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The mitotic PP2A regulator ENSA/ARPP-19 is remarkably conserved across plants and most eukaryotes

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ABSTRACT

Protein phosphatase 2A (PP2A) is a major serine/threonine phosphatase of eukaryotes. PP2A containing the B55 subunit is a key regulator of mitosis and must be inhibited by phosphorylated α–endosulfine (ENSA) or cyclic AMP-regulated 19 kDa phosphoprotein (ARPP–19) to allow passage through mitosis. Exit from mitosis then requires dephosphorylation of ENSA/ARPP-19 to relieve inhibition of PP2A/B55. ENSA/ARPP-19 has been characterized in several vertebrates and budding yeast, but little is known about its presence in plants and the majority of other eukaryotes. Here we show that three isoforms of ENSA/ARPP-19 are present in the *Arabidopsis thaliana* genome with distinct expression profiles across various plant tissues. The ENSA/ARPP-19 proteins, and in particular their key inhibitory sequence FD***S***GDY (FD***S***ADW in plants), is remarkably conserved across plants and most eukaryotes suggesting an ancient origin and conserved function to control PP2A activity.

1. Introduction

Driven by protein kinases and phosphatases, reversible protein phosphorylation regulates a majority of cellular processes. Quantitative phosphoproteomic studies have now demonstrated that in human cells more than 75% of proteins are phosphorylated on at least one residue during their lifetime [1] and ~98% of these events are on the amino acids serine and threonine [2–4]. The majority of the serine/threonine phosphatase activity in eukaryotic cells is carried out by protein phosphatase 1 (PP1) and 2A (PP2A) [2,4,5]. Several specific protein inhibitors for PP1 and PP2A have been identified and it has been proposed that many more remain to be discovered, particularly in plants where only the PP1 inhibitor I2 has been characterized [6]. These inhibitor proteins act to block specific phosphatase activity to keep select substrates highly phosphorylated at certain times and locations in the cell, thus creating a switch like behavior to drive cellular events. Most recently this has been highlighted in animals for the cell cycle regulators α–endosulfine (ENSA) and cyclic AMP-regulated 19 kDa phosphoprotein (ARPP–19) family proteins that inhibit a specific form of PP2A [7,8].

Protein phosphatase 2A (PP2A) is conserved across eukaryotes being composed of a catalytic (C) subunit, a scaffolding A subunit and a variable B subunit with the B-subunits conferring substrate selectivity to the enzyme by controlling active site access. Eukaryotes, including plants, have four B-subunit families known as B (B55), B' (B56), B″ and B‴. Yeast have a single B55 protein (CDC55) and plants two B55 isoforms, while humans have four highly related B55 subunits (α, β, γ, δ) [4,9].

The cell cycle is a tightly coordinated event with multiple checkpoints to ensure appropriate DNA replication and faithful chromosome segregation to daughter cells. The cell cycle is driven by mitotic protein kinases and phosphatases with the core cell cycle components largely conserved in eukaryotes, including Viridiplantae (land plants and green algae) [10]. Homologs of cyclin-dependent kinases (cdks), cyclins and the anaphase promoting complex are found across Viridiplantae, but a recent work has highlighted innovations specific to the plant lineage compared to yeast and animal cell division [10]. During late G2 phase, prior to mitosis (G2/M), the human cyclin-dependent kinase cdk1 is activated and phosphorylates 100s of substrates driving mitotic entry. Full activation of cdk1 alone is not enough to promote entry into mitosis; PP2A/B55 needs to be inactivated to fully maintain cdk1 substrate phosphorylation during mitosis. Depletion of PP2A/B55

List of abbreviations: ENSA, α–endosulfine; ARPP–19, cyclic AMP-regulated 19 kDa phosphoprotein; PP2A, protein phosphatase 2A.

