Identification and Analysis of the Acetylated Status of Poplar Proteins Reveals Analogous N-Terminal Protein Processing Mechanisms with Other Eukaryotes

Chang-Cai Liu1,2, Hang-Yong Zhu1,3, Xiu-Mei Dong1, De-Li Ning1, Hong-Xia Wang4, Wei-Hua Li4, Chuan-Ping Yang1*, Bai-Chen Wang1,5*

1 State Key Laboratory of Forest Genetics and Tree Breeding (Northeast Forestry University), Northeast Forestry University, Harbin, Heilongjiang, China, 2 Laboratory for Chemical Defense and Microscale Analysis, Hubei Nanxing General Chemical Factory, Zhijiang, Hubei, China, 3 Bureau of Garden and Park, Qitaile, Heilongjiang, China, 4 Institute of Basic Medical Sciences, National Center for Biomedical Analysis, Beijing, China, 5 Key Laboratory of Photobiology, Institute of Botany, Chinese Academy of Sciences, Beijing, China

Abstract

Background: The N-terminal protein processing mechanism (NPM) including N-terminal Met excision (NME) and N-terminal acetylation (Nα-acetylation) represents a common protein co-translational process of some eukaryotes. However, this NPM occurred in woody plants yet remains unknown.

Methodology/Principal Findings: To reveal the NPM in poplar, we investigated the Nα-acetylation status of poplar proteins during dormancy by combining tandem mass spectrometry with TiO2 enrichment of acetylated peptides. We identified 58 N-terminally acetylated (Nα-acetylated) proteins. Most proteins (>81%) are subjected to Nα-acetylation following the N-terminal removal of Met, indicating that Nα-acetylation and NME represent a common NPM of poplar proteins. Furthermore, we confirm that poplar shares the analogous NME and Nα-acetylation (NPM) to other eukaryotes according to analysis of N-terminal features of these acetylated proteins combined with genome-wide identification of the involving methionine aminopeptidases (MAPs) and N-terminal acetylation (Nat) enzymes in poplar. The Nα-acetylated reactions and the involving enzymes of these poplar proteins are also identified based on those of yeast and human, as well as the subcellular location information of these poplar proteins.

Conclusions/Significance: This study represents the first extensive investigation of Nα-acetylation events in woody plants, the results of which will provide useful resources for future unraveling the regulatory mechanisms of Nα-acetylation of proteins in poplar.

Introduction

The N-terminal protein processing mechanism (NPM) represents a common protein modification that occurs in eukaryotes, and primarily involves the co-translational processes of N-terminal Met excision (NME) and N-terminal acetylation (Nα-acetylation) [1–4]. In all eukaryotes, the nuclear-encoded protein synthesis machinery requires newly synthesized peptides to begin with methionine (Met), whereas plastid-encoded nascent proteins begin with a Met with an N-formyl group (Fo) [5]. Therefore, NME of the nuclear-encoded proteins requires only methionine aminopeptidase (MAP; EC 3.4.11.18) activity, which proteolytically removes the N-terminal Met [4,6]. NME of the plastid-encoded proteins requires MAP activity and peptide deformylase (PDF) activity [7]. The latter enzymatic activity is required for the removal of the Fo groups, thereby unmasking the amino group of the first Met and allowing the subsequent action of MAP [1,3,8].

Following the synthesis of the peptides in eukaryotes, cytosolic MAPs may remove the first Met residue if the residue at position two has a small enough side-chain, resulting in N-terminal Ala, Val, Ser, Thr, Cys, Gly, or Pro amino acids [9]. Approximately two-thirds of mature proteins undergo NME induced by MAP [1]. Unlike eubacteria, which possess only one type of MAP (MAP1), eukaryotes possess a second type of MAP, MAP2, with similar substrate specificity as found for MAP1 [4]. Experimental data have shown that, in higher eukaryotes, MAP1s are found in mitochondria, plastids, and the cytoplasm, whereas MAP2s are found specifically in the cytoplasm, suggesting that NME occurs in all compartments where de novo protein synthesis occurs [1,5,10].

Nα-acetylation is an enzyme-catalyzed reaction in which the protein α-amino group accepts an acetyl group from acetyl-CoA [9]. Currently, six types of Nats conserved from yeast to humans are responsible for these Nα-acetylation events: each of the three
major Nats, NatA, NatB and NatC contain a catalytic subunit, and one or two auxiliary subunits, whereas NatD, NatE and NatF are composed of only a catalytic subunit [11–12]. Each type of Nats appears to acetylate a distinct subset of substrates defined by the first N-terminal amino acid [13]. NatA is often responsible for the N\(^2\)-acetylation of small N-terminal amino acid residues, including Ser, Ala, Thr, Val, Gly and Cys, following NME induced by MAP [2,14–15]. Interestingly, Nat\(^\text{f}\) has the potential to acetylate these types of N-termini where the Met has not been cleaved [12]. NatB potentially recognizes and acetylates Met-Asp-, Met-Glu-, and Met-Asn- N-termini [12]. Hydrophobic Met-Leu-, Met-Ile- and Met-Phe- are acetylated by NatC. Moreover, these hydrophobic termini are also recognized by NatF and NatE in vitro, suggesting that redundancy in activity also exists between particular Nats [12]. In yeast, NatD was found to acetylate the Ser-N-termini of histones 2A and 4 in vitro and in vivo, whereas no such activity has yet been observed in higher eukaryotes [16]. Furthermore, the entire genes encoding catalytic or auxiliary subunits of NatA-NatF have been identified and described in yeast and humans [12–13]. However, there is still no systematic and comprehensive characterization of Nats in Arabidopsis and poplar.

Previous evidence suggests that NPM possess similar mechanisms across several eukaryotes [1–2]. However, the NPM mechanism present in poplar remains poorly defined. Here, we identified 58 N\(^2\)-acetylated proteins using tandem mass spectrometry combined with TiO\(_2\) enrichment of acetyl peptides in dormant terminal buds of poplar. The site-specific acetylation data provide a wealth of resources for decoding NPM mechanisms present in poplar. As far as we know, this study represents the first extensive investigation of N\(^2\)-acetylation events in woody plants.

Results

Characterization of the Identified Acetylated Proteins in Poplar

The N\(^2\)-acetylation of proteins was investigated to explore NPMs of woody plant proteins. Proteins from poplar were isolated and digested with trypsin in solution and the tryptic peptides were subjected to nanoUPLC-ESI-MS/MS for the identification of acetylation following TiO\(_2\) enrichment. The spectra representing all of these acetylated peptides and the original data collected are listed in the File S1. As outlined in the Table 1 and File S2, we have identified 58 N-terminally acetylated (N\(^2\)-acetylated) proteins. These 58 proteins were divided into two groups: (i) the NME-independent N\(^2\)-acetylation group, where the N-terminal Met residue (iMet) is retained and subsequently acetylated; and (ii) the NME-dependent N\(^2\)-acetylation group, where the N-terminal iMet residue is removed and acetylation occurs at the exposed residue located at position two. In this study, most of N\(^2\)-acetylated proteins (47, 81%) belong to group (i), whereas the remaining proteins (11, 19%) belong to group (ii), suggesting that N\(^2\)-acetylation and NME could represent a common NPM of poplar present in poplar. As far as we know, this study represents the first data provide a wealth of resources for decoding NPM mechanisms of poplar in poplar. Moreover, it could be proposed that NMEs, but not PDFs, represent the only enzymes responsible for NME of the 47 identified acetylated proteins from poplar, subcellular localization of these corresponding genes of identified acetylated proteins were determined by searching the Populus trichocarpa genome database (http://genome.jgi.doe.gov/poplar/). As a result, we found that all identified acetylated proteins were products encoded by nuclear genes. Consequently, it could be observed that MAPs, but not PDFs, represent the only enzymes responsible for NME of the 47 identified acetylated proteins from poplar. However, further efforts are required to determine which MAPs function as NME of these proteins in poplar.

