Two \textit{O-}Linked \textit{N}-Acetylglucosamine Transferase Genes of \textit{Arabidopsis thaliana} L. Heynh. Have Overlapping Functions Necessary for Gamete and Seed Development

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Manuscript received January 14, 2002
Accepted for publication April 22, 2002

ABSTRACT

The Arabidopsis \textit{SECRET AGENT} (SEC) and \textit{SPINDLY} (SPY) proteins are similar to animal \textit{O-}linked \textit{N}-acetylglucosamine transferases (OGTs). OGTs catalyze the transfer of \textit{N}-acetylglucosamine (GlcNAc) from UDP-GlcNAc to Ser/Thr residues of proteins. In animals, \textit{O}-GlcNAcylation has been shown to affect protein activity, stability, and/or localization. SEC protein expressed in \textit{Escherichia coli} had autocatalytic OGT activity. To determine the function of SEC in plants, two \textit{tDNA} insertional mutants were identified and analyzed. Although \textit{sec} mutant plants did not exhibit obvious phenotypes, \textit{sec} and \textit{spy} mutations had a synthetic lethal interaction. This lethality was incompletely penetrant in gametes and completely penetrant postfertilization. The rate of both female and male \textit{sec spy} gamete transmission was higher in plants heterozygous for both mutations than in plants heterozygous for \textit{sec} and homozygous for \textit{spy}. Double-mutant embryos aborted at various stages of development and no double-mutant seedlings were obtained. These results indicate that OGT activity is required during gametogenesis and embryogenesis with lethality occurring when parentally derived SEC, SPY, and/or \textit{O-}GlcNAcylated proteins become limiting.

The Arabidopsis \textit{SPINDLY} (SPY) gene product is an important component of the gibberellin signaling pathway (Jacobsen and Olszewski 1993; Jacobsen et al. 1996; Thornton et al. 1999a; Sun 2000). Gibberellins (GA) are a family of dicyclic terpenoid plant hormones that affect many aspects of plant growth and development including germination, hypocotyl elongation, leaf greening, elongation growth, flowering time, and seed filling (Hedden and Phillips 2000; Lovegrove and Hooley 2000; Sun 2000; Yamaguchi and Kamiya 2000). Mutations in \textit{SPY} suppress the effects of GA deficiency, whether this deficiency is caused genetically or by chemical inhibitors of GA biosynthesis (Jacobsen and Olszewski 1993; Wilson and Somerville 1995; Peng et al. 1997; Silverstone et al. 1997), without restoring GA biosynthesis (Peng et al. 1999; Silverstone et al. 2001). Because \textit{spy} mutations can suppress GA deficiency and because all known \textit{SPY} mutations are recessive, \textit{SPY} negatively regulates GA signaling (Jacobsen and Olszewski 1993; Jacobsen et al. 1996; Swain and Olszewski 1996; Thornton et al. 1999a; Sun 2000). Additional evidence that \textit{SPY} acts as a negative regulator of GA signaling has come from the analysis of double mutants between \textit{spy} and other GA signaling mutations (Wilson and Somerville 1995; Jacobsen et al. 1996; Peng et al. 1997; Silverstone et al. 1997; Peng et al. 1999) and from \textit{SPY} overexpression studies (Robertson et al. 1998; Izhaki et al. 2001; Swain et al. 2001). However, \textit{spy} mutations do not completely suppress mutations in GA biosynthesis genes and \textit{spy} mutant plants are somewhat responsive to exogenously applied GA (Jacobsen and Olszewski 1993; Silverstone et al. 1997; Vivian-Smith and Koltunow 1999). Therefore, a portion of GA signaling may act through a route independent of \textit{SPY}.

There is also evidence that \textit{SPY} has roles beyond its role in GA signaling. Swain et al. (2001) found that \textit{spy} mutants had subtle phenotypes that were not usually observed in GA-deficient mutants or plants treated with excessive GA, including reduced height, deviant phyllotaxy, and absence of leaf serration, and suggested \textit{SPY} could also have a role in GA-independent developmental pathways.