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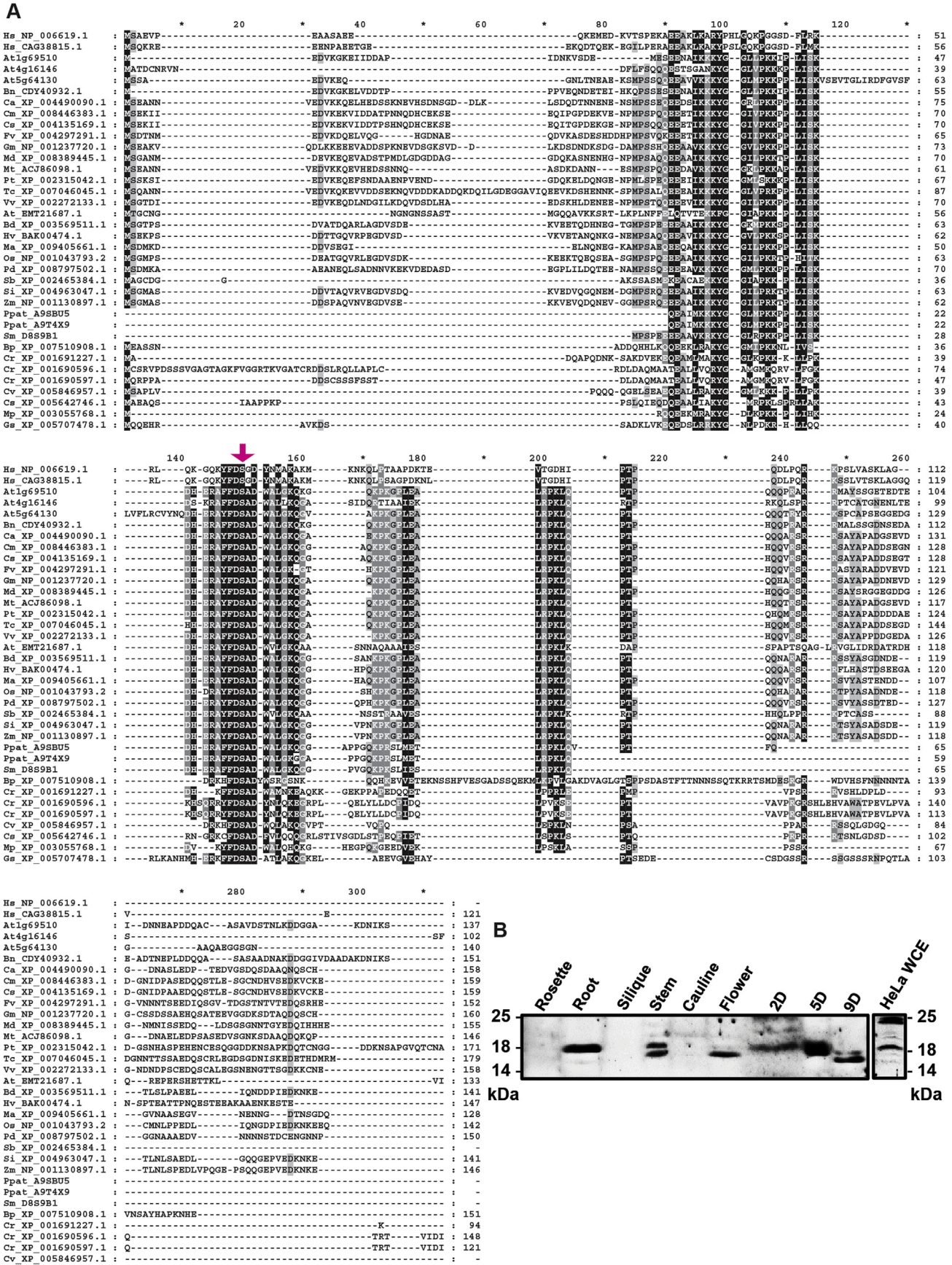
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At1g69510
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Bn_CDY40932.1
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Cm_XP_008446383.1
Cs_XP_004135169.1
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Md_XP_008389445.1
Mt_ACJ86098.1
Pt_XP_002315042.1
Tc_XP_007046045.1
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Hv_BAK00474.1
Ma_XP_009405661.1
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Cr_XP_001690596.1
Cr_XP_001690597.1
Cv_XP_005846957.1
Cs_XP_005642746.1
Mp_XP_003055768.1
Gs_XP_005707478.1
B
Rosette
Root
Silique
Stem
Cauline
Flower
2D
5D
9D
HeLa WCE
25
18
14
kDa