Although there has been systematic and comprehensive characterization of MAPs in Arabidopsis [4–5], until now such information has not been documented in poplar. To clearly obtain all members of the MAP families in Populus, the P. trichocarpa protein sequence data [20] was exploited as a query file for searching across the Conserved Domain Database (CDD) [21]. We found five non-redundant putative MAPs that significantly matched the MetAP1 domain (cd01086), whereas two MAP2s were found to significantly match the MetAP2 domain (cd01088) (File S3 and Table 2). A separate phylogenetic tree was generated from all complete MAP protein sequences of Arabidopsis and poplar (Figure 1). Phylogenetic analysis demonstrated that two distinct clusters are present, including MAP1 and MAP2 clusters, which are respectively encoded by evolutionarily divergent genes (Figure 1). These identified poplar MAPs were denominated in accordance with their MAP orthologues with the closest evolutionary relatedness in Arabidopsis. Consequently, one member of poplar MAP1s (730835) has the closest evolutionary relation with Arabidopsis MAP1A (Ath MAP1A, At2g45240), and was therefore termed poplar MAP1A (Ptr MAP1A) (Figure 1 and Table 2). Notably, another member of poplar MAP1s (588331) was considered a novel member of MAP1s because of the divergence between this protein and other MAP1s (MAP1A-D). This MAP1 was termed Ptr MAP1E (Figure 1 and Table 2). Furthermore, we found that the MAP1 domain of Ptr MAP1E has high sequence similarity with other MAP1s of Arabidopsis and poplar (Figure S1a), whereas the absence of the N-terminal extension was only present in Ptr MAP1E, which could represent its divergence from MAP1A-D of Arabidopsis and poplar (Figure S1a). Surprisingly, Ptr MAP2A and Ptr MAP2B, as well as Ath MAP2A and Ath MAP2B share near-identical amino acid sequences, suggesting a conservation of function (Figure S1b).

In Arabidopsis, three organelle-targeted MAPs (MAP1B, MAP1C and MAP1D), and three cytosolic MAPs (MAP1A, MAP2A and MAP2B) have been characterized as members of the NME machinery [1,4–5]. However, the role of these MAPs in NME of poplar remains unclear. Using TargetP [22], it was predicted that,
| NO. | JGI ID       | Protein ID in NCBI | Description                                                   | Best hits in Arabidopsis | E-value | Subcellular Location | Acetylated N-terminus | Putative Nats Type |
|-----|--------------|--------------------|---------------------------------------------------------------|---------------------------|---------|----------------------|----------------------|--------------------|
| 1   | XP_002301543.1 | S-adenosylmethionine synthetase 2 | At4g01850 0.0 Cytoplasm Ac-Met-Thr-Thr- NatB | | | | |
| 2   | XP_002320949.1 | S-adenosylmethionine synthetase 2 | At4g01850 0.0 Cytoplasm Ac-Met-Thr-Thr- NatB | | | | |
| 3   | XP_002319463.1 | S-adenosylmethionine synthetase 4 | At3g17390 0.0 Cytoplasm Ac-Met-Thr-Thr- NatB | | | | |
| 4   | XP_002316736.1 | NAD(P)-binding Rossmann-fold-containing protein | At2g34460 9e-137 Chloroplast Ac-Met-Thr-Thr- NatB | | | | |
| 5   | XP_002301543.1 | S-adenosylmethionine synthetase 2 | At4g01850 0.0 Cytoplasm Ac-Met-Thr-Thr- NatB | | | | |
| 6   | XP_002328616.1 | Putative PR-10 type pathogenesis-related protein | NA* | | | | |
| 7   | XP_002302922.1 | 1-aminocyclopropane-1-carboxylate oxidase-1 | Atg6620 2e-11 Cytoplasm Ac-Met-Thr-Thr- NatB | | | | |
| 8   | XP_002331268.1 | CAMP-regulated phosphoprotein 19-related protein | At5g64130 1e-55 Cytoplasm Ac-Met-Thr-Thr- NatB | | | | |
| 9   | XP_002311836.1 | Auxin/aluminum-responsive protein, putative | Atg91940 1e-142 Cytoplasm Ac-Met-Thr-Thr- NatB | | | | |
| 10  | XP_002314449.1 | HSBP (heat shock factor binding protein) | Atg415802 2e-41 Cytoplasm Ac-Met-Thr-Thr- NatB | | | | |
| 11  | XP_002338396.1 | Polyphenol oxidase | NA | | | | |
in poplar, the two Ptr MAP2s (Ptr MAP2A and Ptr MAP2B) are specifically targeted to the cytoplasm, whereas Ptr MAP1s is targeted to both the organelles (PtrMAP1B-E) and the cytoplasm specifically targeted to the cytoplasm, whereas Ptr MAP1s is

in poplar, the two Ptr MAP2s (Ptr MAP2A and Ptr MAP2B) are specifically targeted to the cytoplasm, whereas Ptr MAP1s is targeted to both the organelles (PtrMAP1B-E) and the cytoplasm (PtrMAP1A). Due to the absence of any plastid-encoded proteins targeted to both the organelles (PtrMAP1B-E) and the cytoplasm specifically targeted to the cytoplasm, whereas Ptr MAP1s is

The closest relatedness member in Arabidopsis (Table 2), these proteins from the NME-dependent N\(^2\)-acytation group (ii), should be subjected to NME by the three cytosolic NME-independent N\(\text{H}\) orthologs responsible for N\(\text{H}\) motifs reflect the activity of particular Nats. In order to identify the variety of N-terminal sequences and these extracted consensus the N-terminal 14 amino acids from the N\(\text{H}\) motifs of yeast \[2,12,15,24\] (Figure 2A). The last enriched motif (Met-Leu-) was consistent with one of the substrate motifs of NatB, NatC, NatE or NatF identified in yeast and humans (Figure 2A). The third enriched motif (Met-Leu-) was consistent with one of the substrate motifs of NatB, NatC, NatE or NatF identified in yeast and humans [12] (Figure 2A). The last substrate motif, Met-Glu-, was assigned to one of the NatF substrate motifs of yeast [2,12,15,24] (Figure 2A).