The \textit{SPY} protein has a significant level of similarity to animal \textit{O-}linked \textit{N}-acetylglucosamine transferases (OGTs; Kreppel et al. 1997; Lubas and Hanover 2000). OGTs are cytosolic and nuclear localized glycosyltransferases that transfer \textit{N}-acetylglucosamine (GlcNAc) residues from UDP-GlcNAc to serines and threonines via an \textit{O}-linkage (Hart 1997; Hanover 2001; Wells et al. 2001). The activity of the OGT enzyme itself is sensitive to the concentration of UDP-GlcNAc and may therefore serve as a sensor of carbohydrate level within cells (Kreppel and Hart 1999; Han et al. 2000). In mammalian cells, loss of OGT function is lethal (Shaft et al. 2000).

A large number of nuclear and cytosolic proteins are \textit{O-}GlcNAc modified (Hart 1997; Wells et al. 2001).
The $O$-GlcNAc modification of a protein has been shown to affect its stability (Han and Kudlow 1997), subcellular localization (Snow and Hart 1998), and/or interaction with other proteins (Roos et al. 1997). One mechanism by which $O$-GlcNAc addition can affect changes in protein activity is through the competition between $O$-GlcNAcylation and phosphorylation for modification of the same serine/threonine residues. Reciprocal phosphorylation/$O$-GlcNAcylation of specific amino acids has been demonstrated for the murine estrogen receptor $\beta$ (Cheng et al. 2000; Cheng and Hart 2001), the carboxy-terminal domain of RNA polymerase II (Kelly et al. 1993; Comer and Hart 2001), and the transcription factor, c-myc (Chou et al. 1995), and the reciprocal modifications have been shown to differentially affect the activity of these proteins. Thus proteins can exist as the phosphorylated, hydroxylated, and $O$-GlcNAcylated forms, each with different properties. The presence of these three possible states creates an opportunity for regulation of protein activity through the regulated action of kinases, phosphatases, OGT, and/or the enzyme that removes $O$-GlcNAc residues, $O$-GlcNase (Gao et al. 2001; Wells et al. 2002). It should also be noted that not all substrate proteins are regulated via reciprocal phosphorylation/$O$-GlcNAcylation, in some cases, $O$-GlcNAc may directly affect protein activity (Roos et al. 1997; Yang et al. 2001).

Preliminary evidence suggests that SPY has OGT activity in vitro (Thornton et al. 1999b; Thornton 2001) and that spy mutant plants have reduced levels of $O$-GlcNAcylated proteins (Thornton 2001). However, protein $O$-GlcNAcylation is reduced only in spy mutants, raising the possibility of additional OGT(s) in Arabidopsis.

In this article we describe the identification and cloning of the SECRET AGENT (SEC) gene of Arabidopsis. The predicted SEC protein resembles both SPY and animal OGT proteins. When expressed in Escherichia coli, the SEC protein was able to $O$-GlcNAc modify itself, a property exhibited by human OGT. To determine the functional role of SEC, tDNA insertional mutations were identified and their phenotypes were compared to wild type. Although sec insertional mutant lines did not exhibit obvious phenotypes, sec mutations exhibited synthetic lethality when in combination with mutations in spy. These observations indicate that SEC and SPY have overlapping functions and that OGT activity is essential in plants.

**Isolation of SEC-expressed sequence tags and genomic clones:** Twelve expressed sequence tags (ESTs) encoding proteins with similarity to the tetratricopeptide repeat (TPR) domains of rat OGT (U76557; Kreppel et al. 1997) and SPY (U21920; Jacobsen et al. 1996) proteins were identified and ordered from the Arabidopsis Biological Resource Center (ABRC, Columbus, OH, http://www.arabidopsis.org). Three Arabidopsis (H76849, R64973, and W43557) EST clones were sequenced further on the basis of restriction mapping and cross hybridization. One Arabidopsis clone, H76849, predicted to encode a SPY/OGT-like protein (SEC), was used to obtain a cross-hybridizing genomic clone from an Arabidopsis library constructed in pPOCA18 (Olszewski et al. 1988).

**Assembly of full-length SEC cDNA clones:** The H76849 EST clone was not full length and, therefore, the 5’ end of the SEC mRNA was obtained by 5’ random amplification of cDNA ends (5’RACE). RNA was isolated from 1-week-old wild-type plants (Ausubel et al. 1992) and poly(A)-mRNA was purified from total RNA using the Poly(A)-Tract mRNA isolation kit (Promega, Madison, WI). For 5’ RACE, reverse transcription and second-strand synthesis were performed according to the 5’-RACE kit manufacturer’s directions ( Marathon cDNA amplification kit, CLONTECH, Palo Alto, CA) using 1.6 $\mu$g of poly(A)-RNA and the NS4 primer (TGATGAGGATCTGGAGTTTTGTTCTG). The products of the second-strand synthesis reaction were ligated to the AP1 adapter and used as template for PCR (Expand high fidelity PCR system, Boehringer Mannheim, Indianapolis) with the API and NS4 primers. The product that hybridized to 3²-labeled H76849 EST DNA was cloned into pCR2-TOPO (Invitrogen, Carlsbad, CA).

