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FINAL REPORT

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**Manipulation of GA Levels and GA-Signal
Transduction in Anthers to Generate Male Sterility**

D. Weiss, N. Olszewski

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Investigators

Principal Investigator (PI):

David Weiss

Institutions

The Hebrew University of Jerusalem

Co-Principal Investigator (Co-PI):

Neil Olszewski

University of Minnesota

Keywords *not* appearing in the title and in order of importance. Avoid abbreviations.

Arabidopsis, O-GlcNAc modification, Petunia, PhSPY, SPY

Abbreviations commonly used in the report, in alphabetical order:

O-GlcNAc- O-linked *N*-acetylglucosamine

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Signature
Principal Investigator

Signature
Authorizing Official, Principal Institution

Dr. ERAN VARDI
DIRECTOR
AUTHORITY FOR RESEARCH
AND DEVELOPMENT

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AND DEVELOPMENT

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Publication Summary (numbers)

	Joint IS/US authorship	US Authors only	Israeli Authors only	Total
Refereed (published, in press, accepted)	1	2	1	4
Submitted, in review, in preparation		1		1
Invited review papers		1		1
Book chapters				
Books				
Master theses			1	1
Ph.D. theses		1	2	3
Abstracts		8	8	16
Not refereed (proceedings, reports, etc.)				

Postdoctoral Training:

Lynn Hartweck

Cooperation Summary (numbers)

	From US to Israel	From Israel to US	Together, elsewhere	Total
Short Visits & Meetings	-	2	1	3
Longer Visits (Sabbaticals)	-	-	-	-

Description of Cooperation:

The Israeli and American PIs continually shared their results via email. They met twice, once in the US (New Hampshire) and once in Europe (Madrid) and spent few days discussing the results and future plans. In addition a PhD student from Weiss's lab (Gili Ben-Nissan) was sent to Olszewski lab to present her results and study more about the Arabidopsis *SPY*. The American group sent to the Israeli group a genomic clone of the Arabidopsis *SPY* gene and the TPR domain of *SPY*, both fused to the 35S promoter for expression in petunia. The Israeli group sent the petunia *PhSPY* cDNA fused to the 35S promoter to the American group to test complementation of the *spy* mutation in Arabidopsis.

Patent Summary (numbers)

	Israeli inventor (s) only	US inventor (s) only	Joint IS/US inventors	Total
Submitted	-	0	-	-
Issued (allowed)	-	-	-	-
Licensed	-	-	-	-

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Abstract

The original objectives of the research were: i. To study the role of GA in anther development, ii. To manipulate GA and/or GA signal transduction levels in the anthers in order to generate male sterility. iii. To characterize the GA signal transduction repressor, SPY. Previous studies have suggested that gibberellins (GAs) are required for normal anther development. In this work, we studied the role of GA in the regulation of anther development in petunia. When plants were treated with the GA-biosynthesis inhibitor paclobutrazol, anther development was arrested. Microscopic analysis of these anthers revealed that paclobutrazol inhibits post-meiotic developmental processes. The treated anthers contained pollen grains but the connective tissue and tapetum cells were degenerated. The expression of the GA-induced gene, *GIP*, can be used in petunia as a molecular marker to study GA responses. Analyses of *GIP* expression during anther development revealed that the gene is induced only after microsporogenesis. This observation further suggests a role for GA in the regulation of post-meiotic processes during petunia anther development. SPY acts as a negative regulator of gibberellin (GA) action in Arabidopsis. We cloned the petunia *SPY* homologue, *PhSPY*, and showed that it can complement the *spy-3* mutation in Arabidopsis. Overexpression of SPY in transgenic petunia plants affected various GA-regulated processes, including seed germination, shoot elongation, flower initiation, flower development and the expression of a GA-induced gene, *GIP*. In addition, anther development was inhibited in the transgenic plants following microsporogenesis. The N-terminus of SPY contains tetratricopeptide repeats (TPR). TPR motifs participate in protein-protein interactions, suggesting that SPY is part of a multiprotein complex. To test this hypothesis, we over-expressed the SPY's TPR region without the catalytic domain in transgenic petunia and generated a dominant-negative SPY mutant. The transgenic seeds were able to germinate on paclobutrazol, suggesting an enhanced GA signal. Overexpression of *PhSPY* in wild type Arabidopsis did not affect plant stature, morphology or flowering time. Consistent with SPY being an O-GlcNAc transferase (OGT), SPY expressed in insect cells was shown to O-GlcNAc modify itself. Consistent with O-GlcNAc modification playing a role in GA signaling, *spy* mutants had a reduction in the GlcNAc modification of several proteins. After treatment of the GA deficient, *gal* mutant, with GA₃ the GlcNAc modification of proteins of the same size as those affected in *spy* mutants exhibited a reduction in GlcNAcylation. GA-induced GlcNAcase may be responsible for this de-GlcNAcylation because, treatment of *gal* with GA rapidly induced an increase in GlcNAcase activity. Several Arabidopsis proteins that interact with the TPR domain of SPY were identified using yeast two-hybrids screens. One of these proteins was GIGANTEA (GI). Consistent with GI and SPY functioning as a complex in the plant the *spy-4* was epistatic to *gi*. These experiments also demonstrated that, in addition to its role in GA signaling, SPY functions in the light signaling pathways controlling hypocotyl elongation and photoperiodic induction of flowering. A second Arabidopsis OGT, SECRET AGENT (SCA), was discovered. Like SPY, SCA O-GlcNAc modifies itself. Although *sca* mutants do not exhibit dramatic phenotypes, *spy/sca* double mutants exhibit male and female gamete and embryo lethality, indicating that SPY and SCA have overlapping functions. These results suggest that O-GlcNAc modification is an essential modification in plants that has a role in multiple signaling pathways.

