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Final report for DOE Award: DE-SC0002175
Title: Analysis of Proteins Affecting Chloroplast Function
Award Period: 09/01/2009 – 08/31/2016

Our proposal had two specific aims. The first was to complete the characterization of the pfkB family with a focus on the fructokinase-like proteins and the second was to further understand function of FLN1 and 2, two pfkB proteins present in chloroplast transcription complexes.

Summary of accomplishments: In the award period, we have made significant progress on the first aim, with new discoveries reported in one published paper (1) and in one submitted manuscript (2) currently under review. The published manuscript reports on our discovery of plant ribokinase and the metabolic pathway in which it functions; the submitted manuscript is identification and characterization of the plant fructokinase family of enzymes from expression studies, sequence comparisons, subcellular localizations and enzymatic activities of recombinant proteins. Our study of loss-of-function mutants in the fructokinase family members (2) revealed that there were no phenotypic differences observed for the five genes analyzed, so we have adopted the Crispr/Cas9 system to isolate mutants in the two genes for which there are no currently available insertion mutants, and we are generating higher order mutants (double, triples, etc) to discern the relative roles and significance for each fructokinase. These mutants will be an important resource to understand regulation of carbohydrate movement and catabolism in plants. As studies from others indicate, alteration of fructokinases results in changes in cell walls and vasculatures, which have importance relative to biofuel yield and quality. In the second aim, we have characterized the protein-protein interactions for the pkfB proteins FLN1 and FLN2 that are localized to chloroplast transcriptional complexes and have proposed a new model for how chloroplast transcription is regulated. This work has been submitted for publication, been revised and will be re-submitted in December 2016.

REPORT ON PROGRESS

Our long-term goals are to understand biochemical processes important for biofuel production and quality, with a focus on carbohydrate metabolism and chloroplast function. This work has mainly focused on the pfkB family of putative carbohydrate/purine kinases. These proteins are evolutionarily related to a minor enzyme in *E. coli*, but have expanded in plants to consist of 24 predicted members in the model plant species *Arabidopsis thaliana*. We are using this model species to understand the conserved functions of the unknown pfkB proteins and this information will be useful in analyses of homologous enzymes and genes in biofuel species. Only a subset of pfkB proteins had been characterized previously; two are adenosine kinases, four function in co-factor biosynthesis, two are FLN proteins (one focus of our studies-see below), one is a plastid nucleoid protein of unknown function (NARA5) and one pfkB protein phosphorylates myo-inositol. Our work has uncovered biological functions for nine of the fourteen uncharacterized pfkB proteins (1,2) and we performed subcellular localization studies and isolated T-DNA mutants for four of the remaining five.

Specific Aim 1 accomplishments:

Identification of the plant ribokinase and discovery of a role for Arabidopsis ribokinase in nucleoside metabolism: Ribose can be used for energy or as a component of several important biomolecules but in order for it to be used in either capacity it must first be phosphorylated by ribokinase (RBSK). RBSK proteins are part of the phosphofructokinase-B (pfkB) family of carbohydrate kinases. Sequence comparisons of pfkB proteins from the model plant *Arabidopsis thaliana* with the human and *E. coli* RBSK identified a single candidate RBSK, At1g17160 (AtRBSK). AtRBSK is more similar to predicted RBSKs from other plant species and to known mammalian and prokaryotic RBSK than to all other PfkB proteins in Arabidopsis. AtRBSK contains a predicted chloroplast transit peptide, and we confirmed plastid localization using AtRBSK fused to YFP. Structure prediction software verified that the AtRBSK sequence mapped onto a known RBSK structure. Kinetic parameters of purified recombinant AtRBSK were determined to be Km_{ribose} = 153 μM +/- 17 μM, Km_{ATP} = 45.9 μM +/- 5.6 μM, kcat = 2.0 s^{-1}. Substrate inhibition was observed for AtRBSK (Ki_{ATP} = 2.44 mM +/- 0.36 mM), as has been demonstrated for other RBSK proteins. Ribose accumulated in Arabidopsis plants lacking AtRBSK. Such plants grew normally unless media was supplemented with ribose, which led to chlorosis and growth inhibition. Ribose accumulated in plants lacking AtRBSK. Both chlorosis and ribose accumulation were abolished upon the introduction of a transgene expressing AtRBSK-MYC, demonstrating that the loss of protein is responsible for the ribose hypersensitivity. Ribose accumulation in plants lacking AtRBSK was reduced in plants also deficient in the nucleoside ribohydrolase NSH1, linking AtRBSK activity to nucleoside metabolism. Published in (1).