To assemble the full-length SEC cDNA, a NotI-Stul 5’-RACE RT-PCR restriction fragment and a Stul-NdeI EST (H76849) restriction fragment were purified and cloned into the NotI site of pBluescript SK (Stratagene, La Jolla, CA; Ausubel et al. 1992).

**DNA sequencing of cDNA and genomic clones:** The full-length cDNA and a portion of the genomic clone corresponding to the gene were fully sequenced by primer walking at the University of Minnesota Advanced Genetics Analysis Center.

**Expression of maltose-binding protein-SEC and -TPR:** An Xbal fragment from the SEC cDNA clone was cloned into the Xbal site of pMAL 2c (New England Biolabs, Beverly, MA) to create the pMAL-SEC plasmid that encodes a maltose-binding protein (MBP)-SEC fusion protein. While the fusion protein does not contain the first 60 amino acids of SEC, it contains all of the TPRs and the full carboxy-terminal domain. A second fusion-protein expression construct was made to serve as a negative control in experiments examining the OGT activity of MBP-SEC. This construct encodes a protein consisting of only the TPR portion of SEC fused to MBP. The MBP-TPR plasmid was created by self-ligation of pMAL-SEC following digestion with Stul and EcoRV.

**E. coli** (XL1Blue; Stratagene) containing pMBP-SEC or pMBP-TPR were grown at 22°C to an OD600 of 0.6 and protein expression was induced with 0.3 mM isopropyl $\beta$-D-thiogalactopyranoside (Kroll et al. 1993) for 15 min, and then cells were pelleted and frozen. Bacteria were lysed using a French press and fusion proteins were purified by affinity chromatography on a sepharose-bound amylose column (New England Biolabs) according to manufacturer’s directions. Purified proteins were resolved by SDS-PAGE (Laemmli 1970) and visualized by staining with Coomassie or transferred to PVDF membranes (Millipore, Bedford, MA).

**Detection and characterization of protein GlcNAc modification:** The terminal GlcNAc modifications of membrane-bound proteins were labeled with [¹H]galactose as described by Roquemore et al. (1994) and modified by Heese-Peck et al. (1995). For one or two 25-cm² membrane(s) the reaction

**Materials and Methods**

**Plant strains and growth conditions:** All experiments were performed using A. thaliana (L.) Heynh. ecotype Columbia as wild type, the spy-3 mutant in a Columbia background (Jacobsen and Olszewski 1993), and mutants isolated from tDNA-insertion line pools (described below). Plants were grown in a growth chamber with 16-hr light (22°C) and 8-hr dark (20°C) cycles supplied under a mixture of fluorescent and incandescent lights with an intensity of 85 $\mu$mol/m²/sec.

[1] Growth chamber with 16-hr light (22°C) and 8-hr dark (20°C) cycles supplied under a mixture of fluorescent and incandescent lights with an intensity of 85 $\mu$mol/m²/sec.
contained 12 μCi of 60 Gb/mmol [3H]UDP-galactose (American Radiolabeled Chemicals, St. Louis) and 60 milliunits Gal \( \beta (1-4) \) galactosyl-transferase (GaIT, Sigma) in 1 ml of GaIT buffer (10 mM galactose, 10 mM HEPES, 5 mM MnCl\(_2\), pH 7.4).