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Achievements

The main goals of this research were: to study the role of GA in anther development, to test whether inhibition of GA signal or level can arrest anther development and generate male sterile flowers and to study more about GA-signal transduction in general, and the role of SPY in this process, in particular. We also wanted to understand the mechanism by which GA controls flower development. For this study we have used two model systems: petunia (Israel) and Arabidopsis (US). As a result of the cooperative study, a significant progress in this research area was achieved. The following paragraphs describe the major achievements of the study and their significance.

Previous studies have suggested that gibberellins (GAs) are produced in petunia anthers and transported to the corolla to induce growth and pigmentation. In this work, we studied the role of GA in the regulation of petunia anther development. When petunia plants were treated with the GA-biosynthesis inhibitor paclobutrazol, anther development was arrested. Microscopic analysis of the anthers revealed that paclobutrazol inhibits post-meiotic developmental processes. The treated anthers contained pollen grains but the connective tissue and tapetum cells were degenerated. A similar phenotype was found in the petunia GA-deficient mutant *ga*. Expression analyses of the GA-induced gene, *GIP* during anther development revealed induction only after microsporogenesis. This observation further suggests a role for GA in the regulation of post-meiotic processes during petunia anther development.

To learn more about the role of GIP in flower development, we studied the sub-cellular localization of the encoded protein. The non-cleavable hydrophobic N'-terminal sequence of GIP1 suggests endoplasmic reticulum (ER) localization, and indeed transient expression of a GIP1:green fluorescent protein (GFP) fusion in tobacco BY2 cells supports this prediction. On the other hand, anti-GIP antibodies reacted with proteins extracted from both membrane and cell wall fractions. 2D-PAGE analyses suggested the presence of at least five GIP-like proteins, two of which are localized in the membrane. The different GIP-like proteins are regulated differently by GA; some are induced by the hormone while others not. GA induced the expression of both membrane and cell wall GIP-like proteins. We isolated three novel *GIP* homologues, *GIP2* to *4*. The cleavable hydrophobic N'-terminal sequences of GIP2 and 4 suggest cell-wall localization.

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Induction of GIP proteins by GA, their accumulation in elongating tissues and their possible localization in the cell wall suggest a role in GA-induced cell elongation.

To manipulate GA signaling in anthers, we over express the Arabidopsis *SPY* gene in transgenic petunia. *SPY* acts as a negative regulator of gibberellin (GA) action but its mode of action and regulation are still unknown. *SPY* over-expression affected various GA-regulated processes, including seed germination, shoot elongation, flower initiation, flower development and the expression of a GA-induced gene, *GIP*. In addition, anther development was arrested following microsporogenesis. A similar phenotype was obtained when wild-type petunia plants were treated with the GA-biosynthesis inhibitor, paclobutrazol.

The N-terminus of *SPY* contains tetratricopeptide repeats (TPR). TPR motifs participate in protein-protein interactions, suggesting that *SPY* is part of a multiprotein complex. To study more about the mechanism by which *SPY* regulate GA signal we tested the hypothesis that it acts in a complex. We over-expressed the *SPY*'s TPR region without the catalytic domain in transgenic petunia and generated a dominant-negative *SPY* mutant. The transgenic seeds were able to germinate on paclobutrazol, suggesting an enhancement of GA signaling. We hypothesize that the abundant *SPY* TPR domain competed with the endogenous *SPY* protein for interaction with other proteins leading to the generation of inactive complexes and activation of GA signaling in seeds. Yeast two hybrid screens were performed to determine what arabidopsis proteins interact with the TPR domain. This screen identified several interacting proteins including GIGANTEA (GI). The interaction between *SPY* and GI was confirmed using an *in vitro* interaction assay. Genetic experiments were performed to determine the role of the *SPY*/GI complex in arabidopsis. These experiments indicated that *spy* is epistatic to *gi* with respect to circadian regulation of transpiration, light regulation of hypocotyl length and the induction of flowering by long days, indicating that the *SPY*/GI complex functions in these processes. Interestingly, the regulation of these processes by the *SPY*/GI complex did not involve GA signaling suggesting that *SPY* participates in multiple pathways.

