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RESEARCH ARTICLE

Quantitative phosphoproteomics reveals novel roles of cAMP in plants

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Abstract

3′,5′-cyclic adenosine monophosphate (cAMP) is finally recognized as an essential signaling molecule in plants where cAMP-dependent processes include responses to hormones and environmental stimuli. To better understand the role of 3′,5′-cAMP at the systems level, we have undertaken a phosphoproteomic analysis to elucidate the cAMP-dependent response of tobacco BY-2 cells. These cells overexpress a molecular "sponge" that buffers free intracellular cAMP level. The results show that, firstly, in vivo cAMP dampening profoundly affects the plant kinome and notably mitogen-activated protein kinases, receptor-like kinases, and calcium-dependent protein kinases, thereby modulating the cellular responses at the systems level. Secondly, buffering cAMP levels also affects mRNA processing through the modulation of the phosphorylation status of several RNA-binding proteins with roles in splicing, including many serine and arginine-rich proteins. Thirdly, cAMP-dependent phosphorylation targets appear to be conserved among plant species. Taken together, these findings are consistent with an ancient role of cAMP in mRNA processing and cellular programming and suggest that unperturbed cellular cAMP levels are essential for cellular homeostasis and signaling in plant cells.

KEYWORDS
cAMP, cyclic adenosine monophosphate, cyclic nucleotide, kinases, RNA splicing

Abbreviations: AC, Adenylate cyclase; ACN, Acetonitrile; AGCKs, AGC kinases; BY-2, Bright Yellow-2; cAMP, 3′,5′-cyclic adenosine monophosphate; CDKs, Cyclin-dependent kinases; CDPKs, Calcium-dependent kinases; cGMP, 3′,5′-cyclic guanosine monophosphate; CNGC, Cyclicnucleotide gated channel; DAPPs, Differentially abundant phosphosites; DTT, Dithiothreitol; EDTA, Ethylenediaminetetraaceticacid; FASP, Filter aided sample preparation; FIMO, Find individualmotif occurrences; GO, Gene ontology; hnRNPs, Heterogeneousnuclear ribonucleoproteins; KEGG, Kyoto encyclopedia of genes and genomes; KNN, K-nearest neighbors; LC-MS/MS, Liquid chromatography with tandem mass spectrometry; MAPKs, MAP kinases; MCC, Maximal Clique Centrality; MSA, Multiple sequence alignment; NaF, Sodium fluoride; Na_3VO_4, Sodium orthovanadate; NPC, Nuclear pore complex; PCA, Principal component analysis; PDE, Phosphodiesterase; PIN2, Auxin efflux carrier family protein; PKA, Proteinkinase A; PMSF, Phenylmethylsulfonylfluoride; PPI, Protein-proteininteraction; PP1, Proteinphosphatase 1; PPs, Phosphosites; QRILC, Quantile regression imputation of left-censored; RBPs, RNA-binding proteins; RLKs, Receptor-like kinases; RT, Room temperature; SDS, Sodium dodecyl sulfate; SAPs, Spliceosome-associatedproteins; SPE, Solid phase extraction; SR, Serine andarginine-rich; STRING, Search tool for retrieval of interacting genes; TFA, Trifluoroacetic acid; TPS5, Trehalose phosphatase/synthase 5.

1 | INTRODUCTION

The cyclic adenosine monophosphate (3′,5′-cAMP) is an established signaling molecules, both in prokaryotes and eukaryotes. In plants, cAMP acts as second messenger in a number of processes including pollen tube growth [1], cell cycle regulation [2], auxin signaling [3], and stomatal closure [4]. Moreover, increasing evidence suggests that cAMP is also an essential component during the responses to abiotic and biotic stress [5–7]. Nevertheless, cAMP-dependent signaling in plants is still not well understood and molecular and cellular studies will be required to unravel the mechanisms of action as well as the systemic effects of cAMP.

cAMP signaling depends on the activation of adenylate cyclases (ACs) to rapidly increase cAMP levels [8] and phosphodiesterases (PDEs) to decrease these levels by converting cAMP it to AMP [5]. Despite reported AC activities in plant tissue extracts [9–12], to-date, only a few ACs have been identified in plants [8]. Moreover, a moonlighting role for mononucleotide cyclases was recently proposed and >10 *Arabidopsis thaliana* candidate ACs were identified [13]. Until recently, PDE activity was only detected in plant protein extracts [14–16] while PDE domains with cAMP-specific PDE activity were reported e.g. in the CAPE protein of *Marchantia polymorpha* (MpCAPE-PDE) [17].

To-date only a few cAMP-dependent signal transduction mechanisms and pathways have been elucidated and they include direct activation of the cyclic nucleotide gated channels (CNGCs) [8, 18, 19]. It appears that cAMP-dependent signaling may be closely linked to cytosolic Ca^{2+} as well as Na^{+} and K^{+} fluxes [20–22]. At the system level, rapid and reversible post-translational modification by phosphorylation is essential for plant development and adaptation to changing environmental conditions [23]. In mammals, increases in cAMP levels promote the phosphorylation of several intracellular enzymes via the activation of protein kinase A (PKA) [24]. While plant kinases that specifically respond to cAMP concentration changes remain elusive [25, 26], cAMP-dependent changes of phosphorylation are likely given that cAMP, which promotes Ca^{2+} influx, also initiates a protein kinase signaling cascade. This cascade, in turn, leads to changes in the protein phosphorylation status [27]. Furthermore, transcriptome analyses following AC stimulation also suggested that cAMP-dependent phosphorylation does occur in plants [26].