Glycosyl groups can be linked to proteins by either O- or N-linkage. To determine the linkage of the glycosyl groups to SEC, affinity-purified proteins were labeled and subjected to β-elimination or incubated with PNGase F, treatments that hydrolyze O- and N-linkages, respectively (Roquemore et al. 1994). Affinity-purified proteins were precipitated with 8 volumes of acetone, dried, and resuspended in 1% SDS. After [3H]galactose labeling, proteins were separated from unincorporated label by gel filtration chromatography (Roquemore et al. 1994). One-milliliter fractions were collected and the radioactivity in each fraction was quantitated. The labeled protein fractions were pooled, acetone precipitated, and subjected to PNGase digestion or β-elimination (Roquemore et al. 1994). For PNGase digestion, 2500 units of PNGase F (New England Biolabs) was added to the precipitated sample, which CTGATATGTTCACTCTTC primers were used to generate a 490-bp DNA fragment. To differentiate between the wild-type SEC allele corresponding to sec-1, GSP27 (AATGGCGGAGTT GATGAAGCATG) and NS1R (TGCGTCAATACGAAGACCTC CATATTG) primers were used to generate a 490-bp DNA fragment. For sec-2, left tDNA border (GATTCGGTCTC AATGCA) and NS4R (AAACAGACAAAAATCGACATCT CATCA) were used to generate a 128-bp fragment. For detection of the wild-type SEC allele corresponding to sec-2, GSP31 (GTCGCCATCGGCTTTTCA) and GSP33 (ACACTTGGC GTGATGTTTCCTTTC) primers were used to generate an 890-bp DNA fragment.

RESULTS

Identification of SEC: The SEC gene was identified by searching for plant EST sequences with translational similarity to SPY and OGT proteins. This search did not identify any ESTs with identity to the carboxy-terminal catalytic domain, which is diagnostic for OGTs. The search did, however, identify several ESTs with similarity to the TPR domains, which exist in many proteins. Because the sequence information of each EST clone is too short to make a confident identification, the EST clones were sequenced, and the genomic DNA was sequenced with primers corresponding to the ESTs. The longest sequence identified was a 1281-bp fragment that hybridized with probes to the SPY and OGT genes.

Statistical comparisons of different populations: To compare whether plants from two different populations were segregating with different Kan\(^R\)-Kan\(^S\) ratios, chi-square contingency tests were performed (Whitehouse 1973). Two populations were not different when the deviations of the two populations were not greater than the deviations expected for a random mating model. Therefore, 5’ RACE was used to obtain the 5’ end of the SEC cDNA. A full-length SEC cDNA clone was constructed from the RACE cDNA and the H76849 EST.
When the carboxy-terminal domains of SEC are compared, SEC proteins are more similar to animal OGTs than to SPY. The SEC proteins share 53–59% similarity with rat and *C. elegans* OGTs (Table 1 and Figure 2), while SPY shares an equal level of similarity with SEC (35–39%) and animal OGT proteins (33–38%). The similarities between SEC and OGTs are not spread evenly throughout the carboxy-terminal domain. Regions with higher amino acid conservation (Figure 2) have been identified previously and are predicted to play a role in catalysis (Roos and Hanover 2000). In these regions, SEC and animal OGTs share 63% identity.

Searches of GenBank indicate that petunia, soybean, tomato, cotton, *Medicago truncatula*, maize, barley, wheat, and rice have both SEC-like and SPY-like proteins (data not shown), suggesting that they are present in all angiosperms. SEC and the SEC-like protein of maize were more similar to each other than to their corresponding SPY or SPY-like protein (Table 1), suggesting that SPY and SEC arose by gene duplication early in, or prior to, the origin of the angiosperm lineage.

**TABLE 1**

<table>
<thead>
<tr>
<th>% amino acid similarity*</th>
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<tbody>
<tr>
<td><strong>SPY-Zm</strong></td>
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<tr>
<td>------------------------</td>
</tr>
<tr>
<td>SPY-At</td>
</tr>
<tr>
<td>SPY-Zm</td>
</tr>
<tr>
<td>SEC-At</td>
</tr>
<tr>
<td>SEC-Zm</td>
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<tr>
<td>OGT-Rn</td>
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*The carboxy-terminal portions of proteins shown in Figure 2 were compared with the GCG OLDDISTANCES program, using the length of the shorter of the compared sequences, excluding gaps, to calculate the percentage similarity between pairs of sequences.*
Figure 2.—Amino acid alignment of the carboxy-terminal domain of SEC, SPY, and OGT proteins. The SEC-At, SPY-At, OGT-Rn, Caenorhabditis elegans OGT (OGT-Ce, AAB63465; LUBAS et al. 1997), and maize SEC (SEC-Zm, EST accession nos. AI782882 and AW042413) and maize SPY (SPY-Zm; AW061770) proteins were aligned for comparison. The maize EST sequences were obtained through the Z. maize database (http://www.zmdb.iastate.edu) and translated by the authors using GCG software. The sequences were aligned with the GCG PILEUP program with the gap creation and extension penalties set to 20 and 2, respectively. Amino acid residues identical or similar to the consensus are shaded black and gray, respectively. Conserved regions previously identified by ROOS and HANOVER (2000) are overlined and the position of the spy-3 mutation is indicated.

same mobility as one of the less prominently labeled proteins in the MBP-SEC sample.