Removal of the N-terminal Met of nuclear-encoded proteins by cytosolic MAP frequently leads to N\(^2\)-acytation of the resulting N-terminal Ala, Val, Ser, Thr, or Cys residues [9]. We found that similar events were also present for the 47 acetylated proteins of group (ii), based on the alignment of their N-terminus (Figure 2B). As illustrated in Figure 2B, there was an amino acid preference at positions two and three [(Ala/Ser/Gly/Thr/Val]-[Ser/Gly/ Asp/Glu] respectively] with the first position representing the

in poplar, the two Ptr MAP2s (Ptr MAP2A and Ptr MAP2B) are specifically targeted to the cytoplasm, whereas Ptr MAP1s is targeted to both the organelles (PtrMAP1B-E) and the cytoplasm (PtrMAP1A). Due to the absence of any plastid-encoded proteins targeted to both the organelles (PtrMAP1B-E) and the cytoplasm specifically targeted to the cytoplasm, whereas Ptr MAP1s is

The Nats Involved in N\(^2\)-acytation of the Identified Poplar Proteins

Confirmation of acetylation sites are recognized footprints of Nat activities. Eukaryotic proteins subject to N\(^2\)-acytation have a variety of N-terminal sequences and these extracted consensus motifs reflect the activity of particular Nats. In order to identify the Nat orthologs responsible for N\(^2\)-acytating these poplar proteins, the N-terminal 14 amino acids from the N\(^2\)-acytated proteins of NME-independent N\(^2\)-acytation group (i) and NME-dependent N\(^2\)-acytation group (ii) were respectively aligned in a sequence logo plot using WebLogo [23] (Figure 2A and 2B). From the alignment of 11 acetylated proteins belonging to the group (i), we extracted four motifs, Met-Glu- (8/11), Met-Asp- (1/11), Met-Leu- (1/11), Met-Gly- (1/11) (Figure 2A). The first two striking enrichments of acetylation site motifs (Met-Glu- and Met-Asp-) match the previously identified NatB substrate motifs identified in yeast and humans (Figure 2A). The third enriched motif (Met-Leu-) was consistent with one of the substrate motifs of NatB, NatC, NatE or NatF identified in yeast and humans [12] (Figure 2A). The last substrate motif, Met-Gly-, was assigned to one of the NatF substrate motifs of yeast [2,12,15,24] (Figure 2A).

Removal of the N-terminal Met of nuclear-encoded proteins by cytosolic MAP frequently leads to N\(^2\)-acytation of the resulting N-terminal Ala, Val, Ser, Thr, or Cys residues [9]. We found that similar events were also present for the 47 acetylated proteins of group (ii), based on the alignment of their N-terminus (Figure 2B). As illustrated in Figure 2B, there was an amino acid preference at positions two and three [(Ala/Ser/Gly/Thr/Val]-[Ser/Gly/ Asp/Glu] respectively] with the first position representing the

Table 1. Cont.

| NO. | JGI ID | Protein ID in NCBI | Description | Best hits in Arabidopsis | E-value | Subcellular Location | Acetylated N-terminus | Putative Nats Type |
|-----|--------|--------------------|-------------|-------------------------|---------|---------------------|---------------------|-------------------|
| 44  | XP_002315023.1 | 724093 | Translation initiation factor elf-5A | At1g13590 (2e\(^{-101}\)) | Cytoplasm | Ac-Ser-Asp-Glu-NatA |
| 45  | XP_002327184.1 | 740524 | WPP domain-containing protein 2 | At1g24700 (9e\(^{-40}\)) | Cytoplasm | Ac-Ser-Asp-Ser-NatA |
| 46  | XP_002308228.1 | 819223 | Metalloendopeptidase M24 protein | At3g51800 | Cytoplasm | Ac-Ser-Asp-Ser-NatA |
| 47  | XP_002322994.1 | 577003 | Metalloendopeptidase M24 protein | At3g51800 | Cytoplasm | Ac-Ser-Asp-Ser-NatA |
| 48  | XP_002326994.1 | 836390 | T-complex protein 1 alpha subunit | At3g20050 | Cytoplasm | Ac-Ser-ile-Asa-NatA |
| 49  | XP_002308283.1 | 819264 | Nucleosome assembly protein 1/2 | At2g19480 (3e\(^{-176}\)) | Cytoplasm | Ac-Ser-Asn-Asp-NatA |
| 50  | XP_002323796.1 | 736146 | Glucose-6-phosphate dehydrogenase | At5g40760 (9e) | Cytoplasm | Ac-Gly-Ser-Gly-NatA |
| 51  | XP_002298586.1 | 641721 | Glucose-6-phosphate dehydrogenase | At5g40760 | Cytoplasm | Ac-Gly-Ser-Gly-NatA |
| 52  | XP_002323696.1 | 825441 | Phosphoglycerate mutase | At1g09780 (0.0) | Cytoplasm | Ac-Ser-Pro-Asp-NatA |
| 53  | XP_002326437.1 | 739967 | 26S proteasome AAA-ATPase subunit RPT1a | At1g53750 (0.0) | Cytoplasm | Ac-Gly-Ser-Gly-NatA |
| 54  | XP_002309623.1 | 819597 | DEAD-box ATP-dependent RNA helicase 56 | At5g11200 (0.0) | Cytoplasm | Ac-Ser-Asn-Asp-NatA |
| 55  | XP_002324856.1 | 578196 | DEAD-box ATP-dependent RNA helicase 56 | At5g11200 | Cytoplasm | Ac-Ser-Asn-Asp-NatA |
| 56  | XP_002322743.1 | 735121 | Ser/thr protein phosphatase PP2A catalytic subunit | At3g58500 (0.0) | Cytoplasm | Ac-Ser-Thr-Asp-NatA |
| 57  | XP_002326738.1 | 739954 | Putative trehalose-6-phosphate synthase | At1g17770 (0.0) | Cytoplasm | Ac-Val-Ser-Arg-NatA |
| 58  | XP_002303278.1 | 553698 | CASTC3/Barentsz eIF4AIII binding protein | At1g80000 (1e\(^{-98}\)) | Cytoplasm | Ac-Thr-Lys-Val-NatA |

*NA represents “not available”.

doi:10.1371/journal.pone.0058681.t001

doi:10.1371/journal.pone.0058681.t002

Table 2. All predicted MAPs present in Populus trichocarpa genome.

| JGI ACS. number | Protein ID | Domain name in CDD (ID) | The closest relatedness member in Arabidopsis | Novel simplified nomenclature | Subcellular location of the protein |
|----------------|----------|------------------------|---------------------------------------------|-----------------------------|----------------------------------|
| 720677         | XP_00311439.1 | MetAP1 (cd01086) | Ath MAP1 (At1g32540) | Ptru MAP11 | Cytoplasm |
| 720677         | XP_00311439.1 | MetAP1 (cd01086) | Ath MAP1 (At1g32540) | Ptru MAP1B | Mitochondrion |
| 760457         | XP_002306464.1 | MetAP1 (cd01086) | Ath MAP1 (At1g32540) | Ptru MAP1D | Mitochondrion |
| 760457         | XP_002306464.1 | MetAP1 (cd01086) | Ath MAP1 (At1g32540) | Ptru MAP1E | Mitochondrion |
| 552920         | XP_002301676.1 | MetAP2 (cd01088) | Ath MAP2B (At3g59990) | Ptru MAP2B | Cytoplasm |
| 198596         | XP_002305888.1 | MetAP2 (cd01088) | Ath MAP2A (At2g44180) | Ptru MAP2B | Cytoplasm |

*NA represents “not available”.

doi:10.1371/journal.pone.0058681.t002
removed Met. Accordingly, the preference likely represents a combination of consensus motifs for the MAPs and Nats. Furthermore, these acetylated residues are represented by five amino acid residues: Ala (25/47), Ser (13/47), Gly (7/47), Thr (1/47) and Val (1/47), which is consistent with the substrate profiles of NatA in yeast [9,12,24] (Figure 2B). This result suggests that Na-acetylation of these 47 proteins from group (ii) most likely involves the corresponding NatA orthologs in poplar. In summary, the major acetylases involved in the acetylation of these proteins of poplar are NatA (acetylates 47 (81%) proteins) and NatB (acetylates nine (15%) proteins) orthologs (Figure 2A and 2B).