accelerates cdk1 dependent mitotic phosphorylation and mutations in the Drosophila B55 gene result in M-phase arrest [11]. Mitotic exit requires dephosphorylation of cdk1 substrates and in animals this event is performed primarily by PP2A/B55 [11]. First characterized in Xenopus, the specific PP2A B55 subunit was identified as the δ isoform, while in other organisms the sole B55 protein plays this role (for example, Drosophila). Specific inhibition of PP2A/B55 during mitosis is controlled by the ability of Greatwall, a serine/threonine protein kinase, to phosphorylate ENSA or its paralogue ARPP-19, thereby generating a potent inhibitor of this form of PP2A. Phosphorylation by Greatwall at a conserved central motif (FD***S***GDY) increases the inhibitory potency of ENSA/ARPP-19 3800-fold toward PP2A/B55 with no effect on PP2A without its B-subunit or PP2A with any other B-subunit [11]. Given the inhibitory potency, selectivity and key role of the ENSA/ARPP-19 proteins in cell cycle control we asked the question-how conserved is the key PP2A regulator ENSA/ARPP-19 across eukaryotes and specifically Viridiplantae and red algae? Employing bioinformatics we uncovered ENSA/ARPP-19 proteins in all plants examined and in the majority of all other eukaryotes, attesting to a conserved and primeval function for these proteins.

2. Material and methods

Chemicals were obtained through VWR or Bioshop Canada, unless otherwise indicated.

2.1. Bioinformatics

Initial searches for candidate ENSA/ARPP-19 homolog sequences were performed using DELTA-BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastp&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome, [12]) at NCBI, using default parameters, including the BLOSUM62 scoring matrix, and an "Expect" (number of false positive random hits) threshold of 10. In all searches the query was *Homo sapiens* NP_006619.1 (cAMP-regulated phosphoprotein 19). The identities of the target organismal taxa in each search were specified. First, *Arabidopsis thaliana* sequences were searched, then in later searches sequences were specified from taxonomic groups obtained from the Tree of Life (http://www.discoverlife.org/mp/20m?tree=Life&flags=all:). Hits with target sequences, as with the initial query human sequence, were observed with the Endosulfine superfamily sequence model in the PFAM (http://pfam.xfam.org/, [13]) database (PFAM04667). Query-target sequence alignments in the searches were inspected, and target sequences retained as potential candidate homologs if they had a close match to the motif FDSGDY. Initial preliminary alignments were performed using Clustal Omega (http://mobyle.pasteur.fr/cgi-bin/portal.py#forms::clustalO-multialign, [14]) with default settings. These alignments were visualized and edited in GeneDoc (http://iubio.bio.indiana.edu/soft/molbio/ibmpc/genedoc-readme.html, [15]). Edited alignments were then used as input for HHBlits (http://toolkit.tuebingen.mpg.de/hhblits, [16]) searches of the UniProt database, using default parameters except for specifying the return of 200 alignments. This technique provides a "sequence-model vs database-model (i.e. HMM–HMM [Hidden Markov Model])" comparison. Query-model vs target-model alignments were inspected, and constituent database sequences were retained from those target models containing a close motif match. All candidate sequence homologs collected in these two database search procedures were subjected to Batch CD-Search (http://www.ncbi.nlm.nih.gov/Structure/bwrpsb/bwrpsb.cgi, [17]) at NCBI, using default parameters, including an "Expect value" (defined as above) threshold of 0.01. This technique provides a "query sequence to database model" comparison. All sequence-model hits with E values below (closer to zero) the threshold had hits to the PFAM04667 Endosulfine superfamily model. These E values are reported in our data (Table S1). Sequences which failed to achieve a hit with an E value below the threshold are also noted. As the final screening step, all candidate sequence homologs were used as input for HHPred (http://toolkit.tuebingen.mpg.de/hhpred, [18]), specifying the PFAM database as the target sequence models. This technique, as HHBlits above, also provides a "sequence-model vs database-model (i.e. HMM–HMM [Hidden Markov Model])" comparison. A probability ("Prob") value [denoted in our data as "P"] is returned, and quantifies the probability of a true positive hit, its' calculation taking into consideration both conserved secondary structure and the query-model to database-model alignment. Also returned is an E ("Expect") value, which as before is the number of false positive random hits, this depending only on the query-model to sequence-model alignment. All candidate ENSA/ARPP-19 homologs subsequently retained for final analysis had a P value in HHPred of 95% or greater. Finally, alignments were made for presentation using subsets of the total candidate set, using MAFFT Version 7 (http://mafft.cbrc.jp/alignment/server/, [19]) under the BLOSUM45 scoring matrix, and using the L–INS–I (very slow, iterative refinement) option.