To determine if the labeled modifications were O-linked, affinity-purified MBP-SEC and MBP-TPR preparations were labeled with [3H]galactose and then subjected to β-elimination, which removes O-linked but not N-linked modifications (Figure 3B). While the majority (80%) of the labeling to the MBP-SEC preparation was O-linked as indicated by its release with β-elimination, the majority of the labeling in the MBP-TPR preparation was not.
has OGT activity toward itself. spy was refractory to PNGase F, which hydrolyzes an O-glycosyl group eluted just before one column inclusion volume (fraction 5). Free inheritance. In the first set of reciprocal crosses, was quantitated. Proteins with terminally labeled GlcNAc lyzed to determine what factors were influencing this process. Two fractions were collected. The radioactivity within each fraction was determined. In the second set of reciprocal crosses were performed and analyzed. The labeling of the MBP-SEC preparation was then subjected to gel filtration chromatography to remove unincorporated label. The labeled proteins were then subjected to β-elimination to remove O-linked modifications (B) or treated with PNGase F, which removes most N-linked oligosaccharide modifications (C). Following the β-elimination or PNGase treatments, the samples were subjected to gel filtration chromatography and 1-ml fractions were collected. The radioactivity within each fraction was quantitated. Proteins with terminally labeled GlcNAc eluted just after the column void volume (fraction 5). Free glycosyl groups eluted just before one column inclusion volume (fraction 24).

Furthermore the labeling of the MBP-SEC preparation was refractory to PNGase F, which hydrolyzes N-but not O-linkages (Figure 3C). These results indicate that SEC has OGT activity toward itself.

**Isolation of sec insertional mutants:** Two tDNA insertional mutants of SEC were identified and characterized. The site of the tDNA insertion within the SEC gene was determined by sequencing PCR products produced using SEC- and tDNA-specific primers. One allele, sec-1, has a tDNA insertion within the exon encoding the ninth TPR (Figure 1). RT-PCR analysis failed to detect SEC mRNA in sec-1 plants (data not shown). A second allele, sec-2, contains an insertion within an intron adjacent to exons encoding the putative catalytic portion of the protein (Figure 1). Plants homozygous for either sec-1 or sec-2 had no obvious phenotypes. For each allele, the tDNA insertion segregated as a single Mendelian locus (data not shown), indicating that the mutations did not cause any gamete- or embryo-specific phenotypes.

**Reduced transmission of linked sec-spy alleles:** Since SEC and SPY both have OGT activity, we attempted to construct an sec spy double mutant that could be examined for novel phenotypes that would be consistent with these proteins having overlapping functions. Because SEC and SPY are linked on chromosome III, the scheme shown in Figure 4 was used to identify a plant in which recombination had produced a chromosome III containing sec and spy. In the first part of this scheme, plants homozygous for sec-3 were selected by their resistance to the GA biosynthesis inhibitor paclobutrazol (PAC). Plants homozygous for sec mutant alleles were sensitive to PAC (not shown). In the second part of the scheme, KanR plants within the population of sec-3 homozygotes were selected. The genotypes of these plants were determined by allele-specific PCR and CAPs markers. All of the selected plants had the genotype + spy/sec spy.

The + spy/sec spy plants were allowed to self and set seed. It was expected that 75% of the progeny seed would be KanR (indicating inheritance of the sec spy chromosome); however, only 31% of the seedlings were KanR (data not shown). Similar results were obtained in crosses utilizing sec-2 (data not shown). PCR testing of KanR plants indicated that the low KanR/KanS ratio was not due to incorrect phenotyping of sec plants carrying the KanR gene. The observed KanR/KanS ratio was not consistent with the simple models of either lethality of the double mutant or lethality in only one of the gametes.