Identification and biochemical characterization of the fructokinase gene family in *Arabidopsis thaliana*: Fructose is an abundant sugar in plants as it is a breakdown product of both major sucrose-cleaving enzymes. To enter metabolism, fructose must first be phosphorylated by a fructokinase (FRK). Known FRKs are members of a diverse family of carbohydrate kinases known as the phosphofructokinase B (pfkB) family. Protein sequence analysis of the 22 *Arabidopsis thaliana* pfkB members identified eight highly related predicted proteins, including one previously found to possess FRK activity. One gene, At1g50390, was alternatively spliced as compared to its predicted gene model, which led to a premature stop codon suggesting it is likely inactive. The remaining seven proteins were expressed in *E. coli* and phosphorylated fructose specifically *in vitro* leading us to propose a unifying nomenclature (FRK1-7). Substrate inhibition was observed for fructose in all FRKs except FRK1. Fructose binding was on the same order of magnitude for FRK1-6, between 260 μM and 480 μM. FRK7 was an outlier with a fructose Km of 12 μM. ATP binding was similar for all FRKs and ranged between 52 μM and 280 μM. YFP-tagged AtFRKs were cytosolic with the exception of plastidic FRK3. Loss-of-function alleles in five of the seven active FRKs produced no overt phenotype. We extended our analysis to include putative FRKs encoded in sequenced genomes from which we identified a minimal set of three distinct FRKs in plants. We also observed that different subgroups expanded subsequent to speciation. Results presented here provide a starting point for the engineering of specific FRKs to enhance cellulosic biomass production (submitted, 2).

Additional unpublished data: We obtained and verified two independent T-DNA insertion lines in the remaining uncharacterized pfkB-encoding genes: At4g28706, At5g43910, At1g49350 and

At1g06730. The T-DNA insertion mutants have no phenotypic differences from wild type plants. In transients assays we determined their subcellular locations (Table 1). We collaborated with Conrath Laboratory (Aachen University, Germany), giving them our knowledge and T-DNA lines for the uncharacterized pfkB members At5g43910 and At4g28706 and they continue to work on their biological roles. The Witte laboratory (Leibniz University Hannover) is working on At1g19600 and At4g27600; we have coordinated efforts so as to not duplicate work.

We will continue, using undergraduate researchers for immediate future, to generate mutants in the two FRKs for which there are no T-DNA insertion lines using the Crispr/Cas9 system (for FRK4 and 6) and will continue generating higher order mutants using both the T-DNA lines we characterized as well as introducing Crispr/Cas9 vectors into existing T-DNA mutants. We have already made crosses with the single T-DNA mutants that we isolated and characterized (2), focusing on a subset (FRK1-3, 7) with the highest mRNA expression, and will generated double and triple mutants, if possible. We hope to understand the relative contributions of each FRK isozyme to the growth of plants. The biological role for the chloroplast-localized FRK enzyme remains mysterious.

Specific Aim 2:

Arabidopsis FRUCTOKINASE-LIKE PROTEINs as a potential biochemical switch in plastid transcription:

In our work on the fructokinase-like proteins, FLN1 and FLN2, we discovered that ATP modulates their oligomeric organization. This led us to try to further characterize the requirement for this interaction. From this work, we propose a model whereby FLNS are new players in the regulation of chloroplast transcription, but currently it is a model without enough experimental data for complete support. That is why it has been challenging to publish this work. The work in currently being revised for re-submission in Dec 2016. However, we still think these results are highly significant and justify further investigation to provide or disprove our working hypothesis.

***Background*:** Chloroplasts contain two distinct RNA polymerases, a single subunit, nuclear-encoded, eukaryotic-type polymerase (NEP) that typically transcribes housekeeping genes and a multi-subunit, plastid-encoded, prokaryotic-type polymerase (PEP) that transcribes genes related to the photosynthetic apparati. Expression of genes related to photosynthetic complexes is regulated by light at multiple levels. Several metabolites, such as one product of photosynthesis, ATP, also fluctuate in light-dark cycles. This study focuses on two pfkB proteins associated with PEP transcriptional complex, FRUCTOKINASE-LIKE PROTEINS, FLN1 and FLN2. Previously, with DOE support, we characterized their loss-of-function mutant phenotypes and demonstrated that their loss leads to dramatic reduction in PEP-dependent transcription (3). *FLN1* mutants fail to green and cannot grow autotrophically. *FLN2* mutants, on the other hand, are slow to green, but can grow on soil to maturity and set seed.

