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Increasing phosphatidylinositol (4,5) bisphosphate biosynthesis affects plant nuclear lipids and nuclear functions

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Abstract

In order to characterize the effects of increasing phosphatidylinositol(4,5)bisphosphate (PtdIns(4,5)P₂) on nuclear function, we expressed the human phosphatidylinositol (4)-phosphate 5-kinase (*HsPIP5K*) 1 α in *Nicotiana tabacum* (NT) cells. The *HsPIP5K*-expressing (HK) cells had altered nuclear lipids and nuclear functions. HK cell nuclei had 2-fold increased PIP5K activity and increased steady state PtdIns(4,5)P₂. HK nuclear lipid classes showed significant changes compared to NT (wild type) nuclear lipid classes including increased phosphatidylserine (PtdSer) and phosphatidylcholine (PtdCho) and decreased lysolipids. Lipids isolated from protoplast plasma membranes (PM) were also analyzed and compared with nuclear lipids. The lipid profiles revealed similarities and differences in the plasma membrane and nuclei from the NT and transgenic HK cell lines. A notable characteristic of nuclear lipids from both cell types is that PtdIns accounts for a higher mol % of total lipids compared to that of the protoplast PM lipids. The lipid molecular species composition of each lipid class was also analyzed for nuclei and protoplast PM samples. To determine whether expression of *HsPIP5K*1 α affected plant nuclear functions, we compared DNA replication, histone 3 lysine 9 acetylation (H3K9ac) and phosphorylation of the retinoblastoma protein (pRb) in NT and HK cells. The HK cells had a measurable decrease in DNA replication, histone H3K9 acetylation and pRB phosphorylation.

Keywords

plant nuclei; phospholipids; histone; phosphatidylinositol (4,5) bisphosphate; plasma membrane

1. Introduction

The phosphoinositide (PI) pathway is important for many biological processes that affect plant basal metabolism and growth including vesicle trafficking, membrane structure, protein localization, enzyme activity, ion channel regulation, secondary messenger formation and organelle signaling [1–4]. The amount and cellular location of PI pathway lipids and/or enzymes respond to many internal and external cellular cues including heat stress, osmotic stress, cell expansion, cell type, differentiation, and cellular metabolism [1,5–14]. Altering the flux through the plant PI pathway by mutation of genes in the PI pathway,

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overexpression of genes or heterologous expression systems has been shown to affect plant growth and development [3,15–21]; however, the effects of PI metabolism on nuclear processes and nuclear lipids have not been studied in plants.

In yeast, animals, and plants, PI pathway enzymes or enzyme activities and their products have been found in the nucleus [9,12,22–29]. In animal cells, the nuclear localized PI pathway is regulated independently of the PM bound enzymes and lipids creating a novel intra-nuclear signaling mechanism composed of nuclear generated inositol phospholipids and inositol phosphates [5,9,26,30]. For example, the inositol phospholipid PtdIns(4,5)P₂ has a variety of effects on nuclear proteins and processes including, but not limited to, removal of histone H1 from chromatin *in vitro* [31], regulating multiple remodeling complexes similar to the SWI/SNF complex like Brahma associated factor (BAF) [32,33], regulating a novel polyadenylate polymerase [34,35], and interacting with other nuclear proteins like actin binding proteins [7,8]. In *D. melanogaster* the nuclear phosphatidylinositol (4)-phosphate 5-kinase (PIP5K), Skittles, is essential for embryogenesis, and when *skittles* is mutated, histone H1 is hyperphosphorylated and chromatin is compacted [22].

Another inositol phospholipid, phosphatidylinositol(5)phosphate (PtdIns5P), interacts with human INhibitor of Growth 2 (ING2) to regulate a variety of chromatin remodeling complexes [36]. Nuclear localization and activity of phosphatidylinositol-phospholipase C (PI-PLC) β1 was required for cell cycle progression as demonstrated by G2/M blockade by PI-PLC inhibitors [37] and the dominant negative activity of a mutant PI-PLCβ1 without a nuclear localization signal [38]. In animals and yeast many other PI pathway inositol phospholipids, inositol phosphates and enzymes have been shown to modify nuclear functions [9,32,33,39,40] including cell cycle progression [5,41–43], germline development [44], and cell polarity [45].

In contrast, the plant cellular and nuclear PI pathways are still being characterized. Early reports described plant nuclear lipids based on [³H]ethanolamine [5] and [³H] myo-inositol labeling as well as by measuring PIP5K activity with [γ -³²P]ATP labeling [24]. Further analysis of plant nuclei has shown specific nuclear lipid kinase activities including phosphatidylinositol 3-kinase (PI3K) [29] and phosphatidylinositol 4-kinase (PI4K) [46]. More recent data indicate that plant lipids, their modifying enzymes including PIP5Ks, and proteins with lipid binding domains will localize to the nucleus [12,47–53]. However, plant nuclear lipids are not well characterized, and to our knowledge, no one has studied the effects of increasing the flux through the nuclear PI pathway in plants.

We have employed a heterologous expression to assess whether increased cellular PtdIns(4,5)P₂ affected nuclear lipids and functions. Im et al. [54] had shown that N-terminal GFP tagged *Homo sapiens* phosphatidylinositol 4-phosphate 5kinase 1α (E.C. 2.7.1.68) (hereafter denoted *HsPIP5K1α*) localized to the PM of *Nicotiana tabacum* (NT) cells and increased PM PIP5K specific activity and steady state PM PtdIns(4,5)P₂ by 100-fold [54]. *HsPIP5K1α* is known to localize to both the PM and the nucleus in human cell lines [11]. Although in previous studies GFP fluorescence from expressed GFP-*HsPIP5K1α* was primarily detected in the plasma membrane of *HsPIP5K1α*-expressing (HK) cells [54], we hypothesized that expressing *HsPIP5K1α* would affect the plant nuclear PI pathway as well as the plasma membrane PI pathway. We show that expressing *HsPIP5K1α* increased nuclear PIP5K activity and PtdIns(4,5)P₂. Isolated HK nuclei also have altered phospholipid composition compared to NT nuclei. Nuclei were isolated from protoplasts, and the analyzed protoplast PM and nuclei showed distinct differences in lipid classes in the NT and HK cell lines. We also assessed the effects of expressing *HsPIP5K1α* on several nuclear functions. DNA replication measured by Bromodeoxyuridine (BrdU) incorporation, histone

acetylation on histone 3 lysine 9 and phosphorylation of the retinoblastoma protein (pRb) were all decreased in HK cells.