Here we propose the use of an established non-pharmacological approach, consisting of a genetically encoded tool based on the two cAMP-binding domains of the human PKA I regulatory subunit [7, 28, 29]. This cAMP-binding domain lowers the intracellular levels of cAMP in *Nicotiana tabacum* Bright Yellow-2 (BY-2) cells and thereby helps to uncover cAMP-dependent signaling events. This non-invasive modulation of cellular cAMP levels has previously revealed remarkable cAMP-dependent changes in the proteomic profile of tobacco BY-2 cells [6].

To follow on from these experiments, we now address the question of the role of 3′,5′-cAMP on the phosphoproteome with a view to infer mechanisms and systems-level responses that depend on this messenger.

2 | MATERIALS AND METHODS

2.1 | Biological material

Wild type tobacco BY-2 (*N. tabacum* L. cv. Bright Yellow 2; TBY) cell suspensions were routinely propagated and cultured as described elsewhere [30]. The tobacco BY-2 line (termed cAS line) overexpressing a "cAMP sponge" based on the high-affinity cAMP-binding carboxy-terminus of the regulatory subunit of a protein kinase A [28, 29] was propagated in liquid selective medium containing 50 μg/mL kanamycin. For the experiments, cAS cells were cultured in non-selective medium. Two mL of both WT an cAS stationary phase cell suspensions (7 days) were diluted in 100 mL of fresh culture medium in 250-mL flasks and grown at 27°C. After 5 days of culture, cells were collected by vacuum filtration on Whatman 3 MM paper, frozen in liquid nitrogen, and stored at −80°C until the phosphoproteomic analyses. In total, four independent biological replicates were obtained and processed.

2.2 | Phosphoproteomic workflow

Proteins were extracted following SDS/phenol method with minor adjustments [31]. Cells (1 g FW) were ground in liquid nitrogen and then homogenized in extraction buffer (0.15 M Tris-HCl pH 8.8, SDS 1% 1 mM EDTA, 0.1 M DTT, 1 mM PMSF, 0.1 mg/mL Pefabloc 1 mM Na_3VO_4, 1 mM NaF, pH 8.8). After centrifugation (15,000 g for 10 min), the supernatant (SN) was collected and mixed with an equal volume of phenol at room temperature (RT) for 30 min. After centrifugation (15,000 g for 5 min at RT) the phenol phase was collected and proteins were precipitated with five volumes of 0.1 M ammonium acetate in methanol (overnight at −20°C). After centrifugation, the protein pellet was washed once with 0.1 M ammonium acetate in methanol and then with 80% (v/v). After centrifugation (15,000 g for 5 min at 4°C), the pellet was air dried, resuspended in SDS Lysis Buffer (100 mM Tris-HCl pH 7.5, 4% (w/v) SDS, 100 mM dithiothreitol) and quantified with 2D Quant Kit (GE Healthcare).

Proteins were digested with trypsin by filter aided sample preparation (FASP) as described elsewhere [32]. For each sample, six aliquots of proteins (200 μg each) were digested and at the end all peptide fractions obtained were collected. Peptide concentrations were estimated spectrophotometrically assuming that a solution of proteins with a concentration of 1 mg/mL results in an absorbance of 1.1 at a wavelength of 280 nm. Peptides were then desalted using SPE (Phenomenex Strata C18-E). All the procedures were carried out under positive pressure using a vacuum pump (1 drop/s). The column was conditioned with 3 mL 0.1% TFA in methanol and equilibrated with 2 mL Equilibration Buffer (0.1% TFA in H_2O). The sample loading was maximized and flowing 1 mL of Equilibration Buffer. For desalting, 1 mL

of Equilibration Buffer to avoid premature detaching of phosphopeptides. The final elution was performed by loading 1 mL of Elution Buffer (0.1% TFA in 70:30 ACN:H_2O). Phosphopeptides were enriched by the MagReSyn Ti-IMAC microsphere (ReSyn, Biosciences) according to manufacturer's guidelines. The eluted phosphopeptides were analyzed by LC-MS/MS as described elsewhere [6].

2.3 | Data processing

Raw data were searched against the *N. tabacum* Uniprot protein database (version 2019-01, 76,141 entries) with MaxQuant program (v.1.5.3.3) using default parameters including Phospho (STY) in variable modifications. For the quantitative analysis the and "Phospho(STY)sites" output files were processed as detailed previously and modified as follows. Briefly, the inconsistent identifications were filtered out and only phosphosites (PPs) detected in at least three of the four biological replicates (75%) per analytical group (WT and cAS) were considered. Missing values were replaced with the R package imputeLCMD using the hybrid imputation method: imputation of left-censored missing data (missing values ≥ 50% of number of replicas) was done using quantile regression imputation of left-censored (QRILC) method, instead missed at random data (<50% of replicas) were imputed using the k-Nearest Neighbors (KNN) algorithm. Finally, only class I ($p > 0.75$) PPs were included in the following analysis.