Results: Using protein-protein interaction assays with purified proteins after expression in bacteria- glutathione (GST)-FLN fusion protein bound to beads mixed with soluble Flag-tagged FLN protein- we showed that recombinant FLN1 undergoes an ATP-mediated change in binding affinity with itself (Fig 1). More interaction is seen in presence of 3-5 mM ATP (Fig 1A) than no or 1 mM ATP. In contrast, using the same type of assay, recombinant FLN2 hetero-oligomerizes

independently of ATP *in vitro* (Fig 1B). Remarkably, association of FLN1 with its paralog FLN2 is strongly ATP-dependent (Fig 1C). At ATP concentrations that stimulate binding, the FLN1-FLN2 hetero-oligomer is the dominant form *in vitro*, out competing the homo-oligomer of FLN1-FLN1. In this experiment the FLN1-FLN1 homo-oligomer is first formed, then FLN2 is added in various ATP concentrations (Fig 1D). We see a loss of the homo-oligomer FLN1-FLN1 as the hetero-oligomer accumulates (Fig 1D) with increasing ATP. Control experiments showed that these ATP-dependent associations are not seen with the carrier protein GST alone (Fig 1E, F).

We characterized the nature of the ligand required. We tested whether GTP, ADP, AMP, GTP, CTP, UTP were able to substitute for ATP. They were not (Fig 2A-E). We also tested whether non-hydrolyzable ATP analogs would substitute. Surprisingly, they do not (Fig 2F, G). All experiments included a positive control of ATP, and in the presence of ATP all proteins showed ATP-dependent interaction.

We further confirmed that ATP-independent homo-oligomerization of FLN2 and ATP-dependent hetero-oligomerization of FLN1 and FLN2 are both observed *in organello* (Fig 3). Using the split YFP system, we showed that FLN1-FLN2 interact in an ATP-dependent manner in the chloroplast. If the chloroplasts are depleted of ATP, the interaction is lost (Fig 3, glycerate). The localization patterns of both forms of fluorescently-tagged FLN oligomer match that of other known nucleoid-associated proteins. These results indicate that the interaction can be modulated by ATP in the chloroplast.

We also showed that FLN1 (using anti-FLN specific antibodies) is present in a large complex both with and without ATP, suggesting that disassembly of the PEP complex does not occur under changing ATP conditions (Fig 4).

Our current working hypothesis is that Arabidopsis FLN1 and FLN2 act as a molecular switch by changing from a preferred FLN2 homo-oligomer to a FLN1-FLN2 hetero-oligomer in response to changes in ATP concentration both *in vitro* and in isolated chloroplasts (Fig 5). These data suggest that FLN oligomerization responds to light-induced changes in stromal ATP and provides a potential mechanism for the observed up-regulation of PEP activity in response to increasing light intensity. However, we lack any direct data that transcription is affected by these changes and despite some initial attempts, we did not establish a method for testing this specific idea.

Additional unpublished data: No enzymatic activity has been ascribed to FLN proteins despite conservation of key catalytic residues found in active fructokinases. We developed a complementation assay to test for FLN function (3). Expression constructs for modified FLN proteins are introduced into the *FLN1/fln1-1* het background. Heterozygous *FLN1/fln1-1* plants are generated that are homozygous for the introduced gene. Seed from this plant is obtained. Normally, seed from *FLN1/fln1-1* plants segregates ¼ mutant *fln1* white seedlings. If the introduced gene complements the *fln1* mutant phenotype, then no mutant seedlings are observed. We decided to use this assay to test whether the catalytic residue and whether a conserved di-glycine motif found in pfkB proteins and also present in FLN1, are required for FLN1 function. We have had many technical problems with creation of the new expression constructs and the experiments are still in progress. The first set of expression constructs after introduction into

plants did not express protein. Next we could not generate transformants and re-made the expression constructs in a new vector. We currently have plants transformed with FLN proteins without the di-glycine motif, but have not completed the analysis. The catalytic mutant FLN protein expression construct has been introduced into plants and we are currently screening for transformed plants. I plan to complete this experiment. Of great interest is the test of whether the catalytic residue is required. When substituted in active enzymes, the catalytic activity drops dramatically. If the catalytically required residue in FLN1 is not required, this strongly argues that FLN1 (and likely FLN2) are not active enzymes, but have acquired a regulatory role. This result would be consistent with our model that FLN proteins are sensors of metabolic status, linking photosynthetic flux with transcription to coordinate these processes during greening.

Citations

1. Riggs, J., Rockwell, N., Cavales, P. and J. Callis 2016. Identification of the plant ribokinase and discovery of a role for Arabidopsis ribokinase in nucleoside metabolism. J. Biol. Chem 291: 22572.