2. Methods

2.1 Protoplast isolation

Cells were harvested 4 d after transfer by gravity filtration using a 75 μm filter. Cells with a fresh weight (fw) between ~0.7 g to 1.6 g fw per 25 mL of medium were used to produce protoplasts for plasma membrane and nuclei isolation. To digest the cell wall, cells (0.7 to 1 g fw) were incubated at room temperature (RT) with shaking at 75 rpm in a 10 $\times$ 10 mm plastic Petri dish for 1 ½ to 2 h in 10 mL of cell wall digestion buffer (0.4 M sorbitol in 10 mM MES (pH 5.6) with 7.5 mg.mL⁻¹ cellulase and 0.1 mg.mL⁻¹ pectolyase). Cell wall digestion was monitored microscopically and was stopped by addition of 10 mL protoplast wash buffer (PWB: 0.4M D-sorbitol in 10 mM MES (pH 5.6)) when round protoplasts were evident and the remaining cells would release protoplasts when gentle pressure was applied to the microscope cover slip. To remove remaining cells and debris, the protoplasts were filtered through a 75 μm filter followed by 2 rinses with 10 mL of PWB and collected in the 50 mL Falcon tube. Filtered protoplasts were centrifuged at 100 *g* for 5 min. Each protoplast pellet was washed 2 times with 10 mL of PWB by gentle resuspension in PBW followed by centrifugation at 100 *g*.

2.2 Nuclei Isolation

Nuclei were isolated immediately from protoplasts. Protoplasts were incubated on ice for 5 min in PWB, the supernatant was removed with a Pasteur pipette, and protoplasts gently resuspended in 6 mL of ice cold nuclear isolation buffer 1 (NIB1) (0.7 molal D-sorbitol HB (5 mM HEPES, 10 mM MgCl₂, 2 mM EGTA, pH 7) and incubated for 5 min on ice. Protoplasts were centrifuged at 100 *g* for 5 min at RT and returned to ice promptly. NIB1 was removed and protoplasts were gently resuspended in 6 mL of ice cold NIB2 (0.2 molal D-sorbitol HB, 0.025% Triton X-100, 1 $\mu\text{g.mL}^{-1}$ leupeptin, 100 μM PMSF (phenylmethanesulfonyl fluoride) and incubated at least 5 min on ice. Protoplasts in NIB2 were passed through a 26 gauge double-sided needle fitted with 2, 10 mL syringes with rubber tipped plungers, 6 to 7 times. Broken protoplast mixture was filtered through 75 μm mesh and two layers of nylon mesh (150 μm) and rinsed through 2 times with 6 mL of ice cold NIB3 (NIB2 without Triton X-100) and collected into new 50 mL Falcon tubes. The filtered solution was centrifuged 5 min at 100 *g* at RT. A Pasteur pipette was used to carefully remove all the supernatant so as to not disturb the loose pellet. The pellet was gently washed with 6 mL of ice cold NIB3, centrifuged at 100 *g* for 5 min, supernatant was removed with a Pasteur pipette and the isolated nuclei were kept on ice for further procedures.

2.3 Plasma membrane isolation from protoplasts

For plasma membrane isolation, protoplasts were ground 25–30 times in a Dounce ground glass homogenizer on ice and centrifuged at 3,000 *g* for 10 min at 4° C to remove unbroken nuclei and other organelles. The supernatant was centrifuged at 40,000 *g* for 1 h to recover a microsomal pellet as previously described [54]. A plasma membrane-enriched fraction was isolated from the microsomal pellet by aqueous two-phase partitioning as previously described [55].

2.4 Protein quantification

Plasma membrane and nuclei protein was quantified using bicinchoninic acid (BCA) protein assay (microBCA kit from Pierce, Rockingham, IL) with BSA as a standard.

2.5 PtdIns(4,5)P₂ mass measurement

Aliquots of nuclei containing at least 0.5mg of nuclear protein were added to 20% cold PCA (perchloric acid) in a 1:1 v/v ratio. Lipids were extracted from PCA precipitate, the headgroup was hydrolyzed, and Ins(1,4,5)P₃ measured using the Ins(1,4,5)P₃ binding assay (Amersham Inc.) as previously described [56].

2.6 Lipid Kinase Assay

Nuclei were assayed for PIK, PIP5K and DAGK activity with 10 µg of protein for 10 min using the conditions previously described for plasma membrane and microsome activity assays [54,55], with one modification. The ATP concentration was increased to 100 µM with 74 kBq of [γ -³²P]ATP .nmole⁻¹ total ATP per assay because of competing reactions in the nuclear preparations. When exogenous substrate was added to the reaction mixture, the final concentration was 125µM in 0.01% Triton X-100. To monitor the effects of increasing ATP, the specific activity of the [γ -³²P]ATP was held constant (74 kBq.nmole⁻¹ ATP); the reaction was incubated for 30 min and 20 µg of protein was used per assay (Supplemental Figure 2). While 100 µM ATP was not found to be saturating, in the interest of safety we did not use higher concentrations of ATP but rather lowered the amount of protein used for the subsequent kinase assays. The reactions were stopped and lipids were extracted and separated on Partisil LK5D TLC plates (Whatman Inc., Maidstone, England) with chloroform (CHCl₃): methanol (MeOH): concentrated ammonia (NH₄OH):water (90:90:7:22, v/v/v/v) as a solvent as previously described [54]. Incorporation of ³²P was monitored with autoradiography, quantified using a BioScan Imaging scanner and reported as pmol.mg⁻¹.min⁻¹ and % of Total radioactive counts.

2.7 Lipid profiling

Lipids were isolated from 45 to 213 µg of plasma membrane protein and 50 to 4000 µg of nuclei protein using the protocol recommended at the Kansas State Lipidomics Web site for animal tissue samples (<http://www.k-state.edu/lipid/lipidomics/animal-lipid-extraction.htm>). Plasma membrane and nuclei samples were brought up to between 250 and 500 µL in volume with Tris buffer or NIB3, respectively. Briefly, volume of nuclei or plasma membrane used for lipid isolation was set as 0.8 part, and 1 part of CHCl₃ and then 1 part of MeOH were added and the solution was vortexed immediately. An additional 1 part of CHCl₃ was added to the mixture and vortexed briefly. The mixture was centrifuged at 1000 *g* for 10 min. The lower, CHCl₃ layer was removed and placed in a clean test tube. The remaining upper layer was extracted 2 additional times by adding 1 part of CHCl₃ followed by a 5 min centrifugation at 1000 *g*. The CHCl₃ lower phase was removed after each centrifugation. The combined CHCl₃ fraction was back-washed with 0.1 mL of 1M KCl by brief vortexing followed by centrifugation at 1000 *g* for 5 min. The top, aqueous KCl layer was removed. The remaining CHCl₃ fraction was further washed with 0.1 mL H₂O. After the H₂O wash the CHCl₃ layer was removed to a clean test tube and the remaining H₂O was re-extracted with 1 part CHCl₃. The combined CHCl₃ solution was stored at -20°C for up to 1 week. CHCl₃ extractions from nuclear and plasma membrane lipids from the same day were dried under a stream of N₂ on the same day and stored under N₂ at -20°C. Lipids were shipped on dry ice or ice to the Kansas Lipidomics Center for analysis.