$\log_2$ transformed PPs intensities were centered by the Z-score normalization method of Perseus (Version 1.6.10.45, downloaded December 18, 2019; https://www.maxquant.org/perseus/) and subjected to one-way ANOVA testing (FDR < 0.05) in order to discover differentially abundant phosphosites (DAPPs) in cAS versus WT comparisons.

The mass spectrometry proteomics data were deposited in the ProteomeXchange Consortium via the PRIDE [33] partner repository with the dataset identifier PXD040912.

2.4 | Downstream bioinformatic analyses

In order to assess the quality of datasets, $\log_2$ transformed and centered PPs intensities were used for principal component analysis (PCA) by Perseus software version 1.6.10.45 (downloaded December 18, 2019; https://www.maxquant.org/perseus/).

A local BLAST of *N. tabacum* proteins against the *A. thaliana* database (TAIR10, version 2012-05-07) was performed to use bioinformatic tools available for *A. thaliana*. Blast hits with identity <50% and e-value $>10^{-3}$ were filtered out.

The enrichment analysis was performed using the Gene Ontology (GO) enrichment in Panther (December 7, 2022; http://www.pantherdb.org/; [34]) with *N. tabacum* as background. Functionally redundant terms were removed by using REVIGO [35]. The proteins mapping was performed using the Kyoto Encyclopedia of Genes and Genomes (KEGG; https://www.genome.jp/kegg/mapper/search.html) [36].

The analysis of significantly enriched phosphorylation motifs was performed by MOMO tool of MEME suite 5.1.1 (http://meme-suite.org/tools/momo) by using the Motif-X algorithm [37]. The peptide sequences (limited to 13 amino acids) were centered on aligned modification sites (phosphoserine or phosphothreonine). The number of occurrences was set to 20, and the probability threshold was set to $p < 10^{-6}$. The dataset of unchanged peptides was uploaded as background data.

Kinase-target interactions were searched in the Arabidopsis Protein Phosphorylation Site Database PhosPhAt 4.0 (https://phosphat.uni-hohenheim.de) [38]. Known motifs and probable kinases were searched in the PhosphoMotifFinder (http://www.hprd.org/serine_motifs) database and in the literature [39–42].

The scanning for occurrences of cAMP motifs was done by using Find Individual Motif Occurrences (FIMO; MEME suite 4.11.4) with match *p*-value lower than $1E10^{-4}$ [43]. The cyclic nucleotide-binding domain signatures (PS00888; PS00889) obtained from the Prosite database of protein domains (https://prosite.expasy.org/PDOC0069) were used.

The generation of sequence logos was done using the web-based application WebLogo (https://weblogo.berkeley.edu/).

2.5 | Phosphosite conservation analysis

Phosphosite conservation analysis was conducted as detailed previously [44]. Essentially, a multiple sequence alignment (MSA) with each phosphoprotein and its paralogues was performed. For each protein, the best BLAST hit and its paralogues in reference organisms (*A. thaliana*, *B. rapa*, *E. grandis*, *G. max*, *P. trichocarpa*, *V. vinifera*, *S. lycopersicum*, *O. sativa* ssp. Japonica, *A. trichopoda*, *P. patens*, and *C. reinhardtii*) were selected by using PLAZA 5.0 dicots [45]. Conservation of the residues, as well as the window sequences around the residues (−6 or +6), was determined by remapping all residue positions within the *N. tabacum* protein. The percentage of conserved PPs was calculated for every species where the phosphorylated residue was present. The BLOSUM score was used to score the conservation of the sequence within the window around the residue.

2.6 | PPI network construction and essential protein/hub analysis

The search tool for retrieval of interacting genes (STRING) database (https://string-db.org) was used to point to potential interactions between all phosphoregulated proteins in cAS versus WT comparisons [46]. Parameters were set as follows: co-expression as active interaction sources and medium confidence (>0.4). Disconnected nodes were hidden in the network. In order to visualize the protein–protein interaction (PPI) network the Cytoscape software version 3.6.1 was used [47]. The maximal clique centrality (MCC) algorithm of the CytoHubba plugin [48] was used to detect the top hub genes in co-expression

networks. Proteins with the top 10 MCC values were considered hub genes/proteins.

3 | RESULTS AND DISCUSSION

3.1 | Differentially abundant phosphosites (DAPPs) in response to cAMP depletion

In order to assess the dependence of cAMP on the phosphoproteome, we compared WT and cAS TBY cell lines grown for 3 days at 27°C. Plant cell suspensions were chosen since they are a cytologically uniform and reproducible system that is suitable for an efficient induction of changes in the physical environment [49]. In the mutant line, the cAMP content was reduced by approximately 50% due to the sequestration by the sponge which is based on the a PKA I regulatory subunit that specifically binds free cAMP [6]. After 5 days of culture, cAS lines compared to WT showed an inhibition of cell growth equating to an about 35%–40% reduction in fresh weight [6].

By using a Ti-IMAC microsphere-based enrichment approach, we identified 2478 PPs on 2162 unique peptides (mapping to 1551 proteins) (Table S1). The PCA showed that biological replicates plotted very closely in the PCA space, indicating a good correlation between them. It was furthermore noted that the two conditions tested resulted in distinct phosphoproteomic signatures dependent on the cellular levels of cAMP (Figure S1).

