that some key proteins involved in circadian rhythms were also revealed in the phosphoproteome of SNP-treated cotton. The phosphorylated forms of protein LHY (Cotton_D_gene_10035743) and the transcription factor HY5 (Cotton_D_gene_10010118), both important components in clock function, were significantly reduced following treatment with SNP (Figure S9). To our knowledge, this is the first report of a relationship between NO and plant circadian rhythms, especially in terms of phosphorylation regulation. The levels of several other phosphopeptides/phosphoproteins not previously implicated in NO response were significantly also altered by SNP treatment, including translocase of chloroplast 159 (Cotton_D_gene_10024721), stem-specific protein TSJT1 (Cotton_D_gene_10003896), and villin-2 (Cotton_D_gene_10038481). Further exploration of these
Figure 4. Fold changes of the phosphopeptides involved in splicesome significantly altered following SNP. Protein identification numbers refer to the CGP database. To make the figure easier to read, the protein ID is represented only by the numbers in the key (i.e., remove "Cotton_D_gene_").

doi:10.1371/journal.pone.0094261.g004

Figure 5. Fold changes of the phosphopeptides involved in mRNA surveillance significantly altered following SNP. Protein identification numbers refer to the CGP database. To make the figure easier to read, the protein ID is represented only by the numbers in the key (i.e., remove "Cotton_D_gene_").

doi:10.1371/journal.pone.0094261.g005
phosphoprotein targets may provide insight into previously unknown effectors of the NO signaling pathway.

**Conclusions**

This study represents one of the first large-scale phosphoproteomic analyses of the NO response in cotton, providing both a global and comparative analysis of protein phosphorylation regulated by NO and further insight into the dynamics of individual phosphorylation sites. Our results reveal phosphorylation site motifs and proteins not previously observed in plants, which may provide insight into NO signaling in cotton as well as plants in general. In addition, the novel plant phosphorylation site motifs could lead to future discovery of novel kinase-substrate interactions. We have submitted the peptide and protein sequences to the plant protein phosphorylation database (P3DB,
Proteins indicated red were found to be significantly upregulated or downregulated by SNP in our analysis. (TIF)

Distribution of phosphorylation sites. A. Distribution of phosphorylation on serine, threonine, and tyrosine was assessed for all non-redundant localized phosphorylation sites. B. Distribution of single- and multi-phosphorylated peptides showing that the majority of phosphopeptides have only one phosphorylation site. (TIF)

The number of phosphopeptides per protein. The distribution of the number of phosphopeptides per protein is shown for all 1315 unique phosphopeptides. (TIF)

The plant hormone signal-transduction pathway derived from KEGG. Proteins indicated in red were found to be significantly upregulated or downregulated by SNP in our analysis. (TIF)

The splicingosome pathway derived from KEGG. Proteins indicated red were found to be significantly upregulated or downregulated by SNP in our analysis. (TIF)

The mRNA surveillance pathway derived from KEGG. Proteins indicated red were found to be significantly upregulated or downregulated by SNP in our analysis. (TIF)

