Phosphoproteomic studies in *Arabidopsis* and tobacco male gametophytes

Jan Fíla*†, Věra Čapková* and David Honys*[1]

*Laboratory of Pollen Biology, Institute of Experimental Botany, Academy of Sciences of the Czech Republic, Rozvojová 263, Praha 6, Czech Republic
†Department of Plant Experimental Biology, Faculty of Science, Charles University in Prague, Viničná 5, Praha 2, Czech Republic

Abstract

Mature pollen represents an extremely resistant quiescent structure surrounded by a tough cell wall. After its hydration on stigma papillary cells, pollen tube growth starts rapidly. Massive metabolic changes are likely to be accompanied by changes in protein phosphorylation. Protein phosphorylation belongs among the most rapid post-translational modifications. To date, only *Arabidopsis thaliana* and tobacco (*Nicotiana tabacum*) mature pollen have been subjected to phosphoproteomic studies in order to identify the phosphoproteins present. In the present mini-review, *Arabidopsis* and tobacco datasets were compared with each other. The representation of the O-phosphorylated amino acids was compared between these two datasets, and the putative pollen-specific or pollen-abundant phosphopeptides were highlighted. Finally, the phosphorylation sites common for both *Arabidopsis* and tobacco phosphoproteins are listed as well as the phosphorylation motifs identified.

Introduction

Angiosperm mature pollen represents an extremely resistant tissue. Its desiccated cytoplasm is enveloped by a resistant cell wall. Mature pollen represents a quiescent stage that carries the genetic information of the donor plant, so it has to reach the stigma in a viable state. After the arrival on to the papillary cells on the stigma, the pollen cytoplasm rehydrates and pollen grain activates [1]. After a series of communication processes, rapid growth of a pollen tube starts.

To enable metabolic changes associated with the rapid growth of a pollen tube, the synthesis of novel proteins as well as post-translational modifications of the existing ones is of crucial importance. A number of transcripts is stored in complex mRNA storage granules called EPPs (EDTA/puromycin-resistant particles) [2,3]. Upon pollen rehydration, these transcripts are gradually activated and translated, and novel proteins are produced. After all, protein synthesis could not be flexible enough to enable rapid signalling, so post-translational modifications of proteins are performed, especially phosphorylation.

Protein phosphorylation represents one of the most dynamic post-translational modifications. It plays a key role in numerous cellular processes, such as protein synthesis, transcription regulation, cell cycle regulation, signal transduction, cytoskeleton dynamics and protein targeting to the nucleus [4–7]. Protein phosphorylation changes the properties of a modified protein. Because of its negative charge, the attachment of a phosphate group leads to

a decrease in the protein's pI [8]. Such pI changes are likely to change intramolecular and intermolecular interactions among individual domains. Alternatively, the attached phosphate can engage the active site of an enzyme and thus can block its activity (an example of this mechanism was discovered in isocitrate dehydrogenase [9]).

The male gametophyte and phosphoproteomics

Although pollen development, pollen activation and pollen tube growth are likely to be (co-)regulated by protein phosphorylation, only two large-scale pollen phosphoproteomic studies have been published to date. The first phosphoproteome published originated from *Arabidopsis thaliana* mature pollen [10], whereas the second study characterized phosphoproteins present in two time points in tobacco (*Nicotiana tabacum* cv. Samsun), mature pollen and germinating pollen after 30 min of activation *in vitro* [11].

To study the phosphorylated proteins on a large scale, the application of various enrichment techniques is of key importance since phosphoproteins represent only part of the cellular proteins and therefore are difficult to be detected in the mixture with the non-phosphorylated species (reviewed in [12,13]).