2.8 Analysis of lipid profile

Lipids were divided into 11 lipid classes by headgroup (digalactosyldiacylglycerol (DGDG), monogalactosyldiacylglycerol (MGDG), lysophosphatidylcholine, (LysoPtdCho), phosphatidylglycerol (PtdGro), lysophosphatidylethanolamine (LysoPtdEtn), phosphatidylinositol (PtdIns), phosphatidic acid (PtdOH), phosphatidylethanolamine (PtdEtn), phosphatidylcholine (PtdCho), lysophosphatidylglycerol (LysoPtdGro),

phosphatidylserine (PtdSer)) and each lipid class was divided into lipid molecular species by the headgroup and the fatty acid contents (ie. PtdIns 34:1, PtdGro 32:0). The lipid classes of each sample were screened with both the Q test and Grubbs test, and outlier samples for each lipid classes were removed (Supplemental Table 1). Lipid class and lipid molecular species data were analyzed by pair-wise two tailed Student T-tests with the assumption of equal variance similar to previously reported analysis [57] (Supplemental Table 2). Comparisons were made between the NT and HK lipid samples within each sample type (e.g. comparison of NT and HK nuclear lipid analysis) and between different sample types (i.e. PM to nuclei comparison from NT cells). For plasma membrane analysis, five different samples from 3 different biological replicates were averaged. For nuclear lipid analysis, 10 wild type samples from 7 different biological replicates and 12 HK samples from 8 different biological replicates were harvested.

2.9 BrdU labeling

Cells were harvested by gravity on a 75 μ m filter. 0.1g of cells from flasks with similar weights (example flasks with fw between 0.7 to 1.2 g) were placed in 25mL of the same conditioned media (media in which the cells had been growing) and allowed to equilibrate for 30 minutes. BrdU (10 μ M) was added for either 30 min or 1 h at RT at 75 rpm shaking. The cells were harvested by suction filtration to remove media, and divided into 50 μ g aliquots before freezing. DNA was extracted from 50 μ g of cells with Qiagen Plant Genomic DNA extraction kit. DNA concentration was measured and the samples were diluted so that the DNA concentration was equivalent for all cell lines. Three aliquots of increasing amounts of DNA were spotted onto nitrocellulose, cross-linked and the BrdU labeled DNA was detected using a BrdU specific antibody (Sigma, Inc.) and appropriate secondary antibody as previously described [58] and supersignal chemiluminescence (Pierce, Rockingham, IL).

2.10 Western Blot Analysis

Cells were harvested by gravity filtration 4 d after transfer and frozen at -80°C . Nuclear protein was isolated as described above and boiled in SDS-PAGE loading buffer.

2.10.1 Calreticulin and H^{+} -ATPase immunoblots—Nuclei proteins (10 μ g) were separated on 8% SDS-PAGE for H^{+} -ATPase or 10% SDS-PAGE for calreticulin (CRT). Protein was transferred to PVDF membrane with 1x CAPS buffer. H^{+} -ATPase, a marker for plasma membrane, was detected using a polyclonal primary antibody (described in [59]). A polyclonal antibody to the maize CRT was used to detect CRT as a marker for ER as previously described [54]. The antibodies were visualized using anti-rabbit secondary antibody and super signal chemiluminescence (Pierce, Rockingham, IL, according to the manufacturer's directions).

2.10.2 Histone immunoblots—To enrich protein isolations for histones, proteins were acid extracted from frozen cells by grinding 25–30 times in a ground glass homogenizer (10 mM Tris-HCl, pH 7.5, 2mM EDTA, 0.25 M HCl, 5 mM 2-mercaptoethanol, 0.2 mM PMSF), precipitated with 25% TCA and washed 2x with ice cold 100% acetone as described by Tariq et al. (2003)[60]. The dried acetone protein pellets were resuspended in 5 % SDS overnight and protein quantified by BCA assay.

Proteins were separated by 15% SDS-PAGE and transferred to PVDF membranes with Tris-glycine transfer buffer. H3 lysine 9 acetylation (H3K9ac) and H3 were detected using primary antibodies to H3K9ac (Upstate, 07-352) and total H3 antibody (Abcam, ab1791) and were visualized with supersignal chemiluminescence (Pierce, Rockingham, IL). H3K9ac was compared to total H3 to ensure equal histone loading. ImageJ [61] was used to

calculate a ratio of the H3K9 acetylation intensity to the H3 intensity of the same, reprobed blot. The NT H3K9ac /H3 ratio was normalized to 100% to compare HK samples to NT samples.

2.10.3 Retinoblastoma (pRb) immunoblots—Protein was isolated from frozen cell samples by grinding 25–30 times in a ground glass homogenizer in buffer containing (25 mM TRIS-HCl pH 7.6, 15 mM MgCl₂, 15 mM EGTA, 75 mM NaCl, 60 mM β -glycerophosphate, 1 mM dithiothreitol (DTT), 0.1% NP-40, 0.1 mM Na₃VO₄, 1 mM NaF, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 mg.mL⁻¹ protease inhibitors (Complete, Roche, Mannheim, Germany)) as in Abraham et al. [62]. Proteins were separated by 10% SDS-PAGE and transferred to PVDF membranes with Tris-glycine transfer buffer. *Nicotiana tabacum* phosphorylated pRb protein was detected using human polyclonal antibody (Phospho-Rb (Ser807/811) Antibody #9308, Cell Signaling Technology) and visualized with supersignal chemiluminescence (Pierce, Rockingham, IL).

3. RESULTS

3.1 Nuclei isolated from the NT and HK cells appear similar using light microscopy

We hypothesized that expression of *HsPIP5K1 α* in NT cells would affect the PIP5K activity in the nucleus. To test this hypothesis, we first isolated nuclei from NT and HK cells by rupturing protoplasts. Nuclei from both NT cells and HK cells have similar size (Figure 1). The HK cells contained more noticeable starch granules and these co-purify with nuclei (Figure 1). Visible starch granule contamination could be reduced by applying nuclei to Percoll gradients; however, recovered nuclei were less abundant and visually less stable so we did not use gradient purification.