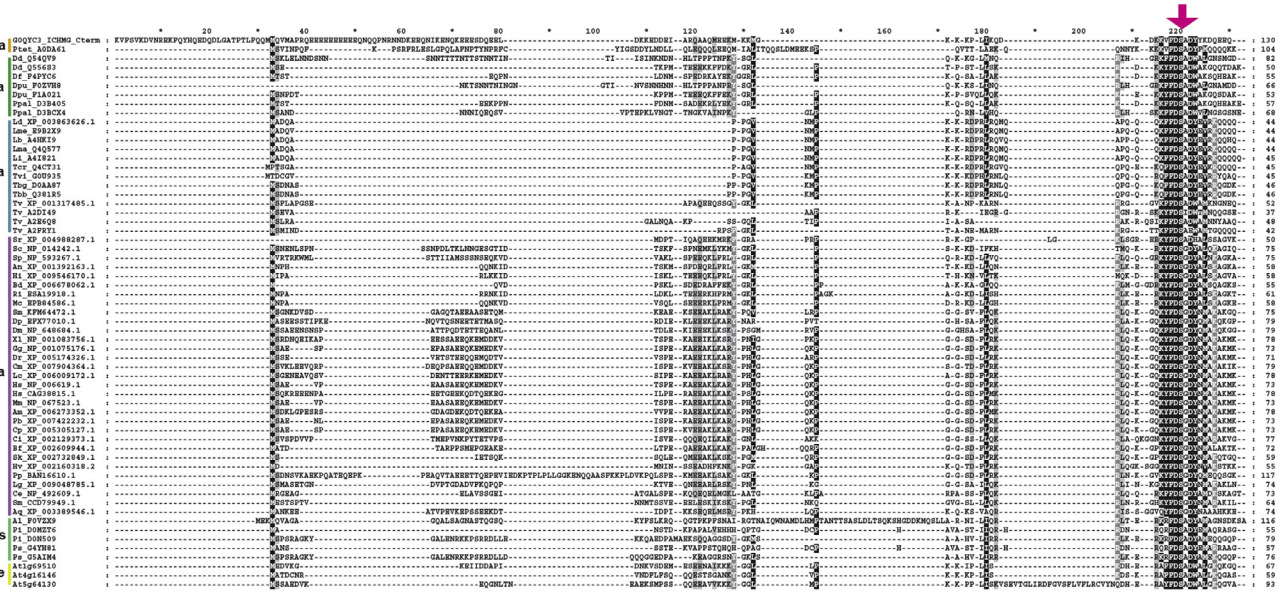
2.2. Plant growth

A. thaliana wild type seeds were surface-sterilized for 4 h, placed in either magenta boxes or 0.6% agar plates containing 0.5× Murashige and Skoog media and stratified in dark at 4 °C for 2 days. Magenta boxes were placed under constant light for 10 days with one media change. Seedlings from plates were transferred to soil and grown in 12hr light/dark conditions for 1.5 months. Each plant tissue (rosette, cauline, stem, flower and silique) and the roots from magenta boxes were harvested, frozen with liquid N_2 and kept at −80 °C until protein extraction [6].