The one-way ANOVA comparison test (FDR < 0.05) allowed the identification of 123 DAPPs between two conditions considered (Table S2). Of those PPs sites, 80 showed increases while 43 decreased in their phosphorylation state. Over 80% of the total DAPPs were serine residues, approximately 15% were threonine residues, and less than 1% were tyrosine residues. Incidentally, this distribution is similar to the one previously reported in a large-scale in vivo phosphorylation site map of *Arabidopsis* cell suspensions [50]. The sequence windows of each of the detected PPs are shown in Table S2 (column AH) and the detected DAPPs were mapped to 115 phosphoproteins of which 105 (91.3%) showed phosphorylation change in one residue and 10 (8.7%) showed two changes.

3.2 | cAMP-dependent phosphorylation is conserved among plant species

Cyclic AMP-sensitive PPs were compared between different species of higher and lower plants to determine the extent of conservation. Overall, 20 (17%) phosphoproteins had phosphorylated orthologs in all eleven selected species. MSAs were performed with each phosphoprotein and its orthologues, and the conservation of the residues, as well as the flanking sequences around the residues (window sequences) were determined (Table S3). Perhaps not surprisingly, we noted the highest degree of conservation of PPs in the closely related *Solanum lycopersicum* (74% of total DAPPs). In the other dicots we found an average of 50 % of the DAPPs conserved and 42% in the early-diverged flowering plant *Amborella trichopoda*. In the monocot *Oryza sativa* the conservation was 37%, and in the moss *Physcomitrella patens* it was 31%. In the single-cell green alga *Chlamydomonas reinhardti* the conservation was markedly lower again (15%; Table S3).

Similarly, the conservation of the of amino acids in the flanking sequences of the phosphorylated residues was also decreasing with increasing distance between the species and approximately 30 % of all flanking sequences showed higher conservation as compared to the whole protein (ratio > 1; Table S3 – column I), indicating that the areas flanking these PPs are likely functionally important. Overall, the presence of evolutionarily-conserved PPs is consistent with an evolutionary constraint on the cAMP-regulated PPs, and much like in animal species, this may be indicative for conserved functional roles.

3.3 | In search of evidence for cAMP-dependent protein kinases

To date, the existence of cAMP-dependent kinases has remained elusive [25, 26]. However, even if no bona fide cAMP-dependent protein kinases have been discovered in plants, a possible role for protein kinase cascades in cAMP-dependent signaling has been proposed [25] and this study lends further support to this notion.

In our dataset, we identified four DAPPs in four annotated kinases (A0A1S3YCC6, A0A1S3XTN9, A0A1S4AUV8, A0A1S4C3G8; Table S2). No specific cyclic nucleotide-binding domain signatures were identified in these kinases that showed altered phosphorylation in cAS lines. This would exclude a direct interaction with a currently annotated cAMP-binding site. It is noteworthy that a serine/threonine-protein kinase, in which we observed a decreased phosphorylation level at S498 (A0A1S3XTN9) has an orthologue in the human proteome (Q96GX5; 59.5% of identity; E-value 2.2 e-47). Interestingly, this orthologue was been reported to be regulated by cAMP-dependent PDEs in human T cells [51] and this is further, albeit indirect evidence for cAMP-dependent plant kinases.

In order to discover further candidate phosphorylation targets of kinases, we searched for over-represented sequence motifs in the differentially phosphorylated phosphopeptides. The over-represented motifs, both in more and less phosphorylated peptides, were similar to a phosphorylation at Ser followed by Pro (…SP…; Figure 1). Incidentally, this site is near identical to previously reported phosphorylation consensus motifs of MAP kinases (MAPKs), receptor-like kinases (RLKs), AFC2 kinases, AGC kinases (AGCKs), cyclin-dependent kinases (CDKs), SnRKs, and calcium-dependent kinases (CDPKs) [39, 52]. Moreover, a PKA kinase motif (…R…S/T…) occurred in seven dephosphorylated and three de novo phosphorylated proteins (Table S4). Among the latter, we found a peptide that mapped on the KH domain-containing protein isoform X1 (A0A1S4D8B9). The human ortholog of this protein is a pre-mRNA-binding protein with a role in mRNA splicing (HNRPK; P61978) and alteration in its phosphorylation status was reported in human T cells [51].

Phosphoregulated proteins in our dataset were further characterized by assessing which regulatory kinases would most likely

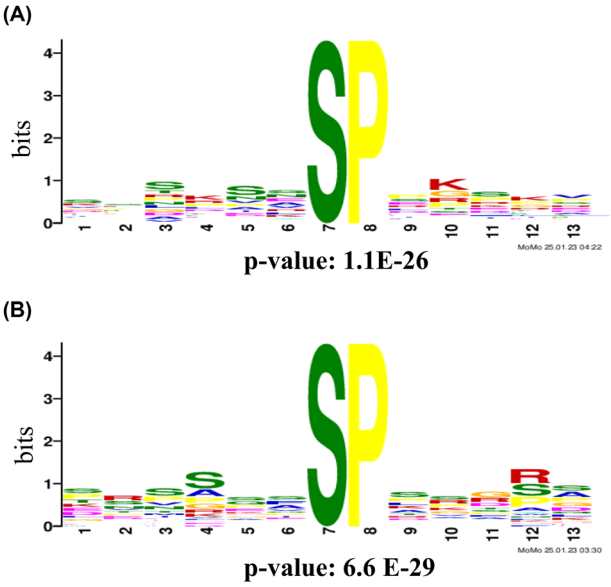

FIGURE 1 Significantly over-represented motifs obtained with the MOMO tool of MEME suite 5.1.1 (http://meme-suite.org/tools/momo) in more (A) and less (B) phosphorylated peptides.