To assess the purity of nuclear preparations we detected plasma membrane contamination using antibodies to the plasma membrane H⁺-ATPase and ER contamination using antibodies to maize calreticulin (CRT) (Supplemental Figure 1A). There was no H⁺-ATPase detected in either the HK or NT nuclei fractions indicating little if any PM contamination. The ER is contiguous with the outer nuclear envelope and invaginations and ER associated proteins have been found within the nucleus [63–65]. Calreticulin (CRT), an ER associated protein, has been found associated with nucleus and outer nuclear envelope by fluorescent protein localization [66–68]. A small amount of CRT was detected in nuclei isolated from both HK and NT cells. The relative amount of CRT recovered increased in nuclei isolations applied to Percoll gradients, where the nuclei were disrupted, and as noted above, these preparations were not used for further experiments (Supplemental Figure 1B).

To assess plastid contamination and determine whether the increased starch granules resulted in increased plastid membrane contamination the lipids, MGDG and DGDG were compared between the two nuclei preparations. As shown in Figure 2 there was no difference in total MGDG or DGDG lipid class from nuclei isolated from the HK and NT cell lines even though the HK line had significantly higher number of starch granules. For comparison MGDG and DGDG were also assayed in the protoplast PM and increased MGDG, but not DGDG, was found in HK protoplast PM. This is in contrast to previous analysis of cell PM which showed decreased MGDG and DGDG in HK cells compared with NT cells [54] and may reflect differences in the plasma membrane lipids after cell wall digestion.

3.2 HK nuclei had increased PtdIns(4,5)P₂ and PIP5K activity

3.2.1 HK nuclei had increased PtdIns(4,5)P₂ based on mass 0measurements—

Mass measurements indicated a steady state increase in PtdIns(4,5)P₂ in the HK nuclei. HK

nuclei had 32.4 ± 6.2 pmol PtdIns(4,5)P₂.mg⁻¹ protein, while a NT nuclei (at least 0.5 mg of protein) did not have detectable PtdIns(4,5)P₂ by mass measurement (Figure 3). Because the PtdIns(4,5)P₂ in the NT nuclei was below the limits of detection with this assay method, we used in vitro PIPK assays to measure the potential to synthesize PtdIns(4,5)P₂ from PtdInsP.

3.2.2 HK nuclei have increased PIP5K activity—PIP5K activity assays were performed using isolated nuclei from HK and NT cells. Using endogenous substrate and 100μM ATP, both NT and HK nuclei had diacylglycerol kinase (DAGK), PtdIns kinase (PIK) and PIPK activity. The HK nuclei produced about 2-fold more [³²P]PtdInsP₂.mg⁻¹ protein compared with NT nuclei. (Figure 4A). The increase is similar in fold-differences to what was reported for plasma membranes from the HK cells with endogenous substrate [54]. Unlike the plasma membrane; however, the PIPK activity did not increase significantly in the nuclei preparations when exogenous substrate (PtdIns4P 125 μM) was added. The lack of detectable increase may be because there were so many competing reactions in the nuclei that used the ATP and PtdIns4P. In support of this hypothesis, there tended to be less [³²P]PtdInsP₂ and more [³²P]PtdOH recovered from HK nuclei when exogenous PtdIns4P was added (Figure 4). Furthermore, when PtdIns was added to the isolated nuclei, [³²P]PtdInsP₂ increased significantly suggesting that the enzyme preferred endogenously synthesized PtdInsP and that there may be a concerted reaction from PtdIns to PtdInsP₂ (Supplemental Figure 3).

3.3 Comparison of lipid classes and lipid molecular species between NT and HK nuclei and protoplast PM lipids

3.3.1 Nuclear lipid classes were different in the NT and HK lines—Nuclear lipids were extracted from NT and HK nuclei and analyzed by Kansas Lipidomics Research Center Analytical Laboratory as previously described [57]. Analysis of NT and HK nuclear lipids revealed significant differences in 5 out of 11 lipid classes (mol %, p-value of 0.05) (Figure 5 A). HK nuclear lipids had significantly decreased mol % of PtdOH, LysoPtdCho and LysoPtdEtn and increased PtdSer and PtdCho compared to NT nuclei. Even though the HK nuclei had high production of PtdInsP₂, the mol % PtdIns was not significantly different.

3.3.2 Changes in lipid molecular species of NT and HK nuclear lipids—The 11 lipid classes analyzed can be subdivided into 153 unique lipid molecular species that have different fatty acid tail/headgroup combinations. Over 25% of the lipid molecular species (47 out of 153) showed significant changes between NT and HK nuclei at p-value of 0.05 (Supplemental Table 2). Half of the significantly changed lipid molecular species (28) were changed between NT and HK nuclei at a p-value of 0.01. In HK nuclei PtdCho 36:1, PtdIns 36:1 and PtdSer 36:1 increased about 2-fold on a mol % basis and PtdCho 32:0 and PtdOH 32:0, LysoPtdCho 18:2 and LysoPtdEtn 18:2 decreased over 2-fold on a mol % basis compared with NT nuclei. Many of the changes in lipid molecular species with similar fatty acid composition (carbon content: double bond number (i.e. 36:1)) had a similar direction and amount of change in the NT and HK nuclei comparison regardless of lipid molecular species headgroup. For example the lipid molecular species PtdCho 36:1 and PtdIns 36:1 have different headgroups, but both lipid molecular species increased about 2-fold based on mol % in HK nuclei compared with NT nuclei.

In general, the unsaturated fatty acids increased and saturated fatty acids decreased in HK nuclei. Many of the lipid molecular species with unsaturated fatty acids increased in HK nuclei compared to NT nuclei with a p-value of less than 0.01. Out of 10 lipid molecular species with saturated fatty acids that were measured, 7 decreased at least 1.5 times on a mol % basis in HK nuclei compared to NT nuclei. Most lipid molecular species derived directly

from plastid fatty acids (16:0-16:0 fatty acid profile) decreased in HK nuclei, suggesting a shift in the origin of the fatty acids for lipid molecular species. The only plastid fatty acid lipid molecular species that increased in the HK nuclei was PtdIns 32:3, which increased 15 fold on a mol % basis, but is found in trace amounts compared to other PtdIns lipid molecular species composed of 16:0 fatty acids.

