FINAL TECHNICAL REPORT:

"Understanding the Role of O-GlcNAc Transferases in Plant Development"; DE-FG02-02ER15353

Reversible posttranslational modification of proteins with O-linked N-acetylglucosamine (GlcNAc) regulates a diverse list of plant processes. However, the list of processes that it regulates is incomplete and how it regulates these processes is not well defined. This project studied the role of O-GlcNAc modification in the model plant *Arabidopsis thaliana*. Arabidopsis has two O-GlcNAc transferases (OGTs) called SEC and SPY. Genetic studies performed prior to this project indicated that SEC and SPY have overlapping functions and that OGT activity is essential during embryogenesis. This project investigated the function O-GlcNAc modification in plants with an emphasis on the function of SEC.

To determine the function of SEC, the phenotypes of two independent T-DNA insertion alleles of *sec* (each allele is in a different ecotype) were characterized in detail (Hartweck *et al*., 2006). The mutants were grown under short days (SD) and long days (LD) and examined for a number of different phenotypic characteristics including germination, root growth, sensitivity to plant hormones, hypocotyl growth, days to flowering, leaf size and number at flowering, plant height, and susceptibility to pathogens. For most of these phenotypes the

mutants and their respective wild type were indistinguishable. Consistent with the known overlap in SEC and SPY function, the traits where *sec* mutants differed from wild type are a subset of the phenotypes exhibited by *spy* mutants. Both *sec* mutants had smaller leaves, reduced plant height and produced fewer leaves before flowering.

SPY is a negative regulator of GA signaling. Thus all GA responses are activated in *spy* mutants. Since SEC and SPY have overlapping function, the hypothesis that SEC acts in GA signaling was tested. Both *sec* mutants exhibited wild-type GA responses suggesting that SEC has little of no role in GA signaling. However, different mutant combinations between *sec*, *spy* and the GA biosynthesis mutant *ga1* caused defects in reproductive development consistent with SEC either acting in the GA pathway or in a parallel pathway to affect reproductive development.

E. coli does not have an endogenous OGT activity but produces UDP-GlcNAc, which is the sugar donor used by OGTs. We expressed SEC in *E. coli* and found that it did not significantly modify any *E. coli* proteins but that it did modify itself. This result suggested that if SEC substrates were expressed together with SEC in *E. coli* SEC would modify the substrates. Therefore, we devised several systems for co-expressing SEC and potential substrate proteins in *E. coli*. Using these systems, we have tested a number of possible substrates. These

substrates were selected either because they were known to interact with SPY in yeast two-hybrid assays or were components of pathways that are affected in *sec* and/or *spy* plants. This has led to the identification of over 20 substrates (manuscript in preparation). Interestingly one of the substrates, GIGANTEA (GI), functions in the long-day flowering pathway, which is affected in *sec* plants suggesting that SEC modification of GI may be important to its role in this pathway.

Since the coat protein of plum pox virus (PPV-CP) was known to be O-GlcNAc modified, we initiated a collaboration with Dr. Juan Antonio Garcia, whose laboratory at Universidad Autónoma de Madrid discovered that PPV-CP was modified, to studying O-GlcNAc modification of PPV-CP. This collaboration developed two lines of evidence that SEC modifies PPV-CP. The coat protein of PPV isolated from wild-type and *spy* plants is modified but the coat protein of virus isolated from *sec* plants is not. Moreover, virus infection of *sec* plants is delayed suggesting that modification of the coat protein has an important role in the infection process. Experiments using the *E. coli* co-expression system, demonstrated SEC modifies PPV-CP. Following on this work, deletion mapping and site directed mutagenesis approaches were used to map the location of the modifications produced in plants and those made by SEC in *E. coli*. This mapping indicates that SEC modifies the same sites that are modified in arabidopsis. These results provide further evidence that SEC is likely to be the OGT that

modifies PPV-CP in arabidopsis. The results also highlight that SEC has the same substrate specificity in arabidopsis and *E. coli*. This work produced three publications (Chen *et al.,* 2005; de Jesus Perez *et al.,* 2006; and, Scott *et al.,* 2006).