2.3. Protein extraction

Frozen tissues and cells were extracted by grinding in presence of extraction buffer (50 mM HEPES-NaOH pH 7.5, 150 mM NaCl, 5%

Fig. 1. The plant PP2A regulator (ENSA/ARPP-19) is conserved across Viridiplantae and red algae and is expressed in various *Arabidopsis thaliana* tissue types. (A) Homologs of human ENSA/ARPP-19 were retrieved from databases for plants and eukaryotic red and green algae as detailed in the materials and methods. Alignments were made for presentation using subsets of the total candidate set, using MAFFT Version 7. The serine phosphorylated by the protein kinase Greatwall is marked with a magenta arrow and resides within the most conserved region or the signature sequence of human ENSA/ARPP-19 (FD***S***GDY in humans; FD***S***ADY in plants). Organism and sequence accession are shown and details are provided in Supplemental Data. Hs, *Homo sapiens*; At, *A. thaliana*; Bn, *Brassica napus*; Ca, *Cicer arietinum*; Cm, *Cucumis melo*; Cs, *Cucumis sativus*; Fv, *Fragaria vesca subsp. Vesca*; Gm, *Glycine max*; Md, *Malus domestica*; Mt, *Medicago truncatula*; Pt, *Populus trichocarpa*; Tc, *Theobroma cacao*; Vv, *Vitis vinifera*; At, *Aegilops tauschii*; Bd, *Brachypodium distachyon*; Hv, *Hordeum vulgare subsp. Vulgare*; Ma, *Musa acuminata subsp. Malaccensis*; Os, *Oryza sativa Japonica Group*; Pd, *Phoenix dactylifera*; Sb, *Sorghum bicolor*; Si, *Setaria italic*; Zm, *Zea mays*; Ppat, *Physcomitrella patens subsp. Patens*; Sm, *Selaginella moellendorffii*; Bp, *Bathycoccus prasinos*; Cr, *Chlamydomonas reinhardtii*; Cv, *Chlorella variabilis*; Cs, *Coccomyxa subellipsoidea C-169*; Mp, *Micromonas pusilla CCMP1545*; Gs, *Galdieria sulphuraria*. (B) Western blotting reveals expression of ENSA/ARPP-19 in *A. thaliana* tissues. Proteins extracted (35 μg) from the tissues indicated were run on 14% SDS-PAGE, transferred to a membrane and probed with anti-ENSA/ARPP-19 antibody. Lanes 7–9 contain protein from *A. thaliana* cells grown in culture with extracts made 2, 5, and 9 days after inoculation into fresh growth medium [6]. Lane 10 is a separate blot with 35 μg HeLa cell (human) extract from an asynchronous cell population.


Alveolata
Amoebozoa
Excavata
Opisthokonta
Stramenopiles
Viridiplantae

glycerol, 0.1% Tween 20, 1 mM PMSF, 1 mM benzamidine) with 25 mM NaF and 0.5 μM microcystin-LR. Suspension cells were french pressed 1X 1000 PSI. All lysates were centrifuged at 20,000 rpm (47,800 g) for 35 min at 4 °C, supernatant protein concentration determined by Bradford reagent using BSA as standard. Extracts were boiled in SDS-cocktail and kept at −20 °C until use.

2.4. Immunoblotting

Proteins from different plant tissues were separated by electrophoresis on 14% SDS-PAGE and transferred to a nitrocellulose membrane for 200 Vh. The membrane was blocked with 10% skim milk in TBS (25 mM Tris–HCl pH 7.5, 500 mM NaCl) with 25 mM NaF for 1.5 h at RT and washed 1× 10 min and 3× 5 min with TBS. Membrane was probed with anti-ENSA/ARPP-19 antibody (Santa Cruz, sc-135145) diluted 1:250 in 0.5% BSA, TBS and 25 mM NaF for 16 h at 4 °C. The membrane was washed with 4× 5 min TBS with 0.05% Tween 20 (TBST) and then probed with goat anti-rabbit antibody diluted 1:5000 in 5% skim milk powder, TBS and 25 mM NaF for 1 h at RT. Finally, membrane was washed 4× 5 min with TBST and 1× 5 min TBS, and developed with ECL prime (Amersham).