Each of the pollen phosphoproteomic studies applied a different set of methods for the identification of phosphorylated proteins present. In tobacco experiments, phosphoprotein enrichment was performed. The total proteins were extracted by TCA (trichloroacetic acid)/acetone, and enriched by MOAC (metal-oxide-affinity chromatography) with an $Al(OH)_3$ (aluminium hydroxide) matrix. The resulting phosphoprotein-enriched fraction was separated in two

distinct ways: by 2D gel electrophoresis and nano-LC–ESI–quadrupole TOF. Collectively, these two approaches led to the identification of 139 phosphoprotein candidates. The phosphoprotein enrichment led to the identification of only a limited number of phosphorylation sites; particularly one. Similar results were obtained in other studies applying phosphoprotein enrichment [14,15] and such results could be considered a disadvantage of phosphoprotein-enriching strategies (recently reviewed in [12]). To enable the identification of more phosphorylation sites, TiO_2 (titanium dioxide) phosphopeptide enrichment was performed on mature pollen protein crude extract in parallel. Such enrichment revealed the exact position of another 51 phosphosites, giving a total of 52 phosphorylation sites identified.

On the other hand, Mayank et al. [10] applied a combination of various phosphopeptide-enriching strategies: IMAC (immobilized metal-ion-affinity chromatography) [16], TiO_2-MOAC [17] and SIMAC (sequential elution from IMAC) [18]. Since direct phosphopeptide enrichment usually leads to a higher number of identified phosphopeptides with their exact phosphorylation sites, it is not surprising that 962 phosphopeptides corresponding to 598 phosphoproteins were identified.

Although the phosphopeptide enrichment usually results in a higher number of identified peptides, it was disadvantageous in the particular case of tobacco pollen since tobacco genomic sequences were not fully available in the public databases. The non-phosphorylated peptides identified upon phosphoprotein enrichment assisted in the proper identification of the phosphorylated proteins that very often relied on non-tobacco homologous sequences.

The conventional enrichment techniques led to the identification of phosphorylated serine, threonine and tyrosine residues. The proportion of all three phosphorylated amino acids was quite similar in both published male gametophyte phosphoproteomes. In *Arabidopsis*, there was 86% serine, 14% threonine and 0.16% tyrosine residues (represented by one phosphorylation site only) [10] (Figure 1A). In the less complex tobacco phosphoproteome, 67% phosphoserine and 33% phosphothreonine residues were identified. No phosphotyrosine was presented in this dataset [11] (Figure 1B). Tyrosine phosphorylation seems to be less abundant in plants, giving a phosphoserine/phosphothreonine/phosphotyrosine ratio of 91.8:7.5:0.7 [19] or 83.81:16.18:0.01 [20], depending on the material studied. This is likely to be caused by the absence of tyrosine-specific kinases from plants, where tyrosine phosphorylation appears to be carried out by dual-specificity kinases instead [21].

Although in most studies, phosphotyrosine was less abundant in plants compared with in animals, in some cases its proportion was comparable; for instance 4.3% phosphotyrosine in the study by Sugiyama et al. [22]. Thus it remains speculative whether tyrosine phosphorylation is really less abundant in plants compared with animals [23].

Phosphorylated histidine remains undetectable when the conventional phosphoproteomic techniques are applied since it is very labile under acidic pH. Thus special

Figure 1 | The proportion of O-phosphorylated amino acids in the published pollen phosphoproteomics data

(**A**) *Arabidopsis thaliana.* (**B**) *Nicotiana tabacum.*
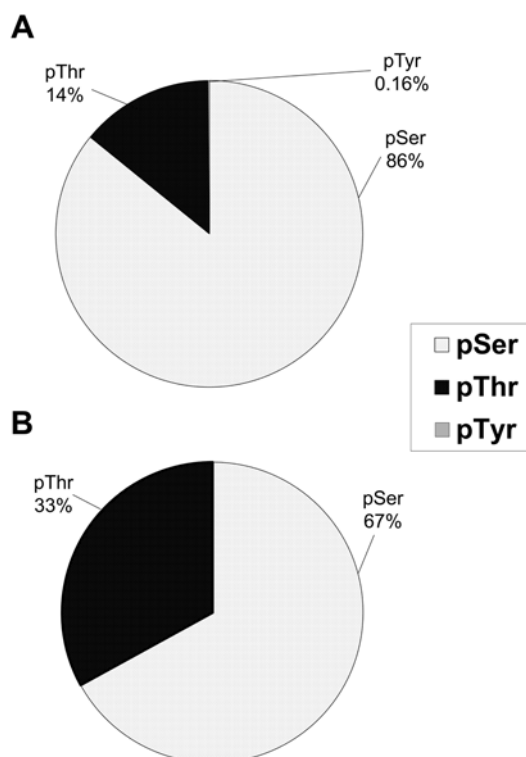
phosphoproteomic methods are required for studies of histidine phosphorylation [24].