3.3.3 Analysis of lipid classes in nuclei and protoplast PM—PtdIns(4,5)P₂ has been characterized as a signaling lipid, and is known to change the curvature of a lipid membrane [2]. Because *HsPIP1α* localizes primarily to the PM and resulted in a 100-fold steady state increase in PtdIns(4,5)P₂ from the PM of cells [54] and because we had to first isolate protoplasts in order to obtain intact nuclei, we wanted to characterize the impact of high PtdIns(4,5)P₂ on the protoplast PM lipid composition. We compared 11 lipid classes from both nuclei and protoplast PM samples from both NT and HK cell lines (Figure 5). Molecular percentages (mol %) of the total lipids recovered of each lipid classes were compared between different nuclei samples and different protoplast PM samples as well as comparison of nuclei to protoplast PM lipids of each cell type.

3.3.4 Comparison of protoplast PM lipid classes between NT and HK cells—Total LysoPtdEtn decreased 5 fold on a mol % basis in HK protoplast PM (Figure 5B) and MGDG increased 1.4 fold on a mol % basis compared with NT protoplast PM (Figure 2). LysoPtdEtn is the only lipid class that was decreased significantly in both protoplast PM and nuclei of the HK protoplasts. Notably, LysoPtdEtn is a lipid with an inverted cone-shaped structure resulting from the loss of one fatty acid while PtdIns(4,5)P₂ has a cone shape because of the large polar headgroup [2,69], so the decrease in LysoPtdEtn may reflect a compensatory mechanism to maintain membrane structure.

3.3.5 Comparison of protoplast PM lipid molecular species between NT and HK cells—The PM of HK protoplasts had 25 lipid molecular species significantly changed from PM of NT protoplasts (Supplemental Table 2). Ten lipid molecular species increased in the HK protoplast PM compared with NT protoplast PM. An increase of 1.5 times or more on a mol % basis was seen in HK protoplast PM PtdCho 36:1 and PtdEtn 36:1 and 7 other lipid molecular species. 15 lipid molecular species decreased in the HK protoplast PM compared with NT protoplast PM. These included a decrease of PtdCho 32:0 of 1.5 fold on a mol % basis in HK protoplast PM and a decrease of 3 fold on a mol % basis in PtdEtn 32:0.

3.3.6 Common and unique changes are found between nuclei and protoplast PM lipids in both NT and HK samples—Lipids in the nucleus and the plasma membrane originate from plastids and the endoplasmic reticulum and changes in the trafficking between the ER and plastids or to either the nucleus or PM could affect the lipid composition. PtdIns(4,5)P₂ is an important molecule for signaling and trafficking, and has been shown to cause changes in cellular trafficking and membrane lipid composition. We have shown that changes in cellular PtdIns(4,5)P₂ can also alter the lipid composition of nuclei and protoplast PM. To further understand how the nuclear lipids and protoplast PM lipids were affected by expression of the *HsPIP1α*, we compared the nuclear and plasma membrane lipids of protoplasts within each cell line.

3.3.7 Comparison of lipid classes between protoplast PM and nuclei for each cell line—Both NT and HK cell lines showed similar differences between the protoplast PM and nuclear lipid profiles. The nuclear MGDG was 1.4 fold lower on a mol % basis and nuclear PtdIns, LysoPtdEtn and PtdOH were over 1.5 fold higher on a mol % basis compared to the protoplast plasma membrane in both cell lines (Figure 5 C, D). The cell

lines also had distinct differences in the lipid comparisons between protoplast PM and nuclei. The NT nuclear samples had significantly more PtdGro and less PtdCho compared to the protoplast PM (Figure 5C) and the HK nuclei had more LysoPtdCho compared to the HK PM (Figure 5D).

3.3.8 Comparison of nuclei and protoplast PM lipid molecular species for both NT and HK cells—Analysis of NT and HK nuclei to protoplast PM lipid molecular species revealed 40 common changes at p-value of 0.05 (Supplemental Table 2). Nuclei had 23 lipid molecular species that account for increased mol % compared to the protoplast PM from both cell lines, including LysoPtdCho 18:2, LysoPtdEtn 18:2, and PtdCho 34:1. NT and HK nuclear lipids have 17 lipid molecular species accounting for a decreased mol % when compared with protoplast PM samples, including PtdIns 32:1 and PtdEtn 32:1.

NT samples had 16 distinct lipid molecular species that were different in the nuclei compared with protoplast PM, including 6 were over 1.5-fold higher on a mol % basis like PtdIns 32:1 and 7 that were over 1.5-fold lower on a mol % basis like PtdSer 40:2 (Supplemental Table 2). HK nuclei had 12 distinctly different lipid molecular species including 4 that were over 1.5-fold higher on a mol % basis e.g., PtdSer 38:2, and 8 that were over 1.5-fold lower on a mol % basis e.g., LysoPtdGro18:2 (Supplemental Table 2).

A comparison of nuclear and protoplast PM lipids showed distinct changes in NT and HK cell lines, but also show common lipid profiles characteristic of the nucleus and PM in both cell lines. Interestingly, trace amounts of very long chain fatty acid lipids with fatty acid composition 42-carbon in PtdEtn and PtdSer and 44-carbon in PtdSer were observed in the nuclei suggesting that, like yeast [70], the plant nuclei may employ very long chain fatty acids for specialized functions (These very long chain fatty acids were previously described in plants [71]). Our lab had previously analyzed PM lipids isolated from cells of the NT and HK lines [54]. To gain insight into the effects of the cell wall digestion process on PM lipid composition of cells with high PtdIns(4,5)P₂, we compared our current protoplast PM data with the previously published cell PM data [54].

3.4 Comparison of lipid classes and lipid molecular species between protoplast and cell PM

3.4.1 Common and cell line specific changes in lipid classes during cell wall digestion of NT and HK cells—Not surprisingly, the cell PM from both NT and HK cell lines had similar changes in lipid classes after cell wall digestion (Supplemental Table 2). Significant changes in the total mol % of PtdCho, PtdEtn and PtdSer are found in both NT and HK protoplast PM lipids compared with cell PM lipids at a p-value of 0.05. For example, compared to the PM from cells, protoplast PM PtdCho was over 2-fold higher on a mol % basis, PtdEtn was over 2-fold lower on a mol % basis and PtdSer was over 9-fold lower based on mol % in the protoplast PM from both NT and HK lines.