3. Results and discussion

To determine how widespread ENSA/ARPP-19 *homologs* are across eukaryotes and specifically plants, we first performed a bioinformatic search for homologs in *A. thaliana*. Here we will refer to the ENSA/ARPP-19 proteins together as both are potent PP2A/B55 inhibitors that share highly conserved sequence identity/similarity. We identified three ENSA/ARPP-19 protein sequences in *A. thaliana* (Fig. 1A) with predicted masses of 15.1, 11.4 and 15.1 kDa, similar to the human proteins (13.3 and 12.3 kDa). Our searches included a requirement for a close fit to the key motif FD***S***GDY (in plants this motif is universally FD***S***ADW), where the ***S*** of this sequence is phosphorylated by Greatwall to generate the potent PP2A/B55 inhibitor. Recently, Mochida demonstrated that a 40 amino acid peptide centered around the phosphorylated FD***S***GDY motif of animal ENSA was nearly as potent a PP2A/B55 inhibitor as the full length ENSA [11], consistent with the conservation observed in this region of the plant proteins (Fig. 1A). Cross-linking studies with the PP2A/B55 complex also demonstrated that ENSA docks both B55 and the catalytic subunit, likely directly blocking the active site [11]. Notably one *Arabidopsis* isoform of ENSA/ARPP-19 (At5g64130) has an insertion just N-terminal to the FD***S***GDW motif that we speculate plays an unknown role in specificity. We identified ENSA/ARPP-19 homologs in all plant genomes searched as well as red and green algae (Fig. 1A and Table S1), supporting the ancient and conserved function(s) of PP2A/B55 in photosynthetic organisms. In addition, transcripts for the three *A. thaliana* ENSA/ARPP-19 genes have been quantified at Genevestigator (data not shown; genevestigator.com/gv/).

We then expanded our search and found ENSA/ARPP-19 homologs spread broadly across eukaryotes (Fig. 2). Fig. 2 shows a selection of ENSA/ARPP-19 proteins covering this vast range of eukaryotic species. We found homologs in Opisthokonts (Animals, Fungi, Choanoflagellates), Viridiplantae, Amoebozoa, Alveolates (Ciliates), Stramenopiles (Oomycetes), and Excavates (Parabasalids) (see Table SI). The only major eukaryotic groups where we failed to find a homolog sequence were Rhizaria, Cryptophytes and Haptophytes. Although our approach did not find obvious ENSA/ARPP-19 homologs in every eukaryotic genome searched, the presence of the PP2A inhibitor in such a wide spectrum of eukaryotes indicates an ancient and highly conserved role for this inhibitor protein. In addition we examined bacterial, archaea and viral genomes. No putative homologs were identified in viral genomes, but we did find two archeal proteins that contained the key inhibitory motif: YFDSWDE from *Haloquadratum walsbyi* and YFDSSDY from *Candidatus Nitrosoarchaeum limnia*. One putative full length homolog of 90 amino acids was identified in the Proteobacteria bacterium JGI 0000113-E04 (data not shown). With the exception of one possible bacterial candidate, the complete lack of ENSA/ARPP-19 homologs in bacterial and archeal genomes suggests that these proteins were eukaryotic inventions, also consistent with no PP2A in prokaryotes.

Western blot analysis of *A. thaliana* suspension cell culture (2, 5 and 9 days after sub-culturing) and a selection of isolated tissues using a human anti-ENSA antibody (generated against the full length human ENSA protein) revealed immunoreactive bands in root, stem, flower and the cultured cells (Fig. 1B). Like human ENSA/ARPP-19, the plant protein(s) displayed masses slightly larger than the predicted masses [11] but near the mass of the human versions.