Identification of Nats in Poplar and Arabidopsis

Although we suggested that NatA, NatB, NatC, NatE and NatF orthologs may be involved in acetylation of the identified proteins according to recognized substrate motifs by known Nats found in yeast and humans [12], it still remains unexplored whether the poplar genome contains genes encoding similar Nat orthologs to those found in yeast and humans. In order to precisely obtain all members of each type of Nat orthologs in Populus, domain files representing subunits of individual types [25] were exploited as queries to identify the Nat orthologs in the P. trichocarpa genome [20]. As a result, we identified 16 non-redundant putative Nat orthologous proteins that were composed of all catalytic and auxiliary subunits of the six types of Nats (NatA-F) (Table 3). Except for the NatD catalytic subunit (Ptr Naa40p), N-terminal amino acid sequences of these identified Nat catalytic subunits orthologs showed that these proteins have the consensus acetyl coenzyme A (AcCoA) binding motif, RxxGxG/A, which is a sequence feature of the N-acyltransferase superfamily (Figure S2). To further characterize the observation in other model plants, we extended the search to the Arabidopsis protein sequence database (http://www.arabidopsis.org/). Similarly, the Arabidopsis genome also contains the genes encoding the six types of Nats (NatA-F) (Table 3). Each Nat catalytic subunit in poplar and Arabidopsis shares high sequence similarity to their counterparts in yeast and humans (Figure S3a–f), suggesting that they are highly conserved from lower to higher eukaryotes. Notably, the AcCoA binding motif RxxGxG/A is also present in catalytic subunit of each NatA, NatB, NatC, NatE and NatF, whereas this motif is absent in catalytic subunit of NatD (Naa40p) from Arabidopsis, poplar, yeast and human (Figure S3a–f).

Although we identified the NatD orthologs of Arabidopsis (Ath Naa40p) and poplar (Ptr Naa40p), which is homologous to yeast Naa4p (Table 3), NatD activities of poplar were not observed in this study since N^3-acetylation status of the NatD substrates, histones H2A and H4 [26], has been not determined in the MS experiment. Therefore, further experiments are required to confirm such activity for Naa40p orthologs in poplar. In summary, it has been suggested that poplar and Arabidopsis...
Discussion

The exact NPM including NME and N\(^{\alpha}\)-acetylation has been well characterized for yeast. This focus on yeast is primarily because of the accessibility of mutants involved in the pathway. In contrast, negligible progress has been made using woody plants, such as poplar. Furthermore, MS for peptide-based proteomics combined with selective enrichment technologies have been widely used for simultaneous identification of acetylated proteins of yeast and humans [27–28]. Based on the large amount of data identifying exact acetylation sites, the substrate spectra of enzymes involved in the pathway process have been well characterized, resulting in the promotion of research targeting the entire NPM [19]. In combination, these techniques and methods enable large-scale identification and analysis of acetylated proteins.

It is noteworthy that TiO\(_2\) column was considered as one of the most effective methods for selective enrichment of phosphopeptides based on the strong specific interaction between TiO\(_2\) and phosphate groups on the molecule of phosphopeptides. For this reason, we had used mass spectrometry combined with TiO\(_2\) phosphopeptide-enrichment strategies to investigate the phosphoproteome of dormant terminal buds in poplar (Populus simonii x P. nigra) [29]. As a result, 161 phosphopeptides with 161 unique phosphorylated sites from 151 proteins were identified [29]. Surprisingly, we identified 51 N-terminally acetylated peptides from 58 proteins with high confidence, among which fourteen N\(^{\alpha}\)-acetylated peptides (27.5%, 14/51) were also occurred on phosphorylation in this study (Table 1 and File S2). To explore and clarify why these N-terminal acetylated peptides and phosphopeptides were together enriched using this approach of TiO\(_2\) affinity, we made in silico analysis of the theoretical pI and acidic amino acid composition (including D and E) for these N\(^{\alpha}\)-acetylated peptides without the occurrence of phosphorylation events using ProtParam tool (http://web.expasy.org/protparam/). We do so mainly because highly acidic peptides or those containing multiple acidic residues tend to absorb with TiO\(_2\) despite the presence of a number of improved TiO\(_2\) phosphopeptide-enrichment procedures [30–32]. It is found that almost all of the N\(^{\alpha}\)-acetylated peptides could be considered as highly acidic peptides or peptides containing multiple acidic residues because of their low theoretical pI or high acidic amino acid composition (File S5). Accordingly, we thought that the enrichment of these N\(^{\alpha}\)-acetylated peptides using TiO\(_2\) microcolumn should mainly be due to the strong specific interaction between their additional phosphate groups or multiple acidic residues and TiO\(_2\). However, it should be very interesting that further experiment are now required to investigate whether interaction between the N-acetyl moiety of protein and TiO\(_2\) was occurred and functioned on this process of enrichment.

Compared with phosphopeptide-enrichment strategies, to date there are still no more suitable methods applied in the N-terminal

**Figure 2.** These identified protein acetylation reaction and sequence alignment of the acetylation sites. A, the acetylation reaction and sequence alignment of the 11 N\(^{\alpha}\)-acetylated proteins that retained their Met residue from group (i). B, the acetylation reaction and sequence alignment of the acetylated sites of the 47 N\(^{\alpha}\)-acetylated proteins where the Met residue has been removed from group (ii). “AC” represents N\(^{\alpha}\)-acetylation, and in B, the blank at position 1 represents the removed of the N-terminal Met residue; sequence alignment of the acetylation sites was made by Weblogo program.

doi:10.1371/journal.pone.0058681.g002
Table 3. All predicted Nats present in *Arabidopsis* and *Populus* genomes.

| Type | Sub-units | Primary | Synonyms | **Arabidopsis** | **Populus** |
|------|-----------|---------|----------|----------------|-------------|
|      |           |         |          | Accession No. | Novel simplified nomenclature | Subcellular Location | JGI protein ID | Refseq protein ID | Novel simplified nomenclature | Subcellular location |
| NatA | CS<sup>a</sup> | Naa10p | Naa1p | AT5G13780 | Ath Naa10p | Secretory pathway | 650021 | XP_002314058.1 | Ptr Naa10p | Secretory pathway |
|      |           | Naa11p | Ard1p |            |             |                   | 641307 | XP_002298415.1 | Ptr Naa11p | Secretory pathway |
|      | AS<sup>b</sup> | Naa15p | Naa16pNaa1p | AT1G0410 | Ath Naa15p | Cytoplasm | 548659 | XP_002299630.1 | Ptr Naa15p | Cytoplasm |
|      |           |         |          |            |             |                   | 553694 | XP_002304180.1 | Ptr Naa16p | Cytoplasm |
| NatB | CS | Naa20p | Naa3p | AT1G03150 | Ath Naa20p | Cytoplasm | 818659 | XP_002307586.1 | Ptr Naa20p | Secretory pathway |
|      |           |         |          |            |             |                   | 643297 | XP_002300841.1 | Ptr Naa21p | Cytoplasm |
|      | AS | Naa25p | Mdm20p | AT5G58450 | Ath Naa25p | Cytoplasm | 571859 | XP_002319956.1 | Ptr Naa25p | Cytoplasm |
| NatC | CS | Naa30p | Mak3p | AT2G38130 | Ath Naa30p | Cytoplasm | 727122 | XP_002317002.1 | Ptr Naa30p | Cytoplasm |
|      | AS<sup>I</sup> | Naa35p | Mak10p | AT2G11000 | Ath Naa35p | Cytoplasm | 560565 | XP_002308056.1 | Ptr Naa35p | Cytoplasm |
|      | AS<sup>II</sup> | Naa38p | Mak31p | AT1G65700 | Ath Naa38p | Cytoplasm | 644178 | XP_002299990.1 | Ptr Naa38p | Cytoplasm |
| NatD | CS | Naa40p | Naa4p | AT1G18335 | Ath Naa40p | Cytoplasm | 729076 | XP_002318313.1 | Ptr Naa40p | Cytoplasm |
| NatE | CS | Naa50p | Naa5p | AT5G11340 | Ath Naa50p | Cytoplasm | 737117 | XP_002324274.1 | Ptr Naa50p | Cytoplasm |
| NatF | CS | Naa60p | Naa15 | AT5G16800 | Ath Naa60p | Cytoplasm | 834607 | XP_002319255.1 | Ptr Naa60p | Cytoplasm |
|      |           |         |          | AT3G02980 | Ath Naa61p | Cytoplasm | 665408 | XP_002325388.1 | Ptr Naa61p | Mitochondria |