2. Riggs, J., Cavales, P., Chapiro SM. and J Callis 2016 Identification and biochemical characterization of the fructokinase gene family in *Arabidopsis thaliana*, Submitted.

3. Gilkerson J, Perez-Ruiz JM, Chory J, Callis J. 2012 The plastid-localized pfkB-type carbohydrate kinases FRUCTOKINASE-LIKE 1 and 2 are essential for growth and development of *Arabidopsis thaliana*. BMC Plant Biology 12. **12**:102 **DOI:** 10.1186/1471-2229-12-102.

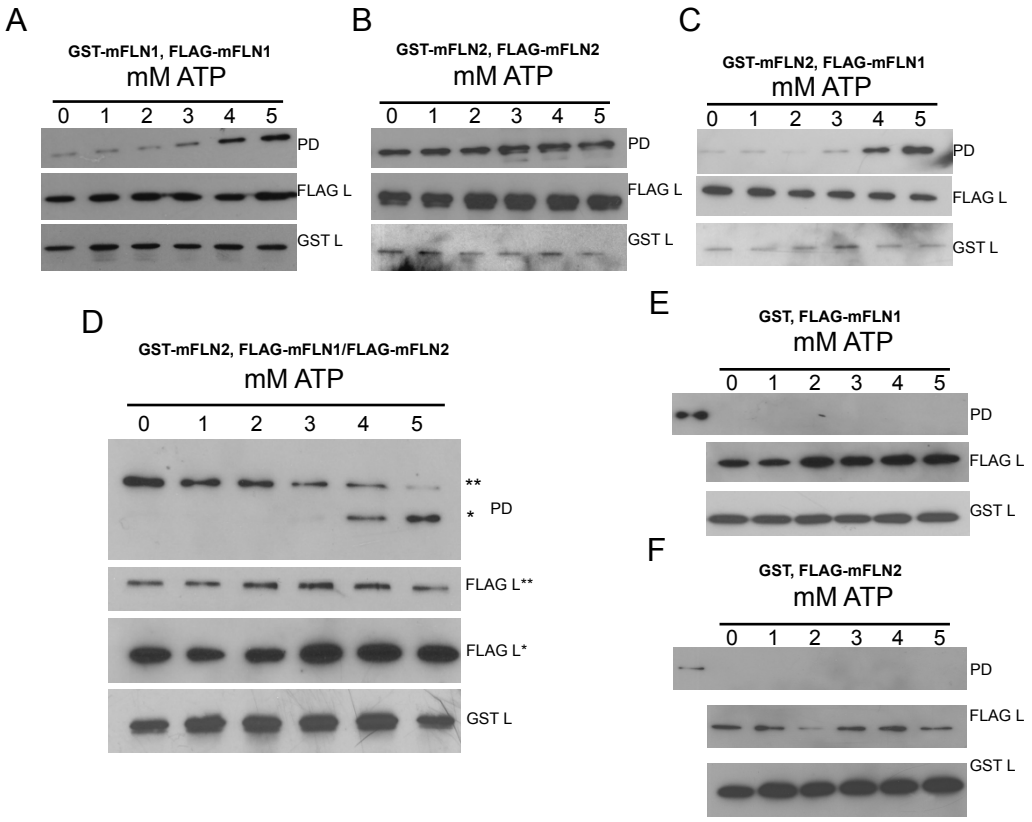

Figure 1. **Interactions between FLN proteins responds to ATP concentration**. **(A-C)** Results of GST pulldown assays between **(A)** FLAG-mFLN1 and GST-mFLN1, **(B)** FLAG-mFLN2 and GST-mFLN2, and **(C)** FLAG-mFLN1 and GST-mFLN2 at the indicated ATP concentrations. Top panels are pulldown (PD) middle and lower panes are representative FLAG (FLAG L) and GST (GST L) loading controls, respectively. **(D)** Results of competition assay between FLAG-mFLN1 and FLAG-mFLN2 for bead bound GST-mFLN2. * denotes FLAG-mFLN1 band, ** denotes FLAG-mFLN2 band. **(E-F)** Results of GST pulldown assay controls between **(E)** FLAG-mFLN1 and **(F)** FLAG-mFLN2 with bead bound GST. All pulldown experiments were performed at least three times, representative images from one experiment are shown.