In support of the role of ENSA/ARPP-19 as a PP2A regulator in plants we noted that the sole B55 proteins (α and β) of *A. thaliana* [9] are most like the δ subunit of the *Xenopus* B55 family that is targeted by ENSA/ARPP-19. It had been previously noted that plants contain a Greatwall homolog (known as Rim15 in *Saccharomyces cerevisiae* and IRE in *A. thaliana*) [11,20] and phosphoproteomic studies have already determined *Arabidopsis* ENSA/ARPP-19 proteins to be phosphorylated at the critical serine of the FD***S***ADY motif [http://phosphat.uni-hohenheim.de/]. This body of data as a whole re-enforces the idea that plant ENSA/ARPP-19 is a critical PP2A regulator in plants and eukaryotic algae, likely during mitosis, and possibly during other events regulated by PP2A. In budding yeast, the ENSA/ARPP-19 homologs Igo1 and Igo2 are phosphorylated by the Greatwall equivalent Rim15, inhibiting PP2A/B55 and allowing cells to exit the cell cycle and enter a quiescent state during glucose starvation [21]. The exact roles of the plant ENSA/ARPP-19 proteins have yet to be defined but the work presented here should provide the impetus to explore their function.

This work highlights a new aspect of plant PP2A regulation that has been neglected up to now and supports a broader view of PP2A control across eukaryotes, the dynamic control of protein phosphatases and the emerging idea of kinase/phosphatase switches to drive cellular events.

Fig. 2. ENSA/ARPP-19 is found across a broad range of eukaryotes. Homologs of human ENSA/ARPP-19 were retrieved and aligned as detailed in the materials and methods. Alignments were made for presentation using MAFFT Version 7. The serine phosphorylated by the protein kinase Greatwall is marked with a magenta arrow and resides within the most conserved region or the signature sequence of ENSA/ARPP-19 (FD***S***GDY). Organism and sequence accession are shown and details are provided in Supplemental Data. Alveolata: G0QYC3_ICHMG_Cterm, *Ichthyophthirius multifiliis* (*strain G5*); Ptet, *Paramecium tetraurelia*. Amoebozoa: Dd, *Dictyostelium discoideum*; Df, *Dictyostelium fasciculatum* (*strain SH3*); Dpu, *Dictyostelium purpureum*; Ppal, *Polysphondylium pallidum*. Excavata: Ld, *Leishmania donovani*; Lme, *Leishmania mexicana* (*strain MHOM/GT/2001/U1103*); Lb, *Leishmania braziliensis*; Lma, *Leishmania major*; Li, *Leishmania infantum*; Tcr, *Trypanosoma cruzi* (*strain CL Brener*); Tvi, *Trypanosoma vivax* (*strain Y486*); Tbg, *Trypanosoma brucei gambiense* (*strain MHOM/CI/86/DAL972*); Tbb, *Trypanosoma brucei brucei* (*strain 927/4 GUTat10.1*); Tv, *Trichomonas vaginalis*. Opisthokonta: Sr, *Salpingoeca rosetta*; Sc, *Saccharomyces cerevisiae S288c*; Sp, *Schizosaccharomyces pombe 972h-*; An, *Aspergillus niger CBS 513.88*; Hi, *Heterobasidion irregulare TC 32-1*; Bd, *Batrachochytrium dendrobatidis JAM81*; Ri, *Rhizophagus irregularis DAOM 181602*; Mc, *Mucor circinelloides f. circinelloides 1006PhL*; Sm, *Stegodyphus mimosarum*; Dp, *Daphnia pulex*; Dm, *Drosophila melanogaster*; Xl, *Xenopus laevis*; Gg, *Gallus gallus*; Dr, *Danio rerio*; Cm, *Callorhinchus milii*; Lc, *Latimeria chalumnae*; Hs, *Homo sapiens*; Mm, *Mus musculus*; Am, *Alligator mississippiensis*; Pb, *Python bivittatus*; Cp, *Chrysemys picta bellii*; Ci, *Ciona intestinalis*; Bf, *Branchiostoma floridae*; Sk, *Saccoglossus kowalevskii*; Hv, *Hydra vulgaris*; Pp, *Patiria pectinifera*; Lg, *Lottia gigantean*; Ce, *Caenorhabditis elegans*; Sm, *Schistosoma mansoni*; Aq, *Amphimedon queenslandica*. Stramenopiles: Al, *Albugo laibachii Nc14*; Pi, *Phytophthora infestans* (*strain T30-4*); Ps, *Phytophthora sojae* (*strain P6497*). Viridiplantae: At, *A. thaliana*.

Conflict of interest

None.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.01.123.

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