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The pea seedling mitochondrial N^{ε}-lysine acetylome

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ABSTRACT

Posttranslational lysine acetylation is believed to occur in all taxa and to affect thousands of proteins. In contrast to the hundreds of mitochondrial proteins reported to be lysine-acetylated in non-plant species, only a handful have been reported from the plant taxa previously examined. To investigate whether this reflects a biologically significant difference or merely a peculiarity of the samples thus far examined, we immunoenriched and analyzed acetylated peptides from highly purified pea seedling mitochondria using mass spectrometry. Our results indicate that a multitude of mitochondrial proteins, involved in a variety of processes, are acetylated in pea seedlings.

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1. Introduction

Among the several hundred protein posttranslational modifications (PTMs), Lys acetylation (herein, Lys acetylation refers exclusively to Lys-N^{ε}-acetylation (KAc)) is notable for having been initially discovered some 50 years ago (Allfrey et al., 1964) and yet only recently has its prevalence been appreciated (Kim et al., 2006). The Lys acetyltransferases (KATs), enzymes responsible for catalyzing the transfer of the acetyl group from acetyl-CoA to the terminal amine of a Lys side chain, have been found in all taxa examined, and a minimum of four groups appear to be common to all eukaryotes (Pandey et al., 2002; Yuan and Marmorstein, 2012). Deacetylation can be performed by Lys deacetylases (KDACs), which are generally separated into three groups (Pandey et al., 2002). Members of the Rpd3/HDA1 group (type I/II) are inhibited by trichostatin A (Rao et al., 2007) and release acetate by hydrolyzing the acetyllysine terminal amide bond. The type III KDACs, known as sirtuins, transfer the acetyl group to NAD^{+}, producing nicotinamide and 2′-*O*-acetyl-ADP (Feldman et al., 2012). The third group, HD2, of which relatively little is known, appears to be specific to plants (Pandey et al., 2002).

In contrast to protein *O*-phosphorylation, which results in an electrically neutral residue becoming negatively charged, lysine acetylation neutralizes a positively charged residue, potentially leading to alteration of secondary and higher order protein structure. As a PTM of histones, KAc contributes to transcriptional activation by relaxing the electrostatic interactions with the DNA backbone (Korolev et al., 2007). Additionally, however, KAc provides a binding site for bromodomain-containing proteins, which notably include several KATs (Chen et al., 2010; Syntichaki et al., 2000), in a manner at least conceptually analogous to the interaction between *O*-phospho-Ser/Thr residues and the 14–3–3 scaffold proteins. Several instances of crosstalk between *O*-phosphorylation and KAc have been reported (Choudhary et al., 2009; Zhang et al., 2012; Zippo et al., 2009), and may be representative of a larger theme (van Noort et al., 2012; Yuan and Marmorstein, 2012).

Aspects of KAc that are only now being characterized in detail include the widespread distribution within the tree of life, and the relatively large number of proteins so modified. It has even been suggested that the extent of KAc might surpass even that of *O*-phosphorylation (Kouzarides, 2000; Lundby et al., 2012). Recent publications have reported many thousands of acetyl-proteins, many at multiple sites, located within all major subcellular compartments, and isolated from diverse taxa. Notable reports have described KAc of proteins from eubacteria (Zhang et al., 2009), archaea (Altman-Price and Mevarech, 2009), fungi (Henriksen et al., 2012), land plants (Finkemeier et al., 2011; Wu et al., 2011) and mammals (Choudhary et al., 2009).

Lys acetylation has been reported to be both a dynamic and an especially prevalent PTM of mammalian mitochondrial proteins (Still et al., 2013). A recent investigation of 16 tissues from rat describes over 2600 mitochondrial acetylation sites, roughly 20% of the total reported

Abbreviations: AmB, ammonium bicarbonate; ACN, acetonitrile; BSA, bovine serum albumin; CID, collision-induced dissociation; FA, formic acid; HCD, higher-energy collisional dissociation; IP, immunoprecipitation; KAc, lysine-N^{ε}-acetylation; KAT, lysine acetyltransferase; KDAC, lysine deacetylase; LC, liquid chromatography; MS, mass spectrometry; MS/MS, tandem mass spectrometry; PSM, peptide-spectrum match; PTM, posttranslational modification; Th, thomson; TFA, trifluoroacetic acid.