Pollen-specific and unknown proteins identified

The phosphoproteomic data from both studies gave the number of phosphorylated proteins and, for some of them, the exact position of phosphorylation site was assigned. The most interesting candidates playing their roles in signalling were likely to be pollen-specific or with higher abundance in pollen. The intriguing unknown proteins can also play such a role.

The *Arabidopsis* phosphoproteome data were searched against the PhosPhAt database that summarizes phosphorylation sites identified in *Arabidopsis* [25,26]. The pollen phosphoproteome presented 240 novel phosphoproteins absent from the database at that time [10], because pollen had never been used as an experimental material for a phosphoproteomic study before. Thus the newly identified proteins were likely to be pollen-specific or at least to be phosphorylated in a pollen-specific manner. This hypothesis was supported at the transcriptomic level. The proteins already present in the database did not show any tissue-specific expression profile. On the contrary, most of the newly identified phosphoproteins showed high or enriched expression in the male gametophyte. Among these

pollen-specific or pollen-enriched proteins, there were several signalling proteins identified, such as protein kinases (e.g. AT1G16760, AT1G78940, AT2G24370 and AT4G31230) or protein phosphatases (e.g. AT1G17720, AT2G33700, AT3G15260 and AT5G10740). Moreover, there were also proteins identified that played a role during exocytosis, such as the members of the EXO70 family (AT5G13150 and AT5G13990). All of these proteins represent candidates for proteins with regulated expression in the male gametophyte. Furthermore, 18 proteins were classified as unknowns, for instance AT1G20770, AT1G30050, AT1G42480, AT2G32980, AT3G30320, AT4G31430, AT5G37550 and AT5G62750.

In contrast, similar information about pollen-specific and/or pollen-enriched proteins was less accessible for tobacco. The data published by Fíla et al. [11] presented the first tobacco phosphorylation sites to be deposited into the P3DB database [27,28], and they still represent the only tobacco data listed in this database. Consequently, on the basis of the P3DB data, it was not possible to distinguish whether there were pollen-specific phosphopeptides among the identified phosphoprotein candidates. Similar difficulties arose when searching for tobacco homologues since its genome sequence was not available in the public databases. It was necessary to identify the phosphopeptides according to homologous sequences from other species. Consequently, the identity of more protein isoforms remained often unclear since it could not be unambiguously stated whether distinctly identified isoforms reflected real isoforms also in tobacco. Moreover, many tobacco pollen-specific proteins may still be unknown and therefore not present in sequence databases. Eight phosphoprotein candidates played a signalling role: several isoforms of 14-3-3 proteins (which regulate many cellular processes [29]) and GDIs (guanine-nucleotide-dissociation inhibitors) of both Rab and Rho GTPases. Surprisingly, no phosphorylated kinases and/or phosphatases were detected. Furthermore, four uncharacterized proteins were identified among the tobacco phosphoprotein candidates.

Phosphorylation sites common to both tobacco and *Arabidopsis*

The intriguing question remained whether the two male gametophyte phosphoproteomic studies led to the identification of phosphorylation sites shared by both species. Again, it has to be kept in mind that both datasets were acquired by different sets of methods and that tobacco proteins were less successfully identified owing to the lack of tobacco sequences in public databases. Therefore the identification mostly relied on non-tobacco homologous sequences present in the database.