The petunia *SPY* homologue, *PhSPY*, was cloned from anther specific cDNA library and its expression was analyzed by RT-PCR. *PhSPY* is expressed in stems, leaves and flowers, at approximately the same level. *PhSPY* expression was constant during corolla

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and anther development. Treatment with either GA or ABA did not affect *PhSPY* expression. Expressing the *PhSPY* gene under the regulation of the 35S promoter in an *Arabidopsis spy* mutant restored a wild type phenotype, indicating that PhSPY functions in the regulation of GA signal transduction. Although overexpression of SPY in *Arabidopsis* causes changes in vegetative and reproductive morphology, and affects flowering time, overexpression of *PhSPY* did not cause these changes suggesting that in *Arabidopsis*, while PhSPY functions in GA signaling, it is either not as active as SPY or does not function in all of the signaling responses mediated by SPY.

The SPY protein has significant similarity to animal O-linked β -*N*-acetylglucosamine transferase (OGT), which adds O-linked β -*N*-acetylglucosamine to cytosolic and nuclear proteins. Experiments were performed to determine if SPY is an OGT and to test the hypothesis that O-GlcNAcylation of proteins plays a role in GA signaling. Similar to OGT, SPY expressed in insect cells is GlcNAc modified. The modification was shown to be O-linked. This modification was shown to be due to SPY modifying itself because a mutant SPY protein, carrying a single amino acid change that disrupts GA signaling, was not modified. These results indicate that SPY is an OGT and that SPY must have OGT activity for it to function in GA signaling. Consistent with SPY negatively regulating GA signaling by modifying proteins, GA treatment of GA deficient *arabidopsis* and *spy* mutations both caused a reduction in the GlcNAc modification of several proteins. While SPY expression is not affected by treatment with GA, GA treatment was found to rapidly induce increased GlcNAcase activity. Based on these results, we hypothesize that SPY negatively regulates GA signaling by GlcNAc modifying proteins and that during GA signaling this inhibition is overcome through the induction of GlcNAcase which removes this modification.

During the course of this work, a second possible *arabidopsis* OGT, SECRET AGENT (SCA) was identified and experiments were performed to determine if SCA was an OGT and what role it plays in the plant. SCA expressed in *E. coli* was found to have OGT activity toward itself. Two *sca* alleles were identified and found to have no obvious phenotypes. However, loss of both SCA and SPY activity caused male and female gamete and embryo lethality indicating that SCA and SPY have overlapping functions and that OGT is essential in plants.

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List of Publication

1. Izhaki A., Swain S.M., Tseng T., Borochov A., Olszewski N.E. and Weiss D. (2001) The role of SPY and SPY's TPR domains in the regulation of gibberellin action throughout the life cycle of *Petunia hybrida* plants. **Plant J.** 28: 181-190.
2. Izhaki A., Borohov A., Zamski E. and Weiss D. (2002) Gibberellin regulates post-microsporogenesis processes in petunia anthers. **Physiol. Plant.** 115: 442-447.
3. Ben-Nissan G., Lee J-Y, Borohov A. and Weiss D. (2002) The *Petunia hybrida* *GIP* gene family: Regulation and subcellular localization in elongating tissues. (submitted)
4. Olszewski N.E., Sun, T.-p. and Gubler, F. (2002) Gibberellin Signaling: Biosynthesis, Catabolism And Response Pathways. **Plant Cell** 14: S61-S80.
5. Hartweck L.M., Scott, C.L. and Olszewski, N.E. (2002) Two O-linked N-acetylglucosamine transferase genes of *Arabidopsis thaliana* L. Heyn. have overlapping functions necessary for gamete and seed development. **Genetics** 161: 1279-1291.
6. Sothorn R.B., Tseng, T.-S., Orcutt, S.L., Olszewski, N.E. and Koukkari, W.L. (2002) GIGANTEA and SPINDLY genes linked to the clock pathway that controls circadian characteristics of transpiration in Arabidopsis. **Chronobiology International** (in press)
7. Tseng, T.-S., and Olszewski, N.E. (2002) Evidence that a complex between SPINDLY and GIGANTEA acts in the light responses controlling photomorphogenesis and flowering time in *Arabidopsis thaliana*. **Plant Cell** (submitted)