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(Lundby et al., 2012). Although there are several mitochondrial sirtuins (Michishita et al., 2005), no mitochondrial KAT has been reliably determined within either the matrix or the intermembrane space. This apparent paradox has led to suggestions that perhaps mitochondrial KAc is the result of non-enzymatic acetylation of a deprotonated Lys ε-amine, which would be accelerated by the elevated pH of actively respiring mitochondria (Wagner and Payne, 2013). While the relevance of non-enzymatic acetylation in vivo has not been established, several studies have raised alternative, but not mutually exclusive, possibilities for enzymatic acetylation of mitochondrial proteins. For example, the report of mitochondrial localization of GCN5L1, which, based on primary structure and genetic analyses, resembles a GNAT-family KAT (Scott et al., 2012) and of a GNAT-type KAT in *Toxoplasma gondii*, Elp3, which is tail-anchored to the mitochondrial outer membrane (MOM) (Stilger and Sullivan, 2013) comprise additional possibilities for the origin of mitochondrial KAc.

In contrast to results from investigations of fungal and metazoan mitochondria, when these experiments were undertaken, only 22 mitochondrial acetylation sites have been reported from plants, approximately 10% of all sites (Finkemeier et al., 2011; Melo-Braga et al., 2012; Wu et al., 2011). Possible explanations for this discongruity include the distinct physiologies and trophic strategies of plants, the particular tissues examined, and limitations of analytical techniques. To investigate this apparent disparity, we have analyzed, using immunoenrichment and extended chromatographic separations coupled with high-resolution tandem mass spectrometry (MS/MS), samples of highly enriched pea seedling mitochondria. In addition to results describing for the first time the occurrence of non-histone N^{ε}-lysine acetylation in garden peas, we present, to the best of our knowledge, the first large scale, and most complete, proteomic analysis of mitochondria from pea, an unsequenced legume (Smýkal et al., 2012).

2. Materials and methods

2.1. Reagents

Unless otherwise specified, all reagents were purchased from Sigma and used without further purification. Percoll was from GE Healthcare Life Sciences and TES (*N*-[tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid) from Research Organics.

2.2. Plant growth and purification of mitochondria

Pea (*Pisum sativum* L. cv Little Marvel) seeds were soaked overnight in running tap water, then germinated in a growth chamber (10 h photoperiod, 250 μE m^{-2} s^{-1}, 18 °C) and grown for 14–17 days before harvesting. Mitochondria were isolated and purified as previously described (Fang et al., 1987), with minor modifications. Everything was done at 4 °C. The homogenizing solution contained: 300 mM mannitol, 50 mM TES–KOH, pH 7.2, 1 mM EDTA, 1 mM $MgCl_2$, 0.2% BSA, 0.5% PVP-40, and 10 mM 2-mercaptoethanol. Green seedlings were homogenized at 0.5 g ml^{-1} using three 10-s bursts with a Braun blender modified to hold single edged razor blades. The homogenate was filtered through four layers of pre-wetted cheesecloth plus one layer of Miracloth, and then centrifuged at 3300 ×*g* for 5 min. The pellet was discarded and the supernatant centrifuged at 18,000 ×*g* for 20 min. The mitochondria-enriched pellet was resuspended in 50 ml of 300 mM mannitol, 20 mM TES–KOH, pH 7.2, 2 mM Na_2HPO_4, 1 mM EDTA, 2 mM $MgCl_2$, 0.1% defatted BSA, and 14 mM 2-mercaptoethanol, first using a fine camel's hair paint brush, then by three passes in a loose-fitting Potter homogenizer.