The phosphorylated peptides with a homologous phosphorylation site detected in both species are listed in Table 1. They were mostly associated with metabolism such as 2,3-bisphosphoglycerate-independent phosphoglycerate mutase, fructose-bisphosphate aldolase and plasma membrane AT-Pase. The other abundant category was translation regulation

represented by translation elongation factor EF1B/ripening-regulated protein and 60S acidic ribosomal protein. Last, but not least, homologous phosphorylation sites were identified in α-tubulin.

The phosphorylation sites shared in both published datasets did not belong to signalling proteins. This occurrence could be due to several reasons: (i) tobacco signalling proteins were not known and/or their degree of homology with known proteins was not high enough, (ii) many signalling proteins become phosphorylated only after pollen activation, but there was no phosphoproteomic study revealing exact phosphorylation sites in activated pollen, (iii) the homology of some tobacco and *Arabidopsis* signalling proteins could be quite low, so they do not share the conserved amino acids to be phosphorylated.

Phosphorylation motifs and kinases

The mature pollen phosphoproteomes were analysed for the phosphorylation motifs identified. The presence of particular motifs should highlight kinases responsible for the phosphorylation at given phosphosites.

The first set of motifs to be mentioned in both *Arabidopsis* and tobacco are MAPK (mitogen-activated protein kinase) or cyclin-dependent kinase motifs XXXS*PXXX and XXXT*PXXX. MAPKs were shown to play key regulatory roles during male gametophyte development [30]. These proline-directed phosphorylation sites were detected, for instance, in 60S ribosomal protein L12-2, eukaryotic initiation factor 4B, ripening-regulated protein, pollen tube RhoGDI2 in tobacco and, for example, RING/U-box superfamily protein, exocyst subunit EXO70 family protein H5 and ribosomal protein L19e in *Arabidopsis*.

The other kinase motifs common to both tobacco and *Arabidopsis* phosphoproteomes were basophilic motif RXXS*XX that is typical for non-plant CaMK2 (Ca^{2+}/calmodulin-dependent protein kinase 2) or 14-3-3 proteins. In *Arabidopsis*, this motif is recognized by other members of the CDPK (Ca^{2+}-dependent protein kinase)–SnRK (sucrose-non-fermenting-related kinase) superfamily since a typical CaMK is lacking [31]. Furthermore, in tobacco, a casein kinase motif S*XXD/E was detected.

Conclusion

The two phosphoproteomic studies presented provided an insight into the post-translational modifications in the male gametophyte. In tobacco, the phosphoproteomic study was limited by the fact that tobacco sequences were not fully available in public databases. So, the assembly of the sequenced contigs into annotated gene sequences will enable the identification of additional proteins from existing data. When the tobacco genome sequence becomes available in the databases, the identification of phosphopeptides will be easier and a combination of enrichment techniques could be applied in order to identify a broader spectrum of phosphorylated peptides [17].

Table 1 | List of homologous phosphopeptides identified in both *A. thaliana* and tobacco phosphoproteomes

After the protein name identified in tobacco, the species name of the sequence is given. The superscript after the accession number in tobacco indicates the database: a, TIGR EST *Nicotiana tabacum*; b, UniProt, 90% homology clusters, Viridiplantae. The asterisk (*) in the peptide sequences indicates the position of phosphorylation site. In cases where no amino acid with an asterisk is given, the exact position of the phosphorylation site could not be assigned.