Since *sec* plants are affected in flowering time and *E. coli*-expressed SEC modifies GIGANTEA (GI), a protein that controls flowering time,, we investigated the role of O-GlcNAc modification in controlling the activity of this protein. We first mapped where SEC modifies GI and then tested the hypothesis that O-GlcNAc modification affects GI activity by testing the ability of mutant GI protein that cannot be modified to rescue *gi* mutants. Using deletion and site-directed mutagenesis approaches, we showed that in *E. coli* SEC modifies T829 of GI. Substitution of T829 with an A (T829A) blocked O-GlcNAc modification. Substitution of the nearby S/Ts results in either a wild-type level of modification (T825A, S828A, T830A, S838A, S840) or reduced modification (T834A, T837A). Substitutions at both T834A and T837A had the same effect on the level of modification as either alone, suggesting that both sites affect the level of modification at T829 but that neither serves as a modification site alone. Modification of the tryptic fragment containing T829 was confirmed by MALDI mass spectrometry. This analysis also showed that SEC makes modifications consisting of a single O-linked GlcNAc demonstrating that the enzymatic product is identical to that of the animal OGTs.

To test whether mutant GI protein that is non-modifiable is functional, we transformed plants with constructs expressing GI with the following substitutions: S828A, T829A, T830A, T834A, or T829D. The T829D mutation mimics phosphorylation of this amino acid and was examined to test the hypothesis that O-GlcNAcylation of this amino acid could function by blocking phosphorylation. All of the constructs including one expressing wild-type GI were transformed into null *gi-2* and *gi-12* mutants. In these constructs, GI was expressed under the control of the CaMV 35S promoter. Overexpression of wild type GI rescued the late flowering phenotype of both mutants. Surprisingly, all of the mutant forms of GI also rescued the late flowering phenotype, indicating that they are active. This result could suggest that GlcNAcylation, rather than being a molecular switch that turns GI on or off, instead, modulates its activity. For each construct a series of lines in which the late flowering was rescued to different extents was obtained. This variation in rescue between the lines occurs because different transformation events express the transgene at different levels. We took advantage of the variation in rescue between the different lines to determine if the specific activity of the mutant GI proteins differed from wild type. The amount of GI RNA in each transgenic plant was quantitated using RT-PCR and compared with the flowering time of the different lines. This analysis did not find any evidence that specific activity of any of the mutants differed from wild type. Therefore either O-GlcNAc modification of GI does not affect its activity or

additional modification sites that were not identified by the mutational mapping studies exist.

We also examined the roles of *SEC, SPY* and *GI* in flowering pathways by genetic analysis. We generated *gi sec-1* and *gi sec-2* double mutants and compared their phenotypes to those of *gi spy-4*. The number of days to flowering, number of rosette and total leaves until flowering of *gi sec* were similar to *gi*. However for other phenotypes, *gi sec* plants appeared to have phenotypes more similar to *sec* single mutants: smaller rosette diameters, shorter leaves, and reduced height of the stem at flower opening than *gi* plants. Therefore, *SEC* appears to be functioning in leaf length and stem height pathways that are independent of *GI,* while *SEC* and *GI* function in the same flowering pathway. Based on the comparisons between the *gi sec* and *gi spy* double mutants, we conclude that SPY participates in two flowering pathways: the autonomous and the long day pathways, while SEC and GI only function in the long day pathway. A manuscript describing the studies with GI is in preparation.

We used the SEC and the *E. coli* expression system to examine the role of the TPR domain in substrate specificity. Like other OGTs, the first half of the SEC is composed of a series of tetratricopeptide repeats (TPRs). The TPR domain is a protein interaction domain and has been hypothesized to have a role in substrate selection and/or to be a site of regulatory interactions. We examined the effect of

deleting SEC's TPRs and substituting heterologous TPR domains on substrate specificity. Reducing the number of TPRs or substituting heterologous TPR domains from SPY and Human OGT did not affect substrate specificity suggesting the catalytic domain is the major determinant of substrate specificity. Interestingly, reducing the number of TPRs increased SEC's activity suggesting that the TPR domain negatively regulates its activity. A manuscript on this work is in preparation.

Publications Resulting From This Grant

Chen D, Juarez S, Hartweck LM, Alamillo JM, Simon-Mateo C, Perez JJ, Fernandez-Fernandez MR, Olszewski NE, Garcia JA (2005) Identification of SECRET AGENT as the *O*-GlcNAc transferase that participates in *Plum pox virus* infection. J Virol 79: 9381-9387.

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Scott CL, Hartweck LM, Perez JJ, Chen DC, Garcia JA Olszewski NE (2006) SECRET AGENT, an *Arabidopsis thaliana O*-GlcNAc transferase, modifies *Plum pox virus* capsid protein. FEBS Lett. 580: 5829-5835.

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