<sup>a</sup>CS denotes catalytic subunit of Nat;  
<sup>b</sup>AS represents auxiliary subunit of Nat.  
doi:10.1371/journal.pone.0058681.t003
proteins were identified and the majority of these proteins (47, showed that the Met-Gly- sequence represents a substrate motif of acetylated. This observation is in accord with a recent study that removed according to the NME rule; however, it was retained and Gly and facilitated NME (File S2). Specifically, the Met of the N-(Table 1); however, the adjacent residue at position two was a terminal Met residue of one PPO (794816) was acetylated the 11 proteins belonging to group (i), we found that the N-acetylation could be provided, especially for woody plants. Although 51 unique acetyl-peptides from 58 proteins were accidentally identified in our study of poplar phosphoproteome since phosphate groups or multiple acidic residues within these acetyl-peptides contributed to their affinity with TiO2 microcolumn, the site-specific acetylation data could also provide a wealth of valuable resources to assist us decoding NPM mechanisms present in poplar.

Removal of the N-terminal Met in Poplar Follows the NME Rule
In this study, we used a proteomics approach to investigate the acetylation status of poplar proteins. Fifty-eight N²-acetylated proteins were identified and the majority of these proteins (47, >81%) undergo N-terminal Met cleavage and subsequent acetylation of the exposed N-terminal residue at position two. Moreover, the residues (Ala, Ser, Gly, Thr and Val) at position two comply with the above-mentioned rule of NME. Surprisingly, of the 11 proteins belonging to group (i), we found that the N-terminal Met residue of one PPO (794816) was acetylated (Table 1); however, the adjacent residue at position two was a Gly and facilitated NME (File S2). Specifically, the Met of the N-terminal motif (Met-Gly-) of the poplar PPO should have been removed according to the NME rule; however, it was retained and acetylated. This observation is in accord with a recent study that showed that the Met-Gly- sequence represents a substrate motif of a newly identified Nat-F type in yeast [12] (Figure 2A).

One Extended Nat Catalytic Subunit System Occurs in the Poplar Genome
Currently, six types of Nats (NatA-NatF) represent the full set of enzymes of the Nats system from yeast to humans [12]. In this study, we found that both Arabidopsis and poplar genomes contain the full Nats system composed of NatA-F (Table 3). Most of the Nat catalytic subunits in poplar exist as two paralogous isoforms, such as the NatA catalytic subunits of Ptr Naa10p and Ptr Naa11p, and the NatB catalytic subunits of Ptr Naa20p and Ptr Naa21p. In contrast, only NatD exists as a single protein, Ptr Naa40p (Table 3). Conversely, no single Nat catalytic subunit of yeast contains paralogous isoforms, only one NatA catalytic subunit in humans contains paralogous isoforms (i.e., Naa10p and Naa11p) and one NatF catalytic subunit of Arabidopsis contains paralogous isoforms (Ath Naa60p and Ath Naa61p) (Table 3 and File S4). This observation suggests that the genes encoding Nat catalytic subunits in poplar have expanded. This expansion, often present on a large number of Populus multi-gene families, could have occurred from multiple gene duplication events, including segmental duplication and tandem duplication events [20]. The presence of more Nat subunit genes in the Populus genome may reflect a greater requirement for acetylation of proteins. A detailed schematic view of the number of paralogous isoforms of each Nat catalytic subunit from the four organisms is provided in Figure S4.

Cytosolic Nat Isoforms Present in Poplar
Following Met cleavage by MAP, the exposed small side-chain amino acid, Ser-, Ala-, Thr-, Val-, Gly- or Cys-, is often further acetylated by Nats, a Nat enzyme present in either the cytoplasm [3,19,36] or the chloroplast of Arabidopsis [6,37-38]. These data indicate that there should be both chloroplastic and cytosolic isoforms of NatA present in Arabidopsis. However, we have identified only one Arabidopsis NatA complex consisting of one catalytic subunit (AT5G13780, Ath Naa10p) and one auxiliary subunit (AT1G80410, Ath Naa15p) (Table 3). Surprisingly, TargetP prediction indicates that Ath Naa10p is secreted, whereas Ath Naa15p is targeted to the cytoplasm in Arabidopsis [10,22] (Table 3). Furthermore, cytosolic isoforms of NatA with N²-acetylation activity are composed of both Ath Naa10p and Ath Naa15p, and the chloroplastic isoforms of NatA only consist of Ath Naa10p [18,22]. Similarly, the secreted catalytic subunits (650021, Ptr Naa10p and 641307, Ptr Naa11p) and cytosolic auxiliary subunits (548659, Ptr Naa13p and 553694, Ptr Naa16p) are also present in poplar (Table 3). Thus, we propose that the single presence of one of the two catalytic subunits “Ptr Naa10p and Ptr Naa11p” combination with auxiliary subunits of Ptr Naa15p and Ptr Naa16p should be the cytosolic isoform forms of NatA in poplar.

According to the analysis of the substrates profile, NatA should be major N-terminal Nats, which could be responsible for acetylating 81% of the identified proteins (Figure 2B and Table 1). Identifying which NatA isoforms carry out the acetylation of these proteins are important. To address this, information about subcellular location of the identified poplar proteins was obtained using TargetP [22], and by a comparison of their best hits in Arabidopsis with the latest plant plastid database (PPDB) [18]. As a result, among the 47 acetylated proteins from group (ii) by NatA, all proteins were targeted to cytoplasm, while no any proteins targeted to the chloroplast were found (Table 1), suggesting that these acetylation events should be carried out by cytosolic NatA isoform and not by chloroplastic NatA isoform. Chloroplastic and cytosolic isoforms of NatB had respectively been found in Chlamydomonas reinhardtii [39] and Arabidopsis [18]. However, it is noteworthy that the two catalytic subunits of NatB in poplar, one is secreted catalytic subunit (Ptr Naa20p) and other one is cytosolic catalytic subunit (Ptr Naa21p), whereas the only one auxiliary subunit of NatB (Ptr Naa23p) in poplar is targeted to cytoplasm (Table 3). Similar to NatA, NatB of poplar should also exist in the forms of either chloroplastic or cytosolic isoforms, where the individual Ptr Naa20p or Ptr Naa21p combination with auxiliary subunit (Ptr Naa23p) could compose cytosolic isoform of poplar NatB.