phosphorylate in a cAMP-dependent manner. The PhosPhAt tool analysis indicated that AGC, CDK, MAPK, and SnRK1 were among the most prevalent kinase families (Table 1A). The serine/threonine kinase (AT2G34650; PID2), a member of the AGC family involved in ABA signaling and auxin transport [53], and the SNF1-related protein (AT3G01090), a SnRK1 family kinase with a role in the sugar signaling [54], are enzymes annotated as capable of phosphorylating multiple residues in the proteins of our dataset (Table 1A). In addition, 10 putative targets of kinases were also found in our dataset (Table 1B). Among them are the auxin efflux carrier family protein (PIN2; AT5G57090, A0A0D4D8G6) and the trehalose phosphatase/synthase 5 (TPS5; AT4G17770, A0A1S4D237), both of which harbor multiple cAMP-dependent PPs. The SNF1-related protein was reported to alter phosphorylation of the TPS5 protein on a residue (S22; [55]) in *A. thaliana* and this site is close to the phosphorylation site altered by cAMP buffering in this study (S17).

3.4 | Candidate cAMP binding-proteins

To date, the only bona fide plant cAMP protein interactors are the cyclic nucleotide-gated channels [56] and 15 additional cAMP interactors in *A. thaliana* [57] that were identified by affinity purification techniques. However, the functional validation of most of the 15 additional cAMP interactors in *A. thaliana* is still outstanding.

To gain further insight into cAMP-protein interactions, all phosphoregulated proteins were assessed for the presence of two cyclic nucleotide-binding domain signatures (https://prosite.expasy.org/PDOC00691). The domain signature 1 (PS00888) was detected in 4 phosphoregulated proteins in our dataset, while domain signature 2 (PS00889) was found in 11 proteins (Table S5). Within the serine/threonine-protein kinase HT1-like protein (A0A1S3YCC6) and the DUF21 domain-containing protein (A0A1S3ZH01) both domain signatures have been found. The motif scan tool predicts two cAMP-binding domains inside the CNNM4 metal transporter (Q6P4Q7) and the human orthologue of the DUF21 domain-containing protein supports this prediction. The CNNM proteins contain domains structurally similar to cyclic nucleotide-binding domains of cyclic nucleotide-gated channels [58], still, the binding to cAMP has yet to be experimentally proven [59].

A cyclic nucleotide-binding domain was also found in the nuclear-pore anchor-like protein (A0A1S3 × 5S0) and its human ortholog, the nucleoprotein TPR (P12270) showed a change in the phosphorylation status in T cells treated with selective inhibitors of cAMP-dependent PDEs [51]. Since PDEs degrade cAMP thereby regulating cellular cAMP levels [60], it is conceivable that the observed TPR phosphorylation (at T2116) is consistent with a reduced phosphorylation of the protein A0A1S3 × 5S0 in the cAS mutant. The high conservation of this protein (75%) among the plant species analyzed points to a conserved role for cAMP in the phosphorylation of components of the nuclear pore complex (NPC). Furthermore, this phosphoproteomic analysis also revealed cAMP-dependent phosphorylation of the nucleoporin interacting component (Nup93/Nic96-like) family protein (A0A1S4DQP1) which is part of the NPC (Table S2). NPC components control the mRNA/protein nucleo-cytoplasmic trafficking [61]. Successful cell cycle completion requires the breakdown and reassembly of the nuclear envelope at the end of mitosis [62]. Many NPC proteins are phosphorylated during these processes [63, 64]. Therefore, the alteration of the phosphorylation status reported here could explain the lower mitotic index previously recorded in cAS lines compared to WT lines under control condition [6].

Moreover, among kinases, CDKs have essential roles in exerting control of cell cycle progression and their activation requires the interaction with specific cyclin partners [65]. The cAMP-dependent phosphorylation of two cyclins (A0A1S3WZH8, A0A1S4A5E4) is further evidence for a role of CDK, as well as the critical role of cAMP in cell cycle regulation.

3.5 | The roles of cAMP- dependent changes in phosphoproteomic signatures

To obtain functional insights into the biological processes affected by cellular cAMP buffering, a GO enrichment analysis was conducted. Several processes associated with RNA processing and splicing are enriched in our dataset (Table 2). Overall, we identified 18 DAPPs assigned to proteins involved in RNA processing and splicing (Table 3). These proteins include several serine and arginine-rich (SR) and spliceosome-associated proteins (SAPs), the Upstream Binding Protein 1 (UBP1)-associated protein (A0A1S4B2A6), and a component of the U2-type spliceosomal complex (A0A1S4BHM7). A schematic

TABLE 1 List of most relevant predicted kinases (A) and targets of kinases (B) retrieved from the Arabidopsis Protein Phosphorylation Site Database (PhosPhAt 4.0).