Cell line specific changes are also detected, for example, NT protoplast PM had decreased DGDG (a 1.6-fold decrease in mol % basis) and PtdIns (3-fold decrease in mol %) compared to NT cell PM. In comparison, HK protoplast PM MGDG is 1.5-fold higher on a mol % basis compared to the HK cell PM.

3.4.2 Comparison of lipid molecular species from cell and protoplast PM—Differences between cell and protoplast PM lipid samples are also reflected in changes in composition of lipid molecular species. Comparison of protoplast PM and cell PM revealed 27 lipid molecular species changes in common between NT and HK samples (Supplemental Table 2). Increases of over 1.5-fold on a mol % basis were found in 7 lipid molecular species in protoplast PM, e.g. PtdEtn 34:1 and PtdCho 34:1, and decreases of 1.4 fold on a

mol % basis were observed in 20 lipid molecular species in the protoplast PM, e.g. PtdEtn 34:2 and PtdSer 34:2.

3.5 Effects of increased PtdIns(4,5)P₂ on nuclear functions

Animal nuclear lipids have been implicated in multiple nuclear processes [7,8,26]. We have shown increased PtdIns(4,5)P₂ and changes in the nuclear lipid profile in HK nuclei, and we wished to assess the effect of the cellular and nuclear lipid changes on other nuclear processes including DNA synthesis, chromatin modification and cell cycle regulation proteins.

3.5.1 BrdU incorporation is less in HK cells—We compared BrdU incorporation into DNA in the NT and HK cells to assess the effect of lipid changes on DNA synthesis. Cells were incubated in equal amounts of BrdU, DNA isolated and three concentrations of DNA spotted on membranes for immunoblot detection of BrdU incorporation. Dot blot analysis of NT and HK BrdU containing DNA after incubation with BrdU showed significantly less BrdU incorporation in HK DNA (Figure 6A). Blot signals were normalized by dividing the intensity of an individual measurement (i.e. NT day 4 lowest DNA concentration) by the average of all three day 4 NT and HK samples to normalize the samples for the blot intensity. Normalized data for each data measurement including over three biological replicates of cell labeling experiments were averaged for NT and HK cells at three different DNA concentrations and plotted along with their standard error (Figure 6B). Significant changes at p-value of 0.05 were detected at the lower two DNA concentrations. Although the chemiluminescent signal saturated at the highest DNA concentrations, the average HK BrdU intensities were always lower than NT intensities at every concentration tested. Similar studies were done on cells at day 2 after transfer when DNA synthesis would be highest. The same trend of HK cells having lower BrdU incorporation compared to NT cells was observed, but a statistically significant difference was not detected.

3.5.2 H3K9ac is decreased in HK cells—Histone acetylation is an integral part of the histone modification code. Previous papers have shown that lipids can effect chromatin remodeling complexes [72] including the Spt-Ada-Gcn5-Acetyltransferase (SAGA) complex which modifies H3K9 with acetylation [73,74]. We employed antibodies specific to acetylated H3K9 (H3K9ac) and reprobed the same blot with antibodies to total H3. Figure 7A shows a representative blot probed with both H3K9ac and H3 antibodies. HK cells have decreased H3K9ac compared to NT cells (arrow Figure 7A). To average multiple blots the ratio of the H3K9ac/total H3 was quantified, and the H3K9ac/H3 ratio of NT samples were normalized to 100%. Figure 7B shows the average relative ratio of H3K9ac/H3 from 3 blots for HK cells compared to normalized NT cells. All blots showed a decreased H3K9ac to H3 ratio for HK cells compared with NT cells.

3.5.3 Retinoblastoma protein (pRb) phosphorylation is decreased in HK cells—Total protein was isolated from NT and HK cells, and western blot analysis with the Ser 807/811 specific antibody (Phospho-Rb (Ser807/811) Antibody #9308) was performed as previously described [62]. Western blots showed decreased pRb phosphorylation (Figure 8 arrow) compared with stained total protein in two different protein isolations. Non-specific bands between 100 and 50 kDa increased and may simply result from non-specific cross reactivity with phosphoproteins in the HK lines or from increased proteolysis of the phosphorylated pRb protein. In either event there was less recovered full-length phosphorylated pRb.

4. Discussion

Expression of *HsPIP5K1α* in NT suspension cell culture increased PM PIPK specific activity [54]. We show here that the total nuclear PtdIns(4,5)P₂ recovered from isolated nuclei increased from levels that were below the limits of detection to 32.4 ± 6.2 pmoles.mg⁻¹ of nuclear protein in the *HsPIP5K1α* expressing cells. Furthermore, the nuclear PIPK activity increased. There was 2-fold higher PIP5K activity in HK nuclei compared with NT nuclei with endogenous lipids as substrate. In the in vitro assays, [³²P]PtdInsP₂ increased even more when exogenous PtdIns was added to the reaction (Supplemental Figure 3) but not when PtdIns4P was added (Figure 4). These data suggest that there was a concerted reaction from PtdIns to PtdInsP₂ or that the exogenously added PtdIns4P was degraded by phosphatases or lipases in the nuclei before it could be phosphorylated by PIPK. The fact that PtdOH increased significantly when PtdIns4P was added to the HK nuclei suggests that the PtdIns4P was being converted to DAG.

Of interest, there was no decreased in mol % PtdIns in the HK nuclei suggesting that the cells were compensating for the increase in PtdInsP₂ biosynthesis by increasing PtdIns production. The changes in the lipid molecular species of the PtdIns (increased unsaturation) in the HK nuclei is also consistent with a change in flux in PtdIns biosynthetic pathways [14,75].

Nuclei were isolated in the presence of 0.025% Triton X-100 and markers for ER showed only minor contamination. The detection of CRT in nuclear protein from intact nuclei is to be expected as it has been found in nuclear fractions [67] and the ER is contiguous with the outer nuclear envelope [63,76,77]. A general increase in CRT has been reported for HK cells [54] so an increase in ER contamination in the HK nuclei would have increased the CRT significantly. Importantly, western blots indicated that the CRT in the HK nuclei was not significantly higher than that in the NT nuclei samples.

A major visual difference in the nuclei isolated from HK cells was the increase in cosedimenting starch grains. We concluded that the starch granules did not contribute to the changes in lipid composition for the following reasons: Vasanthan and Hoover et al. [78] showed that isolated dry starch granules did not yield significant lipids using an extraction method with a chloroform/methanol extraction similar to our method [78,79]. If we had plastid contamination in HK nuclei, the HK nuclear lipid analysis would have shown an enrichment of DGDG and MGDG lipid classes [79]. We did not detect significant differences in DGDG and MGDG in the nuclei samples between NT and HK cells.