**Factors influencing the inheritance of sec spy:** Because the reduced transmission of sec spy could not be explained by a simple model of gamete or embryo lethality, two sets of reciprocal crosses were performed and analyzed to determine what factors were influencing sec spy inheritance. In the first set of reciprocal crosses, + spy/sec spy plants were crossed as females or males to both wild-type and spy plants. In the second set of crosses, + +/sec spy plants were crossed as females or males to both wild-type and spy plants. By examining the inheritance of the sec spy chromosome in these two sets of crosses, it was possible to estimate male and female sec spy gamete inheritance rates and determine whether...
there were any parental influences on the inheritance of the sec spy chromosome.

When + spy/sec spy plants were used as male parents, only 5–7% of the resulting progeny were KanR (Figure 6, A and B), indicating a deficiency in the transmission of the sec spy chromosome through pollen.

Since we wanted to determine if the parental genotype affected the male transmission of the sec spy chromosome, the transmission of the sec spy chromosome from + + sec spy plants was also examined. When + + sec spy plants were used as males, there was a reduction in the number of plants that were KanR (31–36%; Figure 6, C and D). However, the transmission of the sec spy chromosome from + + sec spy plants was not equal to the percentage of KanR progeny because recombination between SEC and SPY loci produces sec SPY chromosomes, which, when transmitted, also confer KanR. Using the observed KanR:KanS ratio from the wild type by + + sec spy cross (Figure 6C) and the expected recombination rate between SEC and SPY, we estimated that the transmission rate of the sec spy chromosome through the male was 26% (Figure 7). This rate of transmission (26%) was higher than that observed when + spy/sec spy plants were used as males (5–7%; Figure 6A and Figure 7; contingency \( \chi^2 = 64.9, P = 7 \times 10^{-16} \)), indicating that the paternal dosage of SPY affected the transmission of the sec spy chromosome.

When female sec spy inheritance was examined, some
similarities with male inheritance were observed. There was reduced transmission of the sec spy chromosome when + spy/sec spy plants were used as females in crosses with either wild-type or + spy+/ spy male plants (Figure 8, E and F). A 50% rate of transmission was expected, but only 30% of the progeny inherited the sec spy chromosome. However, when + +/sec spy females were used, there was no deficiency in the inheritance of the sec spy chromosome (Figure 8, G and H; Figure 9). Therefore, the maternal gene dosage of SPY also affected the transmission of the sec spy chromosome.

Alternatively, the hypothesized parental effect on the sec spy transmission rate could have been due to differential lethality between + spy/sec spy and + +/sec spy embryos. However, no differential lethality was detected (Figure 6, A and B; contingency $\chi^2 = 1.2, P = 0.27$; Figure 6, C and D; contingency $\chi^2 = 1.5, P = 0.22$; Figure 8, E and F; contingency $\chi^2 = 0.03, P = 0.86$; Figure 8, G and H; contingency $\chi^2 = 1.8, P = 0.18$).

**Occurrence of the sec spy/sec spy genotype:** Since SEC or SPY was required for gamete development, we hypothesized that OGT function was also required for seed development and, as a test of this hypothesis, attempted to recover double-mutant seedlings. Although gamete lethality would reduce the recovery of double mutants, we were able to estimate that 12% of the progeny of selfed + +/sec spy plants would be double mutants (Figure 10A). However, when 38 progeny seedlings were genotyped by PCR, none were double mutants, indicating that the double-mutant seedlings did not occur ($P = 0.01$) at the predicted frequency. As an additional test, seeds were germinated on PAC because 70% of the PACR progeny from selfed + +/sec spy plants were expected to be double mutants (Figure 10A). However, none of the PACR seedlings were double mutants (Figure 10B). Furthermore, the observed genotype frequencies were consistent with double-mutant lethality. The failure to recover double mutants in any of these tests suggested a defect in the development of sec spy/sec spy seeds.

In tests to find a viable double mutant, we noted that 14% of the selfed seeds from + +/sec spy plants did not germinate, suggesting that the double mutants might be among these nongerminating seeds. In addition, an equal proportion of the desiccated seeds appeared to be misshapen. This contrasted with the low percentage (2%) of both misshapen and nongerminating seeds produced when + +/sec spy plants had been crossed with spy-3 males. To determine if the misshapen seeds were double mutants with defects in embryo development, we imbibed the seeds overnight at 4$^\circ$C, removed seed coats, examined the embryos, and determined their genotype by PCR. Most of the misshapen seeds either did not have visible embryos (33%) or had a small clump of cells that might have been an embryo that aborted early in development (33%). We were not able to determine the genotype of these aborted embryos. However, a portion of the misshapen seeds appeared to have initiated various degrees of embryo development (15%) and were double mutants (see supplemental figure at http://www.genetics.org/supplemental). Some of these mutants were small and resembled oversized heart-shaped embryos. Others had structures that resembled roots and cotyledonal bumps or cotyledons to various degrees but none resembled wild-type-shaped embryos. If
Figure 7.—Calculating the transmission rate of the sec spy chromosome from a +/+ sec spy male parent. The first column shows each of the four possible genotypes resulting from cross C in Figure 6 and the expected frequencies for each of these genotypes is also given using the parameters defined at the bottom. The second column gives the Kan R or Kan S pheno.