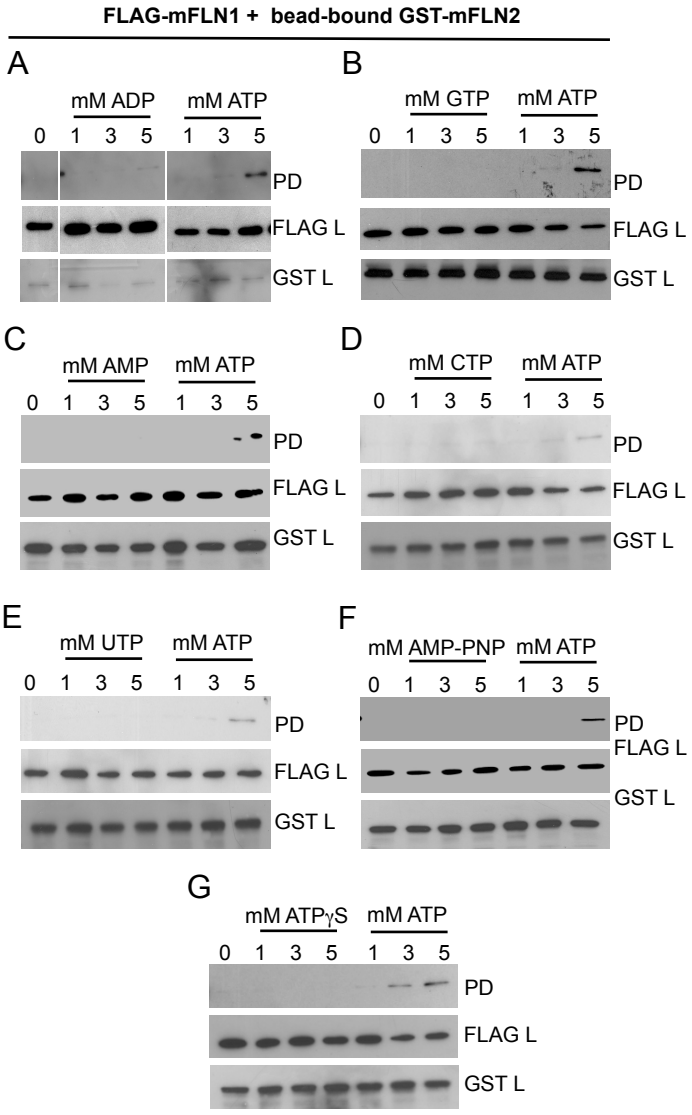

Figure 2. **Interactions between FLN proteins is ATP specific**. **(A-G)** Results of GST pulldown assays between FLAG-mFLN1 and GST-mFLN2 in the presence of the indicated concentrations of ATP (right) and (A) ADP, (B) GTP, (C) AMP, (D) CTP, (E) UTP, (F) AMP-PNP, and (G) ATPγS (left). Top panels are pulldown (PD) middle and lower panes are representative FLAG (FLAG L) and GST (GST L) loading controls, respectively. All pulldown experiments were performed at least three times, representative images from one experiment are shown.