A

Predicted kinase ID	Description	Kinase family	# of predicted kinase target sites
AT2G34650	Encodes a protein serine/threonine kinase	AGC	7
AT3G01090	Encodes a SNF1-related protein kinase	SnRK1	4
AT1G53700	Protein-serine/threonine kinase	AGC	3
AT3G14370	Protein-serine/threonine kinase	AGC	3
AT2G43790	MAP kinase MPK6	MAPK	2
AT3G29160	SNF1-related protein kinase	SnRK1	2
AT3G50530	CDPK-related kinase	CPKRK	2
AT4G01370	cytoplasmically localized MAP kinase	MAPK	1
AT4G23130	Receptor-like protein kinase	other	1
AT4G28980	CDK-activating kinase	CDK	1
AT5G10270	CDKC kinase	CDK	1
AT5G55910	D6PK protein kinase	AGC	1
AT1G76040	Calcium Dependent Protein Kinase	CDPK	1
AT1G30270	CBL-interacting protein kinase 23 (CIPK23)	SnRK3	1

B

Target protein ID	Description	# of predicted kinase target sites
AT5G57090	Auxin efflux carrier	17
AT4G17770	Trehalose synthase (TPS)-like domain protein	6
AT3G44200	Member of the NIMA-related serine/threonine kinases (AtNek5)	2
AT1G64780	Ammonium transporter protein	1
AT2G24590	Serine/Arginine-Rich Protein Splicing Factors (SR proteins)	1
AT2G27100	Single zinc finger containing protein	1
AT3G16270	Adaptor protein (AP)	1
AT3G43300	Immunity associated protein AtMIN7	1
AT4G19600	Cyclin T partner CYCT1	1
AT5G09400	Potassium uptake permease	1

TABLE 2 GO statistically overrepresented categories in cAS versus WT comparison calculated using Panther (http://www.pantherdb.org/) and the *Nicotiana tabacum* as background. Functionally redundant terms were removed with REVIGO.

GO biological process	# *N. tabacum*	# Dataset	# Expected	Fold enrichment	Raw *p*-value	FDR
mRNA splicing, via spliceosome	399	8	0.60	13.36	1.99E-07	7.99E-04
mRNA processing	605	9	0.91	9.91	3.92E-07	3.93E-04
RNA processing	1506	11	2.26	4.87	1.74E-05	9.98E-03
gene expression	3616	16	5.43	2.95	9.33E-05	4.68E-02
mRNA metabolic process	835	10	1.25	7.98	6.03E-07	4.84E-04

representation of the spliceosome activation process including the phosphoregulated proteins, shows the extent of cAMP-dependence (Figure 2A).

The RNA splicing is a pivotal step in gene expression which in turn modulates the messenger RNA population essential for cellular adaptation to continuously changing conditions [66]. Many proteins have been reported to undergo phosphorylation and dephosphorylation during splicing events [67], and RNA splicing is affected by alterations in the phosphorylation status of components of the spliceosome, the molecular components that catalyzes the removal of introns from

TABLE 3 List of phosphoregulated proteins involved in RNA processing and splicing with their respective phosphosites, their conservation (%) and log fold change values.

Phophosite	Nicotiana ID	Annotation	% Conservation	logFC cAS vs. WT
S203	A0A1S4AAX8	Serine/arginine-rich splicing factor RSZ22A-like	83.33	2.19
S189	A0A1S4AAX8	Serine/arginine-rich splicing factor RSZ22A-like	83.33	1.19
S387	A0A1S4B2A6	RNA-binding (RRM/RBD/RNP motifs) family protein	40.00	1.06
S218	A0A1S4DMI2	Serine/arginine-rich splicing factor SC35-like	81.82	0.86
T212	A0A1S4DMI2	Serine/arginine-rich splicing factor SC35-like	45.45	0.77
S674	A0A1S3YBI6	KH domain-containing protein	66.67	0.67
S400	A0A1S3YT22	Serine/arginine-rich splicing factor RS31-like	50.00	−0.96
S234	A0A1S4DDL7	Serine/arginine-rich-splicing factor SR34 isoform X2	58.33	−1.10
S59	A0A1S4BHM7	Splicing factor 3B subunit 2-like	25.00	−1.29
S289	A0A1S4A621	Serine/arginine-rich splicing factor RS31-like isoform X2	58.33	−1.30
S164	A0A1S4CB61	Serine/arginine-rich SC35-like splicing factor SCL30A	70.59	−1.32
S168	A0A1S3ZJ61	Serine/arginine-rich splicing factor SC35-like	100.00	−1.42
S26	A0A1S3Y1I5	Protein BTR1-like	100.00	−1.45
T200	A0A1S4CVZ2	Hyaluronan / mRNA binding family	27.27	−1.67
S30	A0A1S3YP25	Protein BTR1-like isoform X3	23.81	−1.72
S124	A0A1S4CVZ2	Hyaluronan / mRNA binding family	36.36	−1.84
S263	A0A1S4A621	Serine/arginine-rich splicing factor RS31-like isoform X2	66.67	−1.85
S227	A0A1S4C8J5	Serine/arginine-rich splicing factor SR34A-like	82.61	−1.92