References
1. Beligni MV, Lamattina L (2000) Nitric oxide stimulates seed germination and de-etiolation, and inhibits hypocotyl elongation, three light-inducible responses in plants. Planta 210: 215–221.
2. Pagnussat GC, Simonacchi M, Puntarulo S, Lamattina L (2002) Nitric oxide is required for root organogenesis. Plant Physiol 129: 954–956.
3. Garcia-Mata C, Lamattina L (2007) Abscisic acid (ABA) inhibits light-induced stomatal opening through calcium-and nitric oxide-mediated signaling pathways. Nitric Oxide 17: 143–151.
4. Prado AM, Porterfield DM, Feijo JA (2004) Nitric oxide is involved in growth regulation and re-orientation of pollen tubes. Development 131: 2707–2714.
5. Mushina TE, Lamb C, Zeier J (2007) Expression of a nitric oxide degrading enzyme induces a senescence programme in Arabidopsis. Plant Cell Environ 30: 39–52.
6. Yang LM, Tian DG, Todd CD, Luo YM, Hu XY (2013) Comparative proteome analyses reveal that nitric oxide is an important signal molecule in the response of rice to aluminum toxicity. J Proteome Res 12: 1316–1330.
7. Zhao MG, Chen L, Zhang LL, Zhang WH (2009) Nitric reductase-dependent nitric oxide production is involved in cold acclimation and freezing tolerance in Arabidopsis. Plant Physiol 151: 755–767.
8. Zhao MG, Tian QY, Zhang WH (2007) Nitric oxide synthase-dependent nitric oxide production is associated with salt tolerance in Arabidopsis. Plant J 48: 206–217.
9. Lamattina L, Garcia-Mata C (2001) Nitric oxide induces stomatal closure and enhances the adaptive plant responses against drought stress. Plant Physiol 126: 1196–1204.
10. Delledonne M, Xia Y, Dixon RA, Lamb C (1998) Nitric oxide functions as a signal in plant disease resistance. Nature 394: 585–588.

Identification of Phosphoprotein in Cotton

Figure S1 The effect of NO on cotton growth. Cotton seeds were treated with 0, 0.05, 0.1, or 0.5 mM SNP for 24 h. Changes in the morphology (A) and chlorophyll content (B) following the treatment of different concentrations of SNP. (TIF)

Figure S2 The distribution of phosphorylation sites. A. Distribution of phosphorylation on serine, threonine, and tyrosine was assessed for all non-redundant localized phosphorylation sites. B. Distribution of single- and multi-phosphorylated peptides showing that the majority of phosphopeptides have only one phosphorylation site. (TIF)

Figure S3 The number of phosphopeptides per protein. The distribution of the number of phosphopeptides per protein is shown for all 1315 unique phosphopeptides. (TIF)

Figure S4 The plant hormone signal-transduction pathway derived from KEGG. Proteins indicated red were found to be significantly upregulated or downregulated by SNP in our analysis. (TIF)

Figure S5 The spliceosome pathway derived from KEGG. Proteins indicated red were found to be significantly upregulated or downregulated by SNP in our analysis. (TIF)

Figure S6 The mRNA surveillance pathway derived from KEGG. Proteins indicated red were found to be significantly upregulated or downregulated by SNP in our analysis. (TIF)

Figure S7 The RNA transport pathway derived from KEGG. Proteins indicated red were found to be significantly upregulated or downregulated by SNP in our analysis. (TIF)

Figure S8 The plant-pathogen interaction pathway derived from KEGG. Proteins indicated red were found to be significantly upregulated or downregulated by SNP in our analysis. (TIF)

Figure S9 The circadian rhythm pathway derived from KEGG. Proteins indicated red were found to be significantly upregulated or downregulated by SNP in our analysis. (TIF)

Table S1 Phosphopeptide identifications in cotton leaves. (XLS)

Table S2 Phosphorylation motifs identified from cotton (A) and raw peptide data used to extract the given motif of cotton (B). (XLS)

Table S3 Amino acid sequences of 1020 phosphoproteins. (XLS)

Table S4 Quantitative analysis identified significant changes in the content levels of 183 phosphopeptides. (XLS)

Acknowledgments
We would like to thank Shanghai Applied Protein Technology Co. Ltd. for providing technical support.

Author Contributions
Conceived and designed the experiments: SF SY YM. Performed the experiments: YM J. Liu. Analyzed the data: YM HW CP MS. Contributed reagents/materials/analysis tools: SF SZ XZ J. Lan CF. Wrote the paper: YM.

http://p3db.org/download.php [31,83] to help develop future, large-scale cotton proteomic datasets. In this perspective, a better understanding of phosphorylation events may eventually lead to the development of a comprehensive model for the mechanisms of action of NO. Functional analysis of these protein phosphorylation sites by site-directed mutagenesis or reverse genetic approaches will be necessary to further investigate their role in NO signaling.
47. Wang X, Bian YY, Cheng K, Gu LF, Ye ML, et al. (2013) A large-scale protein phosphorylation analysis of cotton leaf response to nitric oxide. J Proteome Res 12: 648.

49. Kravaha Prasad TS, Goel R, Kaulapyrus K, Keerthikumar S, Kumar S, et al. (2009) Human protein reference database-2009 update. Nucleic Acids Res 37: D767–D772.

50. Palmgren MG (2001) Plant plasma membrane H+-ATPases: powerhouses for non-nutrient uptake. Annu Rev Plant Biol 52: 817–843.

48. Palmgren MG (2001) Plant plasma membrane H+-ATPases: powerhouses for non-nutrient uptake. Annu Rev Plant Biol 52: 817–843.

51. Lan P, Li W, Wen TN, Shiuay JY, Wu YC, et al. (2010) ITRAQ protein profile analysis of Arabidopsis roots reveals new aspects critical for iron homeostasis. Plant Physiol 155: 821–834.

52. Eisenhaur EM, Venasales JP, Norton CS, Lansette, D, Boyle S, et al. (2000) An evolutionarily conserved germl cell-specific hnRNP is encoded by a retrotransposed gene. Hum Mol Genet 9: 2117–2124.

53. Zheng GC, Bui AQ, O'Neill SD (1993) Abundance of an mRNA encoding a high mobility group DNase I inhibitor is regulated by light and an abiotic stress: the rosette. Mol Plant Biol 23: 815–823.

54. Alonso JM, Hirayama T, Roven G, Nourizadeh S, Ecker JR (1999) EN2, a bifunctional transducer of ethylene and stress responses in Arabidopsis. Science 284: 2140–2143.

55. Niu Y, Gao F (2012) Nitric oxide regulates dark-induced leaf senescence through eNOS in Arabidopsis. J Integr Plant Biol 54: 516–525.

56. Romanov GA, Lomin SN, Rakova NY, Heyl A, Schmülling T (2008) Does NO play a role in cytokinin signal transduction? FEBS Lett 582: 874–879.

57. Harc C, Zehnder J, Albrecht V, Sceere U, Hummel F, et al. (2006) The response regulator 2 mediates ethylene signaling and hormone signal integration in Arabidopsis. EMBO J 25: 3290–3302.

58. Kim HJ, Ryu H, Hong SH, Woo HR, Lim PO, et al. (2006) Cytokinin-mediated control of leaf longevity by the ABA2 kinase through phosphorylation of ABR2 in Arabidopsis. P Natl Acad Sci USA 103: 814–819.

59. Sheen J (1998) Mutational analysis of protein phosphatase 2C involved in ascorbic acid signal transduction in higher plants. P Natl Acad Sci USA 95: 975–980.

60. Kuhn JM, Boissier-Derrain A, Dizon MB, Makthai MH, Schroeder JI (2006) The protein phosphatase AP2P3CA negatively regulates ascorbic acid signal transduction in Arabidopsis, and effects of abh1 on AP2P3CA mRNA. Plant Physiol 140: 127–139.

61. Zhang A, Zhang J, Zhang J, Ye N, Zhang H, et al. (2011) Nitric oxide mediates brassinosteroid-induced ABA biosynthesis involved in oxidative stress tolerance in maize leaves. Plant Cell and Physiol 52: 181–192.

62. Ma F, Lu R, Liu H, Shi B, Zhang J, et al. (2012) Nitric oxide-activated calcium/calmodulin-dependent protein kinase regulates the abscisic acid-induced antioxidant defence in maize. J Exp Bot 63: 4835–4847.

63. Debarbieux A, Muller P, Guern J (1998) Short-lived and phosphorylated proteins contribute to carrier-mediated efflux, but not to influx, of auxin in suspension cultured tobacco cells. Plant Physiol 116: 833–844.

64. Palusa SG, Ali GS, Reddy AS (2007) Alternative splicing of pre-mRNAs of Arabidopsis serine/arginine-rich proteins: regulation by hormones and stresses. Plant J 49: 1091–1107.