In summary, we confirm that the N-terminal Met residues of these proteins from the NME-independent N²-acetylation group (i), should be directly N²-acetylated by cytosolic NatB, NatG, NatE and cytosolic NatF. And that the second N-terminal residue of these proteins from the NME-dependent N²-acetylation group (ii), should be N²-acetylated by cytosolic NatA following the subjected to NME by three cytosolic MAPs (PtrMAP1A, PtrMAP2A and PrnMAP2B) in poplar (Figure 2A and 2B).

The Biological Significance of N²-acetylation of these Proteins during the Dormancy of Poplar
For many years, it was thought that N²-acetylation protected proteins from degradation [40-41]. On the contrary, N-terminal acetylated Met residues were recently found to be involved in creating degradation signals: a ubiquitin ligase, Doa10, recognizes N²-acetylated proteins and ubiquitinates the protein, thereby marking it for degradation [9]. Although these two hypotheses predict opposite functional outcomes for N²-acetylation and thus
appear to be contradictory, both mechanisms may take place side-by-side in the cell, each functioning to a specific subset of proteins under defined conditions [42]. Accordingly, it was proposed that the functional consequences of N\textsuperscript{\alpha}-acetylation of these identified proteins during dormancy of poplar may be dependent on each specific protein and its cellular state. However, the current major challenge is to determine the specific functions of each individual acetylated protein during the dormancy of poplar.

In conclusion, we have identified 58 N\textsuperscript{\alpha}-acetylated proteins using a tandem MS method combined with TiO\textsubscript{2} acetylpeptide-enrichment strategies. Based on the analysis of the N-terminus of these proteins, we confirm that poplar possesses the analogous NPMs including NME rule and Nat system to other eukaryotes. Furthermore, we also confirm that the acetylation reactions and their involving enzymes of these identified proteins in poplar. Further experiments are now required to confirm that these specific MAP and Nat enzymes interact with the identified acetylation proteins in vivo. A promising way forward is to widely identify and characterize the dynamics of protein acetylation in response to environmental changes, applying specialized targeted quantitative acetylation proteomics tools.

Materials and Methods

Chemicals and Reagents

HPLC-grade acetonitrile (ACN) was purchased from JT Baker (Thomas Scientific, Swedesboro, NJ, USA). HPLC-grade water was produced with a Milli-Q A10 system from Millipore (Billerica, MA, USA). Iodoacetamide (IAA) and dithiothreitol (DTT) were obtained from Acros Organics (Morris Plains, NJ, USA). Modified sequencing-grade trypsin was supplied by Promega (Madison, WI, USA). Phamlyte, protease-inhibitor cocktail and the 2-D Quant kit were obtained from Amersham Pharmacia Biotech (Uppsala, Sweden). All other reagents were purchased from Sigma (St Louis, MO, USA).

Plant Material

Dormant terminal buds were harvested from Populus simonii × Populus nigra trees in Harbin, China, (E126°37' N45°42') at the end of December 2009. The samples were frozen in liquid nitrogen and stored at −80°C until protein extraction was required.

Preparation of Total Protein

The dormant terminal buds were homogenized into a fine powder in liquid nitrogen and resuspended at −20°C with 10% (w/v) trichloracetic acid (TCA) in cold acetone containing 0.07% (v/v) 2-mercaptoethanol for a minimum of 2 h. The mixture was centrifuged at 40,000 × g at 4°C for 1 h and the precipitates were washed with cold acetone containing 0.07% (v/v) 2-mercaptoethanol. The pellets were dried by vacuum centrifugation and washed with cold acetone containing 0.07% (v/v) 2-mercaptoethanol. The mixture was then dissolved in 7 M urea, 2 M thiourea, 20 mM dithiothreitol, 1% (v/v) protease-inhibitor cocktail, 0.2 mM Na\textsubscript{2}VO\textsubscript{4} and 1 mM NaF at room temperature for 2 h before centrifugation at 40,000 × g at 4°C for 1 h. The resulting supernatant was collected and stored at −80°C until further use. The total protein content of the samples was quantified using a 2-D Quant kit.

In-solution Protein Digestion

Total proteins were digested as previously described [43–44]. Briefly, after adjusting the pH of the total protein solution to pH 8.5 with 1 M ammonium bicarbonate, the sample was reduced for 45 min at 55°C by adding DTT to a final concentration of 10 mM, and then carboxamidomethylated in 55 mM iodoacetamide for 30 min at room temperature in the dark. Ca\textsubscript{Cl\textsubscript{2}} was then added to a final concentration of 20 mM. Endoprotease Lys-C was added to a final substrate-to-enzyme ratio of 100:1 and the reaction was incubated at 37°C for 12 h. The Lys-C digestion was added to 1 M urea with 100 mM ammonium bicarbonate and modified trypsin was added to a final substrate-to-enzyme ratio of 50:1. The trypsin digestion was incubated at 37°C for 12 h. After digestion, the peptide mixture was acidified with formic acid for further MS analysis. Samples that were not immediately analyzed were stored at −80°C.

Enrichment of Acetylated Peptides Using a TiO\textsubscript{2} Microcolumn

The TiO\textsubscript{2} microcolumns were packed as previously described [30]. A small plug of C\textsubscript{8} material was stamped out of a 3 M Empore C\textsubscript{8} extraction disk using an HPLC syringe needle and placed at the small end of the GELoader tip. The C\textsubscript{8} disk served only as a frit to retain the TiO\textsubscript{2} beads within the GELoader tip. The TiO\textsubscript{2} beads were suspended in 100% ACN and an aliquot of this suspension (depending on the size of the column) was loaded onto the GELoader tip. Gentle air pressure created by a plastic syringe was used to pack the column. The TiO\textsubscript{2} microcolumn was equilibrated with loading buffer (40 μl; 80% ACN/5% TFA/saturated pthalic acid solution) and the trypsin-digested peptide mixture diluted with loading buffer was then loaded onto the column. The TiO\textsubscript{2} microcolumn was washed once with loading buffer (40 μl) and three times with washing solution (40 μl; 80% ACN/2% TFA). The solvent used for washing and loading the sample onto the TiO\textsubscript{2} microcolumn contained organic solvent (80% ACN), which abrogates the adsorption of peptides to the C\textsubscript{8} material [45]. The bound peptides were eluted twice with 40 μl of ammonium bicarbonate, pH >10.5, and then with 10 μl of 30% ACN. The eluted peptides were lyophilized and dissolved in 1% formic acid before MS analysis.