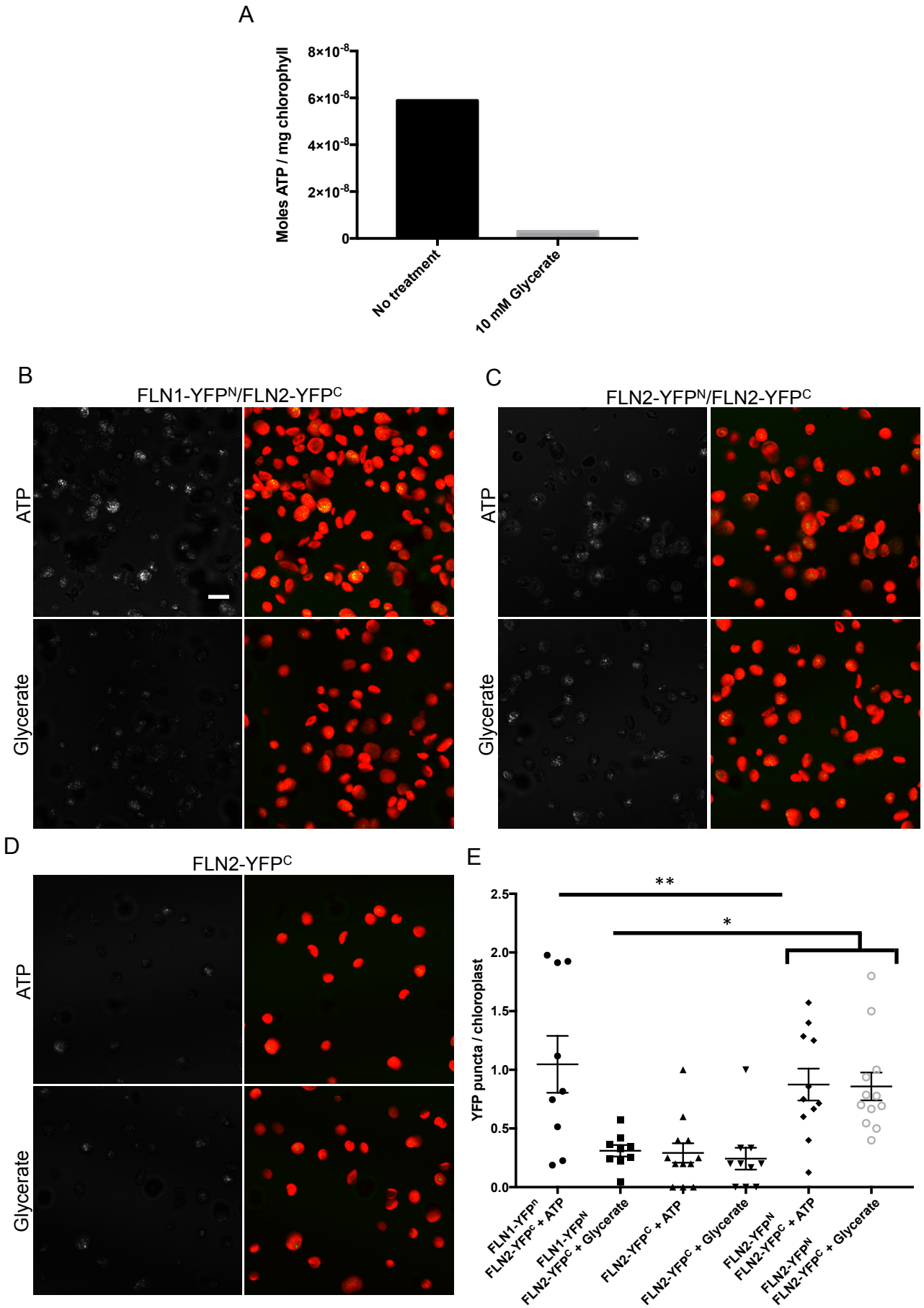

Figure 3. **FLN1 and FLN2 interaction is stimulated by ATP in isolated chloroplasts**. **(A)** Graph comparing the moles ATP/mg chlorophyll in isolated chloroplasts. A standard curve was generated in chloroplast lysis buffer and used to determine the concentration of ATP in the sample by luciferase luminescence. ATP concentrations were normalized to chlorophyll in both conditions for comparison. **(B-D)** Representative images of YFP signal (left) and YFP overlayed with chlorophyll autofluorescence (right) of chloroplasts treated as indicated from *N. benthamiana* leaves expressing **(B)** FLN1-YFP^N and FLN2-YFP^C, **(C)** FLN2-YFP^N and FLN2-YFP^C and **(D)** FLN2-YFP^C only. **(D)** Scatter plot showing quantification of YFP puncta / chloroplast in N≥9 fields. * $p < 0.05$, ** $p < 0.01$. FRET experiments were repeated two times. Fields of chloroplasts for measurement of YFP puncta / chloroplast were selected and quantified from one experiment.

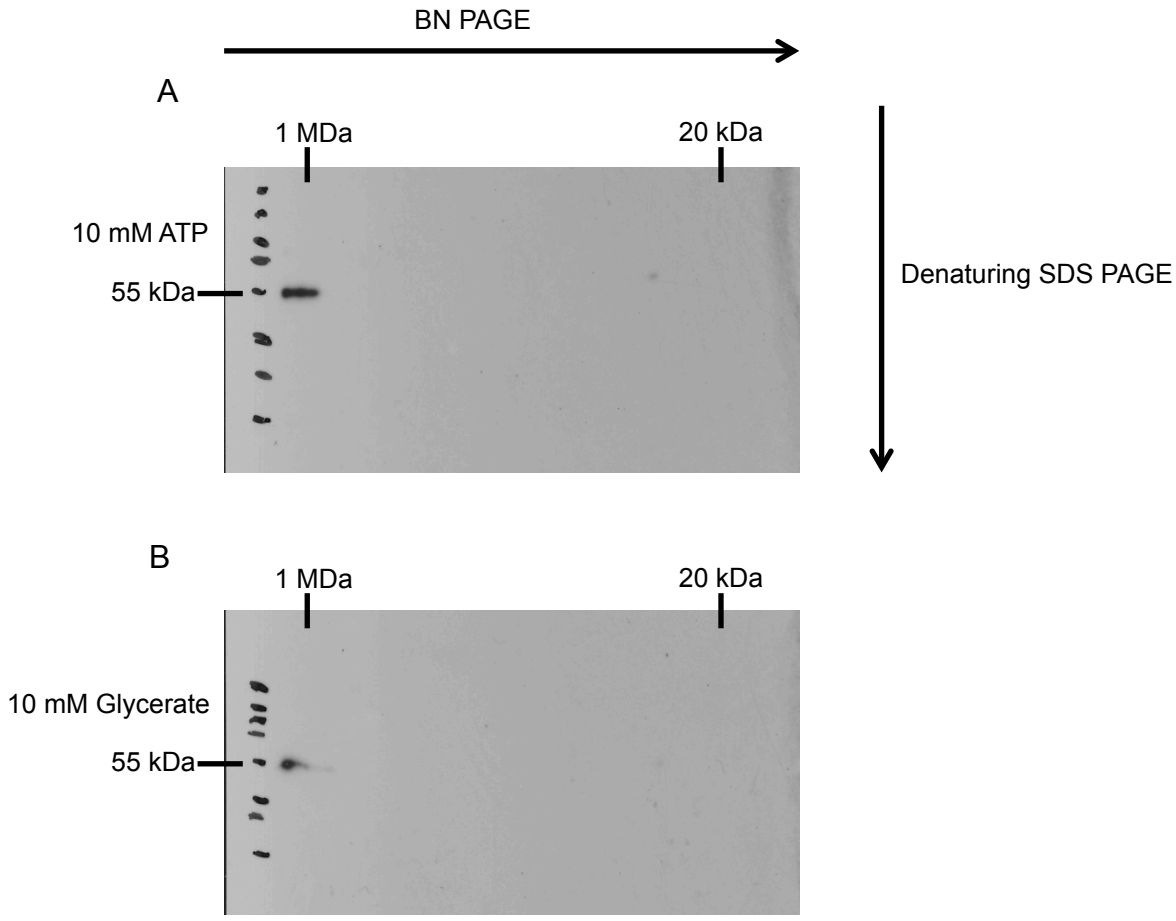

Figure 4. **FLN1 migrates as part of a large protein complex in 2D PAGE regardless of ATP concentration**. Western blots using αFLN1 antibodies showing results of 2D PAGE where lysates from chloroplasts treated with either **(A)** 10 mM ATP or **(B)** 10 mM Glycerate were run first in BN PAGE to separate protein complexes by their native size and then bands were exised and run in a second dimension in SDS-PAGE to separate the protein complexes into their constituents. Diagram showing respective directions of BN and SDS-PAGE migration is shown in a, and MW markers for both dimensions are indicated.