Protein name: *Arabidopsis*	Protein name: tobacco	AGI: *Arabidopsis*	Accession number: tobacco	Peptide sequence: *Arabidopsis*	Peptide sequence: tobacco
H$^+$-ATPase 6	Plasma membrane ATPase 4 (*Nicotiana plumbaginifolia*)	AT2G07560.1	NT_TC82708_1[a]	GLDIDNLNQHY**T***V	GLDIETIQQHY**T***V
Phosphoglycerate mutase, 2,3-bisphosphoglycerate-independent	2,3-Bisphosphoglycerate-independent phosphoglycerate mutase (*Nicotiana tabacum*)	AT1G09780.1	NT_TC77653_1[a] NT_TC80529_1[a]	AHGTAVGLPSEDDMG-N**S***EVGHNALGA-GR	AHGNAVGLPTEDDM-GN**S***EVGHNALGA-GR
Translation elongation factor EF1B/ribosomal protein S6 family protein	Ripening-regulated protein DDTFR10-like (*Solanum tuberosum*)	AT2G18110.1	NT_TC77461_1[a]	ISGVSAEGSGVIVEGSS-PITEEAVATPPAAD-SK	ISGVSGEGAGVTVEGS-APITEEAVA**T***P-PAADTK
Glutathione transferase, C-terminal-like; translation elongation factor EF1B/ribosomal protein S6	Ripening-regulated protein DDTFR10-like (*Solanum tuberosum*)	AT1G30230.1	NT_TC77461_1[a]	ISGVSAEGSGVIVEGSA-PITEEAVATPPAA DSK	ISGVSGEGAGVTVEGS-APITEEAVA**T***PP-AADTK
Tubulin α3	α-Tubulin, fragment (*Gossypium hirsutum*)	AT5G19770.1	Q9SQ71[b]	TVQFVDWCPTGFK	TVQFVDWCP**T***GFK
Tubulin α4 chain	α-Tubulin (*Nicotiana tabacum*)	AT1G04820.1	NT_TC77583_1[a] NT_TC77710_1[a] NT_TC78231_1[a] NT_TC80038_1[a]	TIQFVDWCPTGFK	RTIQFVDWCP**T***GFK
Tubulin α1	α-Tubulin (*Nicotiana tabacum*)	AT1G64740.1	NT_TC77583_1[a] NT_TC77710_1[a] NT_TC78231_1[a] NT_TC80038_1[a]	TIQFVDWCP**T***GFK	RTIQFVDWCP**T***GFK
Tubulin/FtsZ family protein	α-Tubulin (*Nicotiana tabacum*)	AT4G14960.2	NT_TC77583_1[a] NT_TC77710_1[a] NT_TC78231_1[a] NT_TC80038_1[a]	TIQFVDWCP**T***GFK	RTIQFVDWCP**T***GFK
Aldolase superfamily protein	Fructose-bisphosphate aldolase (*Solanum tuberosum*)	AT3G52930.1	NT_TC78239_1[a] NT_TC118911_1[a]	LA**S***INVENVETNR	RF**S***SINVENVESNR
Aldolase superfamily protein	Fructose-bisphosphate aldolase (*Solanum tuberosum*)	AT5G03690.1	NT_TC78239_1[a] NT_TC118911_1[a]	FV**S***INVENVESNR	RF**S***SINVENVESNR
60S acidic ribosomal protein family	60S acidic ribosomal protein-like protein (*Solanum tuberosum*)	AT2G27720.1	NT_TC91479_1[a]	LASVPSGGGGGGVAVA-SATSGGGGGGGAP-AAESK	LASVPCGGGGGGGVA-VAAPAGGAAAA-A**S***AAEEKK

After the tobacco phosphoproteomic dataset is broadened, it will be possible to also compare the regulatory phosphoproteins present. Possibly, the differences could reflect different regulatory strategies in bicellular pollen (represented by tobacco) and tricellular pollen (represented by *Arabidopsis* [32]). However, in order to reveal such differences in a more general way, it will be necessary to perform phosphoprotein identification in other species (ideally with sequenced genome) shedding both bicellular and tricellular pollen as well.

Finally, '-omic' techniques can bring high-throughput data without knowing function and other details about any single identified protein. So, in order to reveal the function of the proteins themselves and of their phosphorylation sites, more detailed studies will be required.

Funding

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