65. Lorková ZJ, Lopato S, Pexa M, Lehner R, Barta A (2004) Proteomics analysis of cotton leaf response to nitric oxide. J Proteome Res 10: 5416–5432.

66. Romanov GA, Lomin SN, Rakova NY, Heyl A, Schmülling T (2008) Does NO play a role in cytokinin signal transduction? FEBS Lett 582: 874–879.

67. Lopato S, Kalyna M, Dorner S, Kobayashi R, Krainer AR, et al. (1999) Interactions of Arabidopsis RS domain containing cyclophilins with SR proteins and U1 and U2 small nuclear ribonucleoprotein-specific proteins suggest their involvement in pre-mRNA splicing. J Biol Chem 274: 2148–2152.

68. Reddy AS (2004) Plant serine/arginine-rich proteins and their role in pre-mRNA splicing. Trends Plant Sci 9: 541–547.

69. de la Fuente van Bentem S, Anrather D, Roittinger E, Djamai A, Hufnagl T, et al. (2006) Phosphoproteomics reveals extensive in vivo phosphorylation of Arabidopsis proteins involved in RNA metabolism. Nucleic Acids Res 34: 3267–3278.

70. Tanner NK, Linder P (2001) DExD/H box RNA helicases: from generic motors to specific dissociation functions. Mol Cell 8: 251–262.

71. Arenas J, Abejon J (1991) Requirement of the RNA helicase-like protein PRP22 for release of messenger RNA from splicing complexes. Nature 347: 487–493.

72. Makarov EM, Makarova OV, Uralshb H, Getznel M, Will CL, et al. (2002) Small nuclear ribonucleoprotein remodeling during catalytic activation of the spliceosome. Science 298: 2205–2208.

73. Zhang Y, Mald T, Bagulj K, Kern T, Kang HS, et al. (2013) Structure, phosphorylation and U2AF65 binding of the N-terminal domain of splicing factors on the 3′ splice site. Nucletic Acids Research 41: 1345–1354.

74. Kunz JB, Neu-Yilik H, Hentze MW, Kulozik AE, Gehring NH (2006) Functions of hUPF3a and hUPF3b in nonsense-mediated mRNA decay and translation. RNA 12: 1015–1022.

75. Ohtsuki T, Yamashita A, Kashima I, Schell T, Anders KR, et al. (2003) Phosphorylation of hUPF1 induces formation of mRNA surveillance complexes containing hSMG-5 and hSMG-7. Mol Cell 12: 1177–1200.

76. Sun C, Todorovic A, Querol-Audín J, Bai Y, Villa N, et al. (2011) Functional reconstitution of human eIF3: a versatile scaffold for translation initiation factor 3 (eIF3). P Natl Acad Sci USA 108: 20473–20478.

77. Hinnebusch AG (2006) Identification of Phosphoprotein in Cotton Proteomics. PLoS ONE 12 April 2014 | Volume 9 | Issue 4 | e94261
78. Gingras AC, Raught B, Sonenberg N (1999) eIF4 initiation factors: effectors of mRNA recruitment to ribosomes and regulators of translation. Annu Rev Biochem 68: 913–963.
79. Farley AR, Powell DW, Weaver CM, Jennings JL, Link AJ (2011) Assessing the components of the eIF3 complex and their phosphorylation status. J Proteome Res 10: 1481–1494.
80. Asai S, Yoshioka H (2009) Nitric oxide as a partner of reactive oxygen species participates in disease resistance to necrotrophic pathogen Botrytis cinerea in Nicotiana benthamiana. Mol Plant Microbe In 22: 619–629.
81. Rothman JE (1989) Polypeptide chain binding proteins: catalysts of protein folding and related processes in cells. Cell 59: 591–601.
82. Koizumi N (1996) Isolation and responses to stress of a gene that encodes a luminal binding protein in Arabidopsis thaliana. Plant Cell Physiol 37: 862–865.
83. Gao J, Agrawal GK, Thelen JJ, Xu D (2009) P3DB: a plant protein phosphorylation database. Nucleic Acids Res 37: D960–D962.