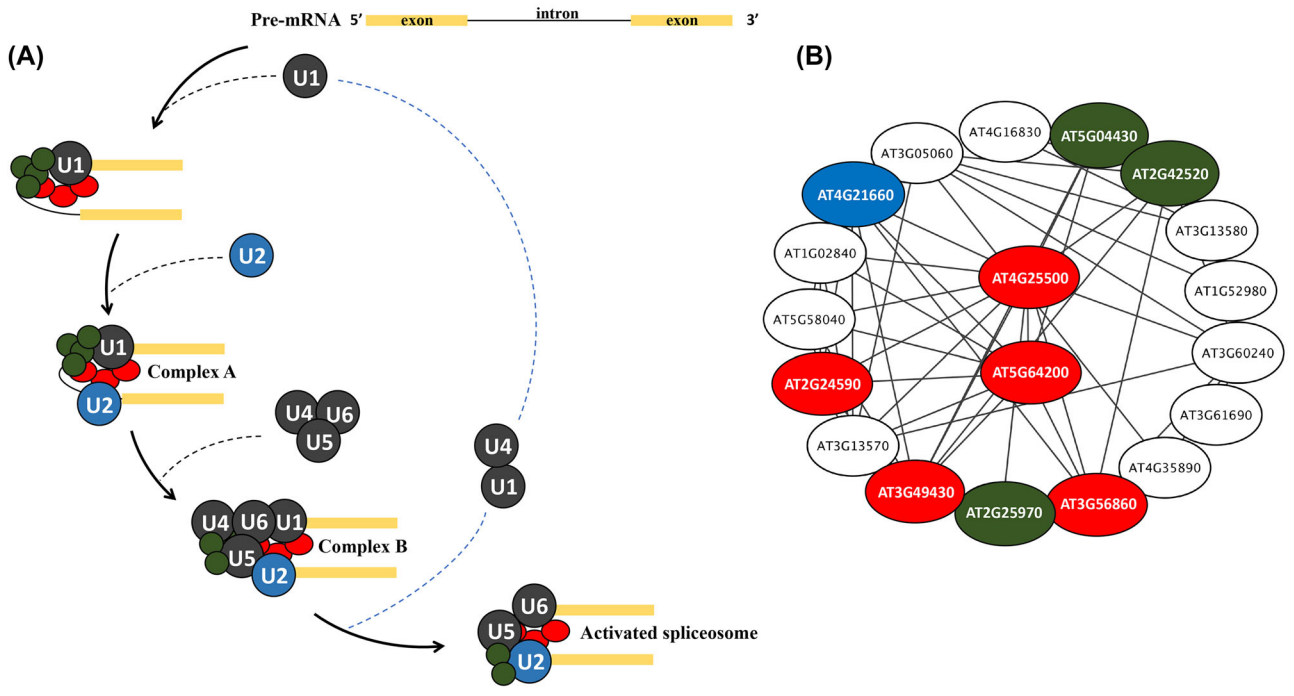

FIGURE 2 (A) Schematic representation of the spliceosome activation process including the phosphoregulated proteins. (B) Protein–protein interaction networks of phosphoregulated RNA-binding proteins constructed using the search tool for retrieval of interacting genes (STRING) database and visualized in Cytoscape. Common spliceosome elements are showed in red (A0A1S4B2A6, AT3G56860; A0A1S3YT22, AT4G25500; A0A1S3ZJ61, AT5G64200; A0A1S4AAX8, AT2G24590; A0A1S4C8J5, AT3G49430; A0A1S4A621, AT4G25500; A0A1S4DMI2, AT5G64200), while spliceosome-associated proteins (A0A1S4CVZ2, AT4G16830; A0A1S3Y1I5, AT5G04430; A0A1S3YBI6, AT2G25970; A0A1S3YP25, AT5G04430) and the U2 component (A0A1S4BHM7, AT4G21660) in olive green and blue, respectively.

nuclear pre-mRNA, as well as proteins with regulatory functions this process [68]. In mammalian cells, several stimuli that increase the intracellular cAMP level affect alternative splicing through phosphorylation of both SR proteins and heterogeneous nuclear ribonucleoproteins (hnRNPs) by PKA [24].

In this study, the phosphorylation status of several RNA-binding proteins (RBPs) is shown to be responsive to cAMP buffering (Table S6; Table 3). Plant RBPs have a role in post-transcriptional processes essential for plant adaptation to environmental stimuli [69]. The activity and fate of RNA transcripts are critically dependent on RBPs [70] which enable and modulate RNA processing in the nucleus, export out of the nucleus, and metabolism in the cytoplasm.

The topological network of the RBP PPI (Figure 2B) points to two serine/arginine-rich splicing factors, the RS40 (AT4G25500, A0A1S4A621) and the SC35-like (AT5G64200, A0A1S4DMI2) as top hub genes/proteins (Table S7). In the RS40 protein, the phosphorylated residue (S263) shows a high degree of conservation (66.67%) in the species investigated, and this also holds true for the S218 in the SC35-like protein (81.82%) (Table S3), again pointing to a conserved functional role of splice factor phosphorylation.