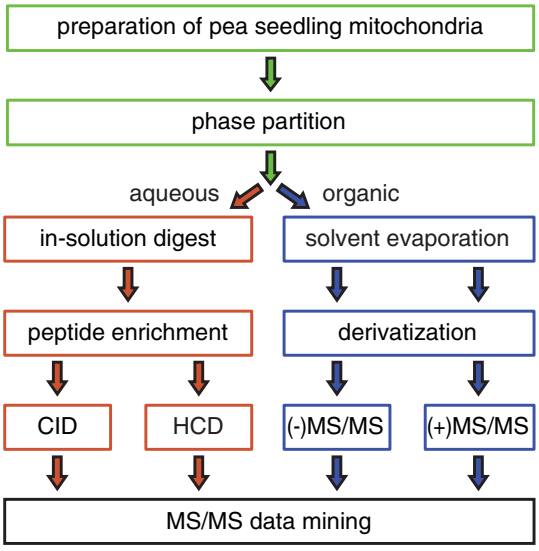
Percoll solutions (v/v) all contained 250 mM sucrose, 10 mM TES–KOH, pH 7.2, and 0.1% defatted BSA. Eight ml of resuspended mitochondria was loaded onto a discontinuous gradient consisting of 6 ml 21% Percoll, 12 ml 26%, and 10 ml 47%, then centrifuged at 65,000 ×*g* for 45 min. The mitochondria, which banded at the 26/47 interface, were collected, diluted 1:1 with 300 mM mannitol containing 20 mM TES, pH 7.2, 1 mM EDTA, 2 mM $MgCl_2$, 0.1% defatted BSA, and 2 mM DTT (RMA), loaded onto a second gradient consisting of 10 ml 26% and 10 ml 47% Percoll, and centrifuged at 45,000 ×*g* for 30 min. Pure mitochondria, which band at the 26/47 interface, were collected, slowly diluted 1:5 with RMA, and concentrated by centrifugation at 18,000 ×*g* for 30 min. The final pellet was resuspended in 2 ml of homogenization buffer, shell frozen, and stored at −70 °C until analyzed. A conceptual overview of the preparatory and analytical methods described herein is presented in Scheme 1.

Our results are based upon independent analysis of three biological replicates. Biological replication starts with isolation and purification of mitochondria from a separate group of pea seedlings. While not integral to our experimental design, each of the biological replicates described herein was prepared more than 30 days after its predecessor.

2.3. Protein delipidation, digestion, and peptide enrichment

Purified mitochondria were thawed on ice and brought to 5 ml with a solution of the KDAC inhibitors nicotinamide, 5 mM, and trichostatin-A, 1 μM, in 25 mM Tris–HCl, pH 6.8. Diluted suspensions were delipidated according to the method of Cham and Knowles (1976). Each of these was combined with two volumes of 40:60 (v/v) butanol:diisopropylether, vortexed briefly, and mixed for 30 min with rocking. Following centrifugation at ambient temperature for 5 min at 2000 ×*g*, organic phases were removed, aqueous phases added to four volumes of cold acetone, and left at −20 °C overnight. Protein precipitates were subsequently pelleted at 8 °C for 15 min at 10,000 ×*g*, washed once with cold acetone, and subjected to a final centrifugation for 5 min at 8000 ×*g*. Protein pellets were allowed to dry at room temperature, covered with 1 ml of 10 mM HEPES, pH 7.5, and stored at −20 °C until use.

Urea deionized with mixed bed resin was immediately either stored at −80 °C or used to make 1.25× resuspension buffer (7.5 M urea and 2.5 M thiourea in 12.5 mM HEPES, pH 8.0). Protein pellets were reconstituted in one volume 1× resuspension buffer and disulfides were reduced for 45 min with 5 mM DTT and alkylated for 30 min with 10 mM iodoacetamide. DTT was again added to 5 mM, total protein was quantitated using the EZQ kit (Molecular Probes), and samples were frozen at −20 °C until digestion.



Scheme 1. Flowchart of experimental design. Isolated mitochondria were phase partitioned to remove lipids for separate analysis. Results in the present study were obtained from immunoenriched and unbound fractions of proteins precipitated from the aqueous phase.