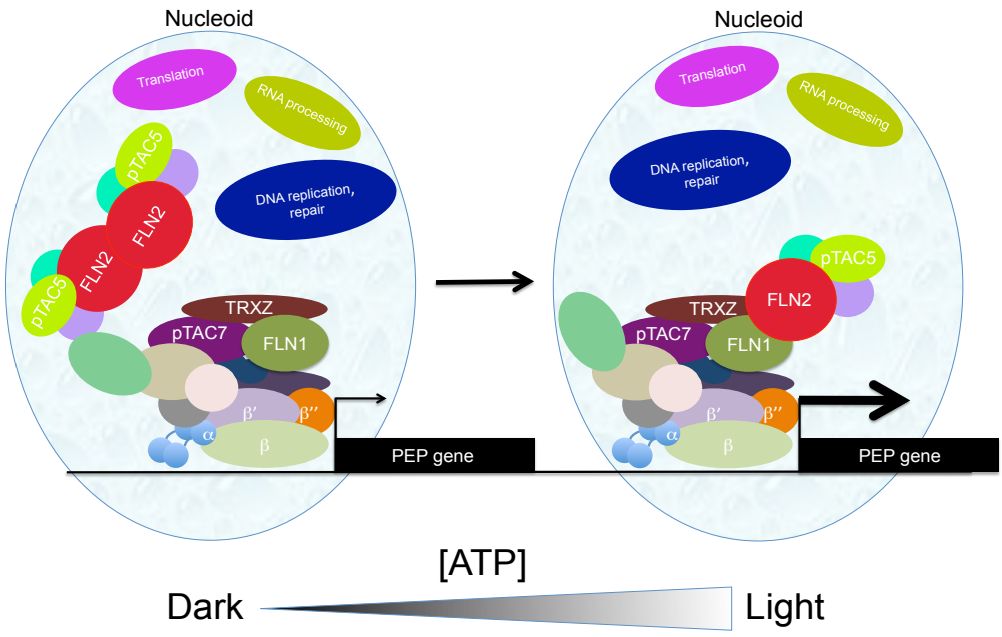

Figure 5. **Model depicting the activation of TAC transcription by the ATP mediated change in FLN binding.** Cartoon of nucleoid containing proteins related to translation, DNA replication and repair (likely also bound to DNA but not depicted doing so here due to limited space), RNA processing, and specifically TAC components bound to DNA. Note FLN1 and FLN2 are not associated (left), though FLN2 is associated with another FLN2 protein due to low ATP levels in chloroplasts of dark grown plants. As ATP is synthesized in the presence of light, FLN2 is recruited to FLN1 via their ATP mediated switch in binding affinities leading to the rearrangement and activation of the TAC complex (right), leading to the increased transcription of PEP-dependent genes.

TABLE 1. List of pfkB proteins: includes uncharacterized proteins, FLNs, NARA and annotated fructokinases

unknown pfkBs: focus of original proposal	Gene (blue= FRK-like clade, green are chloroplast nucleoid associated proteins)	hmz T-DNA line available in Callis lab?	# of indep. hmz lines	WT mRNA in mutant? (determined in Callis lab)	localization predicted	localization (if previously published, citation is noted) In red- from tobacco transient assay in Callis lab	nomenclature for FRK proteins	enzyme activitiy	others working on these proteins
1	At1g06020.1	yes	3	no, for 2; #3 not tested	TargetP:no prediction WoLFPSORT:cytosol	cytosol	**FRK5** (our work)	yes fructokinase	
2	At1g06030.1	no	none aval.	N/A	SubLoc : cytosol WoLFPSORT:cytosol	cytosol	**FRK6** (our work)	yes fructokinase	
3	At1g06730.1	yes	2	no for 2	TargetP:plastid WoLFPSORT: plastid	plastid			
4	At1g17160.1	yes	2	no for 2	TargetP:plastid WoLFPSORT: plastid	plastid, punctate	RBSK1 (our work)	yes, ribokinase (our work)	
	At1g19600.1*	N/A	N/A	N/A	TargetP:no prediction WoLFPSORT: plastid	N/A			Witte lab
5	At1g49350.1	yes	2	no for 2	iPSORT:plastid TargetP:no prediction WoLFPSORT:unclear	cytosol			
6	At1g50390.1/ not functional gene-incomplete ORF	N/A	N/A	N/A	N/A	N/A	none, pseudogene (our work)	no	
	At1g66430.1	IP	2	no for 2	TargetP: mito WoLFPSORT: plastid	plastid	FRK3 (Plant Cell 22:1498) activity shown; **FRK3** (our work)	yes, fructokinase	
	At1g69200.1/FLN2	yes	2	no for both, BMC Plant Biol.12:102	published	plastid, nucleoid (Plant Cell 22:1498)		none detected to date	
in this proposal	At2g31390.1	possible	2	no for 2	TargetP: no prediction WoLFPSORT: cytosol	cytosol	FRK2 (Trends in Plant Sci 5:531 and Plant Cell 22:1498) activity reported as data not shown; **FRK2** (our work)		
	At3g54090.1/FLN1	yes	1	no, BMC Plant Biol.12:102	published	plastid, nucleoid (Plant Cell 22:1498)		none detected to date	
7	At3g59480.1	yes	1	no	TargetP:no prediction WoLFPSORT:cytosol	cytosol	**FRK7** (our work)		
in this proposal	At4g10260.1	no	none aval.	N/A	TargetP:no prediction WoLFPSORT:cytosol /secreted	cytosol	FRK3 (Trends in Plant Sci 5:531) **FRK4** (our work)	yes, fructokinase	
	At4g27600.1/ NARA5	yes	1	no, Plant Physiol 151:114	published	plastid, nucleoid (Plant Physiol 151:114)			Witte lab
8	At4g28706.2+	yes	2	no for 2	TargetP:plastid WoLFPSORT: plastid	plastid			Conrath lab
9	At5g43910.2	yes	2	no for 2	TargetP:no prediction WoLFPSORT:plastid	cytosol			Conrath lab
in this proposal	At5g51830.1	possible	1	no	TargetP:no prediction WoLFPSORT: none	cytosol	FRK1 (Trends in Plant Sci 5:531) but FRK4 in (Plant Cell 22:1498) activity reported as data not shown; **FRK1** (our work)	yes, fructokinase	

Abbrev: N/A, not applicable

*under investigation in Hanson laboratory

+ all gene models correspond to that in TAIR10, with this exception.

For At4g28706, .4 is the gene model in TAIR, but our data indicate .2 likely represents the major expressed mRNA