NanoUPLC-ESI-MS/MS

NanoUPLC-ESI-MS/MS was performed with a splitless nanoUPLC (10 kpsi nanoAcquity; Waters) coupled to a Synapt high-resolution mass spectrometer with a nanospray ion source (Waters). The program MassLynx (version 4.1; Waters) was used for data acquisition and instrument control. A symmetric C\textsubscript{18} 5-μm, 180-μm × 20-mm pre-column and a BEH C\textsubscript{18} 1.7-μm, 75-μm × 250-mm analytical reversed-phase column (Waters) were used. The mobile phases were (A) 100% H\textsubscript{2}O/0.1% formic acid and (B) 100% ACN/0.1% formic acid. The samples were initially transferred in an aqueous 0.1% formic acid solution to the pre-column with a flow rate of 5 μl/min for 3 min. The peptides were separated by a gradient of 5%–40% mobile phase B over 90 min at a flow rate of 200 nl/min, followed by a 10-min rinse with 90% mobile phase B. The column was re-equilibrated using the initial conditions for 20 min. The lock mass was delivered from the auxiliary pump of the NanoAcquity pump with a constant flow rate of 400 nl/min at a concentration of 100 fmol/μl of (Glu1) fibrinopeptide B to the reference sprayer of the NanoLockSpray source of the mass spectrometer. All samples were analyzed in triplicate. Data-dependent acquisition was performed in the positive ion mode. MS spectra were acquired for 1 s from mass-to-charge ratios (m/z) of 350 to 1990. Two of the most intense precursor ions that were doubly or triply charged were selected from m/z 350 to 1990. MS/MS spectra generated with collision-induced dissociation were acquired for 2 s from m/z 30 to 1990. The collision energy was automatically calculated based on the peptide charge and m/z; a dynamic exclusion window was set at 1800 s and the exclusion mass was set at 30 m/z.
applied that prevented the same m/z from being selected for 2 min after its acquisition. The mass tolerance in the MS and MS/MS modes was 15 and 50 ppm, respectively. The candidate acetylated peptides were initially assigned by ESI-MS/MS using 42-Da mass increments per acetyl moiety relative to the unmodified peptides.

Data Analysis and Mascot Database Search

The MS/MS data were converted to a pk file format using the ProteinLynx software (Waters) and the resulting pk file was searched against the JGI Populus trichocarpa v1.1 (http://genome.jgi-psf.org/Poptr1_l1/Poptr1_l1.home.html) protein sequence database using an in-house Mascot server (version 1.8). Two missed cleavage sites were allowed: acetylation, carbamidomethylation, methionine oxidation and phosphorylation of serine/threonine/tyrosine of the N-terminus of the protein were accepted as variable modifications. The FDR is 0.00% for peptide matches above the identity threshold and 0.36±0.85% for peptide matches above the homology or identity threshold.

Bioinformatics

The complete protein sequence database of poplar was downloaded from Populus trichocarpa v1.1 (www.jgi.doe.gov/poplar). Using a custom Perl program, all the acetylated protein sequences were extracted from the protein databases by their protein ID identifiers. These protein sequences with the conserved domains of MetAP1 (cd01086) and MetAP2 (cd01085) were respectively considered as the family members of MAPs from poplar and poplar. Sequence conservation is highest in the region of the MetAP1 domain (unmarked), and these MAPs have various N-terminal extension sequences (grey box above sequence alignment). In particular, the N-terminal extension is absent in Ptr MAP1E. a amino acid sequence alignment of MAP2s from poplar and Arabidopsis. MAP2s from poplar and Arabidopsis share near-identical amino acid sequences, indicating that these MAP2s have a conserved function. The identifiers of the proteins are shown in Table 2.

Supporting Information

Figure S1 Alignment of the amino acid sequence of MAPs from Arabidopsis and poplar. Color shading represents 70% identical residues among the sequences. Gaps were introduced to ensure maximum identity. a amino acid sequence alignment of Ptr MAP1E with MAP1A-D of Arabidopsis and poplar. Sequence conservation is highest in the region of the MetAP1 domain (unmarked), and these MAP1s had various N-terminal extension sequences (grey box above sequence alignment). In particular, the N-terminal extension is absent in Ptr MAP1E. b amino acid sequence alignment of MAP2s from poplar and Arabidopsis. MAP2s from poplar and Arabidopsis share near-identical amino acid sequences, indicating that these MAP2s have a conserved function. The identifiers of the proteins are shown in Table 2.

Figure S2 Amino acid sequence alignment of all predicted Nat catalytic subunits from poplar. The consensus acetyl coenzyme A (AcCoA) binding motif sequence RxxGxG/A, where x can be any amino acids, is boxed (red). The identifiers of the proteins are shown in Table 3.

Figure S3 Amino acid sequence alignments of each Nat catalytic subunit from several eukaryotes. The consensus acetyl coenzyme A (AcCoA) binding motif sequence RxxGxG/A, where x can be any amino acid, is indicated within the red boxes. Gaps were introduced to ensure maximum identity. Color shading represents 70% identical residues among the sequences. a amino acid sequence alignment of the NatA catalytic subunits from poplar, Arabidopsis, human and yeast. b amino acid sequence alignment of the NatB catalytic subunits from poplar, Arabidopsis, human and yeast. c amino acid sequence alignment of the NatC catalytic subunits from poplar, Arabidopsis, human and yeast. d amino acid sequence alignment of the NatD catalytic subunits from poplar, Arabidopsis, human and yeast. e amino acid sequence alignment of the NatE catalytic subunits from poplar, Arabidopsis, human and yeast. f amino acid sequence alignment of the NatF catalytic subunits from poplar, Arabidopsis and human. The identifiers of the proteins are shown in the Supplemental Table 3.

Figure S4 Schematic view of the number of paralogous isoforms of each Nat catalytic subunit from the four organisms.

File S1 A file that contains all the original MS/MS spectra of acetylated peptides identified in this study.

File S2 The detailed information for these identified N-terminal acetylated peptides in poplar.

File S3 Predicted poplar MAPs containing MetAP1 and MetAP2 domains.

File S4 All previously identified Nats present in yeast and human.

File S5 A file containing the complete amino acid sequences of all the poplar proteins identified and their homologues in Arabidopsis.
Acknowledgments

We thank Prof. Li Li (Northeast Forestry University) for helpful discussions. We thank Hua-Hua Li and Wei Li at Northeast Forestry University for collection of samples.

Author Contributions

Conceived and designed the experiments: C-PY B-CW. Performed the experiments: C-CL H-YZ N-MD H-XW W-HL. Analyzed the data: C-CL H-YZ D-LN. Contributed reagents/materials/analysis tools: H-XW W-HL. Wrote the paper: C-CL C-PY B-CW.