Among the RBPs, SR proteins are splicing factors rich in arginine-serine dipeptide repeats (RS domains) involved in spliceosome formation and splice site recognition [71, 72]. Changes in the phosphorylation status of RS domains can interfere with the ability of SR proteins to interact with RNA and other splicing-related proteins [73]. Despite this central function, mechanism by which SR protein phosphorylation is occurring and regulated in plant cell is not yet well understood. In humans, several kinase families involved in the splicing-related phosphorylation have been reported and the growing list includes the SRPKs (SR-specific protein kinases) and CLKs (Cdc2-like kinases) which are the best characterized [74]. SRPKs phosphorylate the RS domains in SR proteins and in turn, RS domains contain prolines with flanking serines that are phosphorylated by CLKs. The proline-directed phosphorylation of these sites affects SR protein conformation and splicing [75]. CLKs are conserved kinases with members such as the human CLK1-4, the Saccharomyces cerevisiae KNS1, the Drosophila melanogaster DOA and *A. thaliana* AFC1-3 [76, 77]. It is noteworthy that CLK homologs (AFC2s) were reported to participate in alternative splicing regulation under heat stress conditions [78].

We also investigated the amino acid sequence patterns flanking the phosphorylated residues in RNA splicing-associated proteins and found that the Ser-Pro motif was the most prevalent (Figure S2) and again, this is consistent with a role of cAMP-dependent phosphorylation in the functionalization of AFC2 in the SR protein.

In support of a role of cAMP in the RNA splicing regulation is the finding that PPs appear conserved among 11 different plant species investigated (Table 3, Table S3, Figure S3). Overall, 11 out of 18 sites showed conservation of the central S/T of over 50%. The presence of several evolutionarily-conserved targets is also an indicator for the reliable quality of the data as well as supporting the functional assignment of the site [79, 80].

3.6 | Specificity of the cyclic nucleotide monophosphate (cNMP) response

In addition to cAMP, the plant cyclic nucleotide signaling system also includes 3′,5′-cyclic guanosine monophosphate (cGMP). Both cAMP and cGMP elicit different plant physiological processes, ranging from cell cycle progression to perception of external abiotic and biotic stimuli [8, 16]. Both cNMP signals act through cellular effectors such as CNGCs and my affect Ca^{2+} [81], Na^{+} [82], and K^{+} fluxes [83, 84].

At the systems level, the case is less clear. Cyclic GMP-dependent protein phosphorylation has been demonstrated in *A. thaliana* cell suspension culture cells [42]. Exogenous administration of a membrane permeable cGMP analogue causes specific cGMP-dependent phosphorylation of spliceosome components and of proteins involved in cell-size regulation. Although different experimental approaches were adopted in the two studies, a comparison between them can provide some information on systemic roles of cNMPs and specificity of cAMP and cGMP. The comparative analysis reveals that, despite the limited number of common regulated phosphoproteins (9 proteins; Figure S4), RNA processing was the most enriched process in both studies. This finding therefore indicates that cAMP and cGMP signals may operate through phosphorylation of spliceosome components and further experiments will elucidate the nature of their complementary roles in the dynamic reorganizations of the spliceosome assembly system.

Among the common targets are two RNA-binding family proteins (AT3G56860, A0A1S4D8B9; AT5G15270, A0A1S4B2A6) that showed an increased phosphorylation both when the cAMP and cGMP levels decrease and increase, respectively. Therefore, we speculate that the two messengers may intervene in the splicing process regulating these proteins in an antagonistic way.

Interestingly, a phosphoprotein phosphatase inhibitor (A0A1S3ZHZ3; AT5G52200.1) showed cNMP-dependent phosphorylation. This protein, also known as protein GLC8-like, prevents or reduces the activity of protein phosphatases. The Arabidopsis homologue AtI-2 inhibits all plant protein phosphatase 1 (PP1) isoforms [85].

4 | CONCLUDING REMARKS

Here we present a large-scale phosphoproteomic study to assess the role of 3′,5′-cAMP dampening in tobacco BY2 cells. Overall, the phosphoproteome is severely affected following cAMP buffering as a total of 2478 PPs mapping on 2162 PPs of 1551 proteins show alterations in their phosphorylation status. Our findings reveal that the cAMP-dependent changes in phosphoregulations depend on MAP kinases, RLKs, and CDPKs which makes these proteins plausible cAMP-dependent kinase candidates. Furthermore, the data are consistent with a systemic role of cAMP since it affects RNA-binding proteins involved in RNA splicing, including several RS proteins, which are differentially phosphorylated in response to cAMP depletion. The over-representation of a Ser-Pro motif among target

phosphorylation sites in SR proteins may implicate the presence and operation of cAMP-dependent AFC2 kinases.

Taken together, our results provide a repertoire of cAMP-responsive PPs in the proteome that will allow to infer the role of cAMP-dependent phosphorylation in plant responses at the molecular and systems level. The dataset will also serve as a useful baseline for the study of developmental and stimulus specific cAMP-dependent changes in protein phosphorylation plants.

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The authors have nothing to report.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The mass spectrometry proteomics data were deposited in the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD040912.

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SUPPORTING INFORMATION

Additional supporting information may be found online https://doi.org/10.1002/pmic.202300165 in the Supporting Information section at the end of the article.

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