PtdIns(4,5)P₂ and PtdIns5P are important lipids in nuclear functions in animal cells [11,80]. A transient increase in PtdIns(4,5)P₂ and/or an unregulated, continuous increase in PtdIns(4,5)P₂ production during cell growth may have influenced phospholipid biogenesis and nuclear processes and thereby affected the lipid composition of the nucleus and PM. We found multiple changes in nuclear lipids and protoplast PM lipids when *HsPIP5K1α* expressing cells were compared with wild type, which is not surprising because of the interconnected lipid biosynthesis pathways [81–85].

Along with increased PtdCho, increased PtdSer and decreased lysolipids were characteristic of the HK nuclei. Both PtdSer and lysolipids have been hypothesized as signaling molecules in animals and plants, although the intracellular localization and effects of these signaling molecules are still not well understood [5,86,87]. Like PtdIns(4,5)P₂, lysolipids are predicted to have an inverted-cone shape in a lipid bilayer [2,69]. It is likely that the decrease in lysolipids help compensate for the over accumulation of PtdIns(4,5)P₂.

Major fatty acid differences were identified between the nuclei and protoplast PM. Total saturated fatty acids decreased in HK nuclei and protoplast PM and total unsaturated fatty acids increased. These changes in lipid saturation may reflect the increased flux through the PI signaling pathways because the PI signaling pools were observed to have increased unsaturated fatty acids [14]. Increased turnover of signaling PtdIns(4,5)P₂ would also be predicted to increase unsaturation of other phospholipids through changing the PtdOH and DAG fatty acid backbones [14,53]. Increased PtdIns(4,5)P₂ and PI pathway signaling may also change the localization, activity or substrate preference of lipid metabolizing enzymes, lipid binding proteins or change the types of isozymes that are active. Endogenous plant PtdIns(4,5)P₂ signaling is increased during stress responses like heat and osmotic stress [12,88,89], and lipid remodeling, lipid isozyme changes, and changes in fatty acid compositions are known responses to heat and osmotic stress [90,91].

Changes in fatty acid composition between the NT and HK nuclei also may indicate that changes in the carbon metabolism in HK cells are affecting multiple cellular compartments. Fatty acid transport among the chloroplast, the mitochondria and the ER affects the fatty acid composition of phospholipids. The decrease in 16:0-16:0 fatty acid containing lipids suggests a change in chloroplast fatty acid trafficking. Lipids and lipid precursors with 16:0-16:0 fatty acids are synthesized in the chloroplast, and the decrease of multiple different lipids with 16:0-16:0 in the nucleus may indicate a general cellular decrease in utilization of plastid lipids and more of a reliance on the ER for lipid precursors. Schneiter et al. [70] identified a unique PtdIns lipid molecular species with a 26:0 very long chain fatty acid that was important for nuclear membrane formation and is hypothesized to be important for nuclear pore stability. Like the changes in lipid classes, the changes in nuclear lipid molecular species between the HK and NT nuclei also may be compensating for the effect of increased nuclear PtdIns(4,5)P₂ in the HK nuclear membrane.

To isolate intact nuclei we used protoplasts which involved enzymatic digestion of the cell wall. Increased lipid kinase activity has been measured in embryogenic carrot cells during enzymatic cell wall digestion [92] and because others had shown that the lipid composition of PM isolated from protoplasts isolated with enzymatic digestion was different from that of cells in both plants and oomycetes [93,94], we compared the lipid composition of the protoplast PMs with that previously reported for cell PMs [54]. The NT and HK lines had multiple common changes when comparing their respective protoplast PM and cell PM lipid classes and lipid molecular species (Supplemental Table 2). In addition, multiple unique changes were found in comparing the protoplast to the cell PM within each cell type (Supplemental Table 2). The changes in the lipid molecular species composition of HK protoplast PM compared with the NT protoplast PM could have arisen from a differential response to cell wall digestion [13]. Changes in lipid saturation and lipid molecular species in HK protoplast PM, for example, the decrease in the lipid classes LysoPtdEtn and some LysoPtdCho lipid molecular species could help compensate for the effect of increased PtdIns(4,5)P₂ on membrane fluidity [17] and stabilize the newly formed protoplast.

We hypothesized that changes in cellular and nuclear lipids could influence nuclear functions in HK cells since phospholipids have been shown to influence a variety of nuclear processes including DNA synthesis, chromatin remodeling and cell cycle progression [7]. For example eukaryotic DNA polymerases α , β and γ and type I and II Topoisomerases are inhibited by a variety of anionic phospholipids [95]. Chromatin remodeling complexes like the animal BAF complexes are influenced by the lipid PtdIns(4,5)P₂ and its signaling products [32].

We found a decrease in DNA replication in HK cells as determined by BrdU incorporation. Decreased BrdU incorporation could have resulted from differences in cell cycle regulation

or from DNA synthesis defects; however, a major defect in cell cycle was not observed as the cells divided and increased in fresh wt. Lewis et al. [96] recently identified direct binding of human Topoisomerase II α to PtdIns(4,5)P₂ and showed decreased topoisomerase activity when PtdIns(4,5)P₂ was present [96]. PtdInsP₂ or PtdSer in the nucleus of the HK cells could have inhibited the DNA polymerase activity and/or Topoisomerase activity, thus slowing down DNA polymerase progression. More extensive studies are needed to understand the exact mechanisms behind the changes in DNA replication in the HK cells.

H3K9ac was decreased in HK cells. We cannot rule out that changes in the H3K9ac may be responding to other metabolic cues in the *HsPIP5K1 α* expressing cells, but there is evidence from both plants and animals to suggest a direct effect of nuclear lipids on chromatin remodeling complexes and their histone modification. A variety of chromatin remodeling complexes like the BAF are known to interact with PtdIns(4,5)P₂, the phosphoinositide lipids and secondary signaling molecules produced by PtdIns(4,5)P₂ hydrolysis like Ins(1,4,5)P₃ in animals [72], and data for lipid-chromatin remodeling interactions in plants is growing [97–99].

One chromatin remodeling protein in plants that has been shown to be sensitive to the PI pathway lipid PtdIns5P is the histone trimethyltransferase ATX1. Exogenously added PtdIns5P causes relocalization of ATX1 from the nucleus to the PM and subcellular vesicles. The result is a decrease in ATX1-dependent H3K4 trimethylation [98]. Studies of the interaction of chromatin modification in plants have shown that H3K4 trimethylation at different genes enhances H3K9 acetylation [100]. It is possible that expression of *HsPIP5K1 α* and increasing PtdIns(4,5)P₂ indirectly increased plasma membrane PtdIns5P as a result of the dephosphorylation of PtdIns(4,5)P₂ by a PtdIns(4,5)P₂ 4-ptase. In addition, other chromatin remodeling complexes in the nucleus could respond to different lipid class changes in the nucleus of HK cells. For example, increased PtdSer in HK nuclei may inhibit the plant GCN5 homologue H3K9 acetylation activity through one of its co-activating Ada2 homologues similar to the mammalian SAGA complex [74].