described in the first column and each value was then divided however, SPY has two insertions between adjacent TPRs. is calculated by \[ \frac{(1 - \theta)/2 + \theta/2}{1 - \theta/2} \], which can be simplified to tion in the number of TPRs, but all sequences have[\[\frac{(1 - \theta)/2 + \theta/2}{1 - \theta/2}\]]. Using these equations, the estimated values for \( \theta \) and \( \beta \) were used in the equations described in the first column and each value was then divided by \( M \) to give the frequencies shown in the third column.

DISCUSSION

This article describes the discovery and genetic character-
ization of SEC, a gene with predicted translational similarity to SPY and animal OGT proteins. Because the analysis of spy mutants suggested that plants might contain additional OGTs, we initiated a search for additional OGTs.fixes to determine if, like animal OGTs, each modification of SEC consists of a single GlcNAc. In addition,
Figure 8.—The sec spy chromosome is transmitted at reduced rates through female gametes. For the indicated crosses, the inheritance of the sec was scored on the basis of KanR and compared to the inheritance expected if there is no gamete lethality. The expected and observed numbers of KanR and KanS progeny from the crosses were compared using \( \chi^2 \) goodness-of-fit tests.

<table>
<thead>
<tr>
<th>Cross</th>
<th>Parents</th>
<th>Progeny</th>
<th>Observed (%KanR)</th>
<th>Expected (%KanR)</th>
<th>( \chi^2 )</th>
<th>(p)</th>
</tr>
</thead>
<tbody>
<tr>
<td>E.</td>
<td>( + ) spy/sec spy \times \sigma \quad + + + + + + + +</td>
<td>+ + + + + + + +</td>
<td>61:143 (30%)</td>
<td>1:1 (102:102)</td>
<td>32.9</td>
<td>(p &lt; 0.01)</td>
</tr>
<tr>
<td>F.</td>
<td>( + ) spy/sec spy \times \sigma \quad + + + + + + + +</td>
<td>+ + + + + + + +</td>
<td>72:183 (31%)</td>
<td>1:1 (117.5:117.5)</td>
<td>35.2</td>
<td>(p &lt; 0.01)</td>
</tr>
<tr>
<td>G.</td>
<td>( + + + + + + + + ) \times \sigma</td>
<td>+ + + + + + + +</td>
<td>142:147 (49%)</td>
<td>1:1 (144.5:144.5)</td>
<td>0.1</td>
<td>(0.75)</td>
</tr>
<tr>
<td>H.</td>
<td>( + + + + + + + + ) \times \sigma</td>
<td>+ + + + + + + +</td>
<td>129:167 (44%)</td>
<td>1:1 (148:148)</td>
<td>4.8</td>
<td>(0.02)</td>
</tr>
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</table>

experiments are in progress to determine if it has activity toward other proteins.

To determine the function of SEC in plants, two tDNA insertional mutants of sec were identified, but these mutants did not have obvious phenotypes. Only when a chromosome III containing mutations in both sec and spy was identified did we detect phenotypes. When a + spy/sec spy plant was selfed, there was a deficiency in the frequency of progeny inheriting the sec spy chromosome (Figure 5). The mechanism for this deficiency was investigated in a series of reciprocal crossing experiments that indicated that the transmission of the sec spy chromosome through both male and female gametes was reduced (Figures 6–9). Furthermore, the dosage of SPY in the parent strongly affected the penetrance of gamete lethality. We hypothesize that the SEC or SPY proteins or O-GlcNAcylated products needed for gamete development can be supplied by parental tissues and that parents with a higher dosage of SEC and SPY provide more of the limiting factor(s), thereby reducing the penetrance of the synthetic lethal phenotype.

Parental suppression of lethal gametophytic mutations may be a common phenