Development of Constructs and Characterization of Five *Arabidopsis thaliana* HAD-like Phosphatase Gene Isoforms

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Phosphate (P_i) is essential in all organisms, with some critical functions including serving as the backbone of DNA and storing energy as adenosine phosphates. Cells constantly need P_i to maintain their DNA and remain metabolically active, therefore when plant cells are limited by external P_i it has to be obtained from internal reserves instead. It is hypothesized that *A. thaliana* can increase free internal P_i status by use of haloacid dehalogenase (HAD)-like phosphatases to cleave P_i from cellular components such as sugars, nucleotides, and glycolytic intermediates. 21 such phosphatase genes have been identified in *A. thaliana* of which five: At2g38740, At5g02230, At5g59490, At3g62040, and At2g32150 are being characterized by sub-cellular localization, promoter regulation, and overexpression/silencing. These five are predicted to be cytosolic and were chosen due to the likelihood of cytosolic P_i status being chiefly vital to overall P_i homeostasis. PCR primers were designed to fuse GFP genes to the 5' terminals of our genes of interest through Gateway cloning methods. The clones were inserted into pDONR221, then recombined into destination vector pKWGF2, and transformed into *E. coli*. The plasmid DNA is currently purified and will next be transformed into agrobacteria and then transfected into *A. thaliana*.

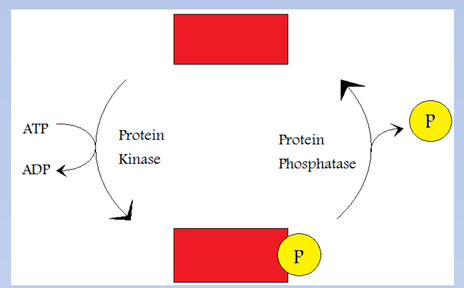

Fig. 1: Kinase/Phosphatase cycle. Free phosphate status can potentially be regulated by the activity of kinases/phosphatases, which determine when phosphate are attached to larger molecules.

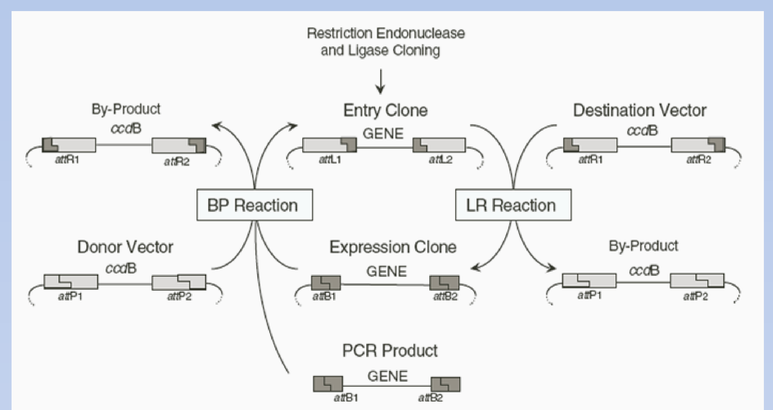

Fig. 2: Overview of Gateway cloning. The PCR product is put through the BP and LR reactions to create the destination vector[2].

Results/Discussion:

Currently the constructs for overexpression and N-/C-terminal GFP fusion have been completed for all five genes.

Promoter constructs for At2g38740, At3g62040, and At5g59490 have been completed through the LR reaction but the constructs are not yet in a purified state.

The promoter construct for At5g02230 is currently in the BP reaction stage and the promoter construct for At2g32150 will have to be restarted from the PCR stage, due most likely to inefficient PCR primers and the DNA template used containing too many impurities.

The RNAi constructs have not been attempted yet.

Table 1: The 21 identified *Arabidopsis* HAD-like phosphatases and their predicted cellular localizations. The five highlighted genes are the subject of this study.

Localization	HAD-like Phosphatase Genes
Chloroplast (8)	At1g79790
	At5g45170
	At4g25840
	At3g48420
	At4g39970
	At1g56500
	At4g11570
	At3g10970
Cytosol (8)	AtFHy
	At5g57440
	At2g38740
	At5g02230
	At5g59490
	At5g59480
	At3g62040
	At2g32150
Possibly Chloroplast or Cytosol (1)	At5g10460
Mitochondria (2)	At2g33255
	At1g14310
Secreted (2)	At2g41250
	At5g44730

Materials and Methods:

PCR amplification: PCR primers were individually designed for all five genes but all carried the same extension coding for the first portion of the adapters for the BP reaction. A second PCR reaction completed the entire addition of the adapters.