PtdIns(4,5)P₂ has also been found to be important for cell cycle regulation including mitotic spindle formation, cell plate formation, and regulation of retinoblastoma protein. We hypothesized that increased PtdIns(4,5)P₂ could affect the phosphorylation of the retinoblastoma protein. Total protein isolated from HK cells showed decreased pRb phosphorylation with human pRb Ser 807/811 specific antibodies previously shown to detect plant phosphorylated pRb [62]. Decreased phosphorylated pRb could slow cell cycle progression, change DNA replication timing, and affect chromatin remodeling during cell division [101].

In summary, we have shown that expressing *HsPIP5K α* in plants affected nuclear lipid PIPK activity and the composition of nuclear lipids. DNA replication, histone acetylation and retinoblastoma protein phosphorylation were all decreased. These results indicate that increasing PtdIns(4,5)P₂ and altering nuclear lipids in plants can have pleiotropic effects on nuclear function. The effects of lipids on nuclear function in plants is in agreement with the research of lipids on animal nuclei. Lewis et al. [96] recently identified multiple nuclear proteins that bind directly to PtdIns(4,5)P₂ thus increasing the possible protein targets in animals. Additional *in vitro* and *in vivo* studies targeting and identifying specific nuclear PtdIns(4,5)P₂ binding proteins in plants are necessary to elucidate the direct effects of PtdIns(4,5)P₂ and other lipids on the plant nuclear functions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

ATX1	Arabidopsis homolog of Trithorax 1
BAF	Brahma associated factor
BCA	bicinchoninic acid
Bromodeoxyuridine	5-bromo-2'-deoxyuridine or BrdU
CRT	calreticulin
fw	fresh weight
H3K4	histone H3 lysine 4
H3K9ac	histone H3 lysine 9 acetylation
HsPIP5K1α	<i>Homo sapiens</i> phosphatidylinositol 4-phosphate 5kinase 1 α
HK	<i>HsPIP5K1α</i> -expressing
NIB	nuclear isolation buffer
PCA	perchloric acid
PI	phosphoinositide
PMSF	phenylmethanesulfonyl fluoride
plasma membrane	PM
PWB	protoplast wash buffer
pRb	retinoblastoma protein
SAGA	Spt-Ada-Gcn5-Acetyltransferase

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Figure 1. Nuclei isolated from NT and HK cells are intact and have similar size. NT (A) and HK (B) cells. HK cells are slightly smaller. NT (C) and HK (D) nuclei after isolation. Note Nucleolus denoted by arrows.

Figure 2.

DGDG and MGDG from NT and HK nuclei and protoplast PM samples. NT nuclei (white), NT protoplast PM (white dotted), HK nuclei (grey) and HK protoplast PM (grey striped). Nuclei data are represented by the average mol % $\pm$ standard error (SE). NT nuclei data is the average of 10 samples from 7 different biological replicates and HK nuclei data is the average of 12 samples from 8 different biological replicates. Protoplast PM data are the average of 5 lipid extractions from 3 different biological replicates. Significant changes in the MGDG and DGDG were not found when comparing NT to HK nuclear lipid samples, but a significant increase was seen in the HK protoplast PM MGDG compared to the NT protoplast PM MGDG. In addition, there is a 1.4 fold increase in MGDG on a mol % basis in the PM of both types of protoplasts compared to the respective nuclei.

Figure 3.

HK nuclei have increased steady state PtdIns(4,5)P₂. Lipid headgroup analysis of steady state PtdIns(4,5)P₂ was measured from NT (white) and HK (grey) nuclei. Measurements are the average of 3 independent nuclei isolations $\pm$ Stdev. ND = not detected. At least 0.5 mg of nuclear protein was used per sample.

Figure 4.

HK nuclei have increased endogenous PIP5K activity. NT (white) and HK (grey) nuclei were assayed for endogenous lipid kinase activity without PtdIns4P added (-) and added PtdIns4P (+). [³²P]PtdInsP₂ (A, D), [³²P]PtdInsP (B, E) and [³²P]PtdOH (C, F) are reported as pmol.mg⁻¹.min⁻¹. Measurements are the average of 3 independent experiments ± SE.

Figure 5. Lipid classes from NT and HK nuclei and protoplast PM show significant differences between cell types. A. Comparison of lipid classes from NT (white) and HK (grey) nuclei. B. Comparison of lipid classes from NT (white dotted) and HK (grey striped) protoplast PM. C. Comparison of lipid classes from NT nuclei (white) and NT protoplast PM (white dotted). D. Comparison of lipid classes from HK nuclei (grey) and HK protoplast PM (grey striped). * indicates a changed lipid classes at a p-value of 0.05. NT nuclei data are the average of 10 lipid extractions from 7 independent biological replicates. HK nuclei data are the average of 12 samples from 8 independent biological replicates. NT and HK protoplast PM data are the average of 5 lipid extractions from 3 independent biological replicates. All data are the average mol % $\pm$ SE.

Figure 6.

BrdU incorporation is decreased in HK cells compared with NT cells. A) Example of an immunoblot detecting BrdU incorporation in DNA isolated from NT and HK cells after BrdU treatment. Three different DNA concentrations are shown. B) A comparison of BrdU incorporation between NT and HK cells, normalized to total signal intensity (L=low, M=medium and H=high DNA, e.g. 50, 75 and 100 ng of DNA). Values are the average of more than three separate experiments $\pm$ SE.

Figure 7. Histone H3 lysine 9 acetylation is decreased in HK cells compared with NT cells. A) Example of an immunoblot with H3K9ac-specific antibodies and H3-specific antibodies with NT and HK protein samples. B) Graph of the average ratio of H3K9ac/H3 ratio from HK cells compared to the NT H3K9ac/H3 ratio which was normalized to 100%.

Figure 8. Phosphorylation of retinoblastoma protein (pRb) is decreased in HK cells. Immunoblot is shown using pRb phosphoSer807/811 specific antibodies of two biological replicates of NT and HK cell samples. Arrow indicates the migration of pRb at ~100kDa.