References

1. Giglione C, Boulatov A, Meinmel T (2004) Protein N-terminal methionine excision. Cellular and Molecular Life Sciences 61: 1453–1474.
2. Polevoda B, Sherman F (2003) N-terminal acetyltransferases and sequence requirements for N-terminal acetylation of eukaryotic proteins. Journal of Molecular Biology 325: 395–622.
3. Martinez A, Traverso JA, Valot B, Ferro M, Espagne C, et al. (2008) Extent of N-terminal modifications in cytosolic proteins from eukaryotes. Proteomics 8: 2809–2831.
4. Ross S, Giglione C, Pierre M, Espagne C, Meinmel T (2005) Functional and Developmental Impact of Cytosolic Protein N-Terminal Methionine Excision in Arabidopsis. Plant Physiology 137: 625–637.
5. Giglione C, Sereer A, Pierre M, Boisson B, Meinmel T (2000) Identification of eukaryotic peptide deformylases reveals universality of N-terminal protein processing mechanisms. The EMBO Journal 19: 3916–3929.
6. Pesaresi P, Gardner NA, Masiero S, Dietmann A, Eichacker L, et al. (2003) Cytoplasmic N-terminal protein acetylation is required for efficient photosynthesis in Arabidopsis. Plant Cell 15: 1817–1832.
7. Liu CC, Liu BG, Yang ZW, Li CM, Wang BC, et al. (2012) Genome-Wide Identification and in Silico Analysis of Peptidic Peptide Deformylases. International Journal of Molecular Sciences 13: 5112–5124.
8. Giglione C, Vallon O, Meinmel T (2003) Control of protein life-span by N-terminal methionine excision. The EMBO Journal 22: 13–23.
9. Hwang CS, Shemorry A, Varshavsky A (2010) N-Terminal Acetylation of Cellular Proteins Creates Specific Degradation Signals. Science 327: 973–977.
10. Sereer A, Giglione C, Sardini A, Martinez-Sanz J, Meinmel T (2003) An unusual peptide deformylase feature in the human mitochondrial N-terminal methionine excision pathway. Journal of Biological Chemistry 278: 52951–52963.
11. Arnsten T (2009) Protein N-terminal acetylation. NAT 2007-2008 Symposia 2009. BioMed Central Ltd. 81.
12. Dan Van Damme P, Hole K, Pimenta-Marcues A, Helsens K, Vandekerckhove J, et al. (2011) NatF Contributes to an Evolutionary Shift in Protein N-Terminal Acetylation and Is Important for Normal Chromosome Segregation. PLoS Genetics 7(6): e1001269.
13. Polevoda B, Arnsten T, Sherman F (2009) A synopsis of eukaryotic N2-terminal acetyltransferases: nomenclature, subunits and substrates. 2009. BioMed Central Ltd. 82.
14. Gauhsi M, Just S, Miu A, Ross S, Rücknagel P, et al. (2003) The Yeast N2-Acetyltransferase NatA Is Quantitatively Anchored to the Ribosome and Interacts with Nascent Polypeptides. Molecular and Cellular Biology 23: 7483.
15. Polevoda B, Sherman F (2000) N-terminal acetylation of eukaryotic proteins. The Journal of biological chemistry 275: 36479–36482.
16. Polevoda B, Hoskins J, Sherman F (2009) Properties of Natf, an [alpha]-Acetylated Acetyltransferase from Saccharomyces cerevisiae That Modifies N-Termini of Histones H2A and H4. Molecular and Cellular Biology 29: 2913–2924.
17. Michel H, Hunt D, Shabanozij J, Bennett J (1998) Tandem mass spectrometry reveals that three photosystem II proteins of spinach chloroplasts contain N-acetyl-O-phosphophenylpeptide at their NH2 termini. The Journal of biological chemistry 263: 1123–1130.
18. Zhyballov B, Rutschow H, Fries G, Rudella A, Emanuelsson O, et al. (2008) Sorting Signals, N-Terminal Modifications and Abundance of the Chloroplast Proteome. PLoS One 3: 1–19.
19. Arnsten T, Van Damme P, Polevoda B, Helsens K, Sorensen R, et al. (2009) Proteomics analyses reveal the evolutionary conservation and divergence of N-terminal acetyltransferases from yeast and humans. Proceedings of the National Academy of Sciences 106: 8157–8162.
20. Tuskan G, Difazio S, Jansson S, Bohlmann J, Grigoriev I, et al. (2006) The Arabidopsis thaliana Chloroplast Proteome Reveals Pathway Abundance and Novel Protein Functions. Current Biology 14: 334–362.
21. Giglione C, Serero A, Meinmel T, Sherman F (2006) Impact of the N-terminal amino acid on N-terminal acetylation of yeast ribosomal proteins and its effect on protein synthesis. Journal of Proteome Research 5: 431–441.
22. Kleffmann T, Russenberger D, von Zychlinski A, Christopher W, Sjo¨lander K, et al. (2010) N[alpha]-Acetyltransferase NatA Is Quantitatively Anchored to the Ribosome and Interacts with Nascent Polypeptides. Molecular and Cellular Proteomics 8: 190–200.
23. Kariau M, Yasui K, Ino Y, Kamp R, Polevoda B, et al. (2010) N[alpha]-Acetylation of yeast ribosomal proteins and its effect on protein synthesis. Journal of Proteome Research 7: 2947–2955.
24. Van Damme P, Arnsten T, Gevaert K (2011) Protein alpha-N-acetylation studied by N-terminomics. FEBS Journal 278: 3832–3834.
25. Dorweyer W, Mohammad S, Breukelen B, Krijgsveld J, Heck AJR (2007) Targeted analysis of protein termini. Journal of Proteome Research 6: 4634–4645.
26. Sautilas N, Aletas A, Fu M, Aumann MM, Helbig AJ, Mohammed S, et al. (2009) Strong cation exchange-based fractionation of Lys-N-generated peptides facilitates the targeted analysis of post-translational modifications. Molecular & Cellular Proteomics 8: 190–200.
27. Jensen N (2004) Modification-specific proteomics: characterization of post-translational modifications. Nature Biotechnology 21: 253–261.
28. Lu CC, Lu G, Wang HX, Shen ZY, Yang CP, et al. (2011) Identification and analysis of phosphorylation status of proteins in dormant terminal buds of poplar. BMC Plant Biology 11: 158.
29. Larsen M, Thingholm T, Jensen O, Roepstorff P, Jørgensen T (2005) Highly Selective Enrichment of Phosphorylated Peptides from Peptide Mixtures Using Titanium Dioxide: Microcolumns*. Molecular & Cellular Proteomics 4: 873–886.
30. Varga A, Larsen MB, Helbig AJ, Mohammed S, et al. (2006) Novel protein phosphorylation site identification in spinach stroma membranes by titanium dioxide microcolumns and tandem mass spectrometry. Journal of Proteome Research 5: 973–982.
31. Van Damme P, Arnsten T, Gevaert K, et al. (2011) Protein alpha-N-acetylation studied by N-terminomics. FEBS Journal 278: 3832–3834.
32. Dorweyer W, Mohammad S, Breukelen B, Krijgsveld J, Heck AJR (2007) Targeted analysis of protein termini. Journal of Proteome Research 6: 4634–4645.
33. Sautilas N, Aletas A, Fu M, Aumann MM, Helbig AJ, Mohammed S, et al. (2009) Strong cation exchange-based fractionation of Lys-N-generated peptides facilitates the targeted analysis of post-translational modifications. Molecular & Cellular Proteomics 8: 190–200.
34. Kariau M, Yasui K, Ino Y, Kamp R, Polevoda B, et al. (2010) N[alpha]-Acetylation of yeast ribosomal proteins and its effect on protein synthesis. Journal of Proteome Research 7: 431–441.
35. Kleffmann T, Russenberger D, von Zychlinski A, Christopher W, Sjo¨lander K, et al. (2010) The Arabidopsis thaliana Chloroplast Proteome Reveals Pathway Abundance and Novel Protein Functions. Current Biology 14: 334–362.
36. Kleffmann T, Von Zychlinski A, Russenberger D, Hirsch-Hoffmann M, Gehrig P, et al. (2007) Proteome dynamics during plastid differentiation in rice. Plant Physiology 143: 912–923.
37. Bienvenut WV, Espagne C, Martinez A, Majeran W, Valot B, et al. (2011) Dynamics of post-translational modifications and protein stability in the stroma of Chlamydomonas reinhardtii chloroplasts. Proteomics 11: 1734–1750.
38. Meinmel T, Sereer A, Giglione C (2006) Impact of the N-terminal amino acid on N-terminal acetylation of yeast ribosomal proteins and its effect on protein synthesis. Journal of Proteome Research 5: 431–441.