BP reaction: The PCR product was inserted into pDONR221 (Fig. 3) by an established Gateway cloning protocol to create entry clones. The vector was transformed into competent *E. coli* cells and the entry clones were purified after sufficient bacterial growth. Selectivity was determined by kanamycin resistance of pDONR221.

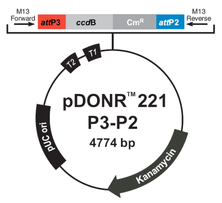

Fig. 3: Vector map of pDONR221 used for the BP reaction[3].

LR reaction: The entry clones were recombined with their corresponding destination vectors (Fig. 4) to yield the final expression clones. The resulting vectors were transformed into competent *E. coli* cells and were purified after sufficient bacterial growth. Selectivity was determined by spectinomycin resistance of all four destination vectors.

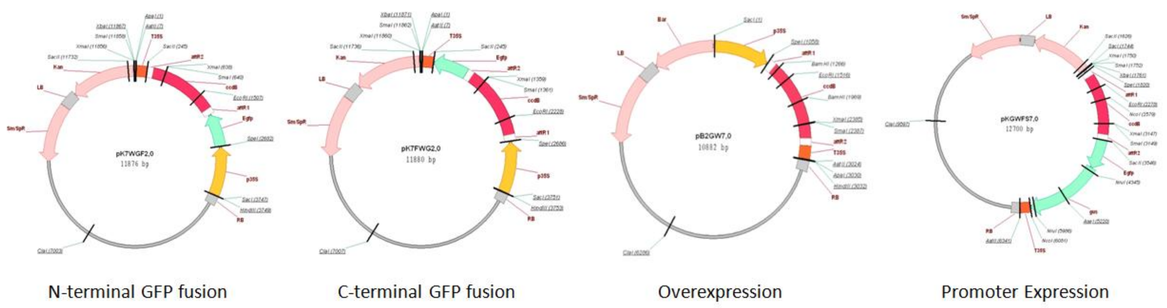

Fig 4: Vector maps of the destination vectors used for the LR reaction (not including the RNAi vector)[1].

Table 2: State of Constructs to be Transformed into Agrobacteria

Genes	N-terminal fusion	C-terminal fusion	Overexpression	Promoter	RNAi
At2g38740	Complete	Complete	Complete	LR	Not initiated
At5g02230	Complete	Complete	Complete	BP	Not initiated
At5g59490	Complete	Complete	Complete	LR	Not initiated
At3g62040	Complete	Complete	Complete	LR	Not initiated
At2g32150	Complete	Complete	Complete	PCR	Not initiated

Future Work:

The next immediate step is to finish the promoter constructs and beginning the RNAi constructs.

Once all 25 constructs have been completed they will be transformed into agrobacteria cells. These cells are able to transfect their DNA into plant cells and will thus transfer the phosphatase genes into *A. thaliana*. Sub-cellular localization of the proteins will be identified by the GFP tags from the N-/C-fusion constructs. Promoter expression will be indicated by GUS fusions, which transforms the GUS's substrate blue.

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1. Gateway Vectors. 2011. Department of Plant Systems Biology and Bioinformatics, Universiteit Gent. 31 July 2011 <http://gateway.psb.ugent.be/vector/show/pK7WGF2/search/index/> <http://gateway.psb.ugent.be/vector/show/pK7FWG2/search/index/> <http://gateway.psb.ugent.be/vector/show/pB2GW7/search/index/> <http://gateway.psb.ugent.be/vector/show/pKGWFS7/search/index/> 2. Invitrogen. "Gateway Cloning Technology Instruction Manual." The Wolfson Centre for Applied Structural Biology. 23 Sept. 2007. The Hebrew University of Jerusalem. 31 July 31, 2011 <http://wolfson.huji.ac.il/expression/gatewayman.pdf> 3. Invitrogen. 2011. Invitrogen Corporation. 31 July 2011 <http://tools.invitrogen.com/content/sfs/vectors/pDONR221P3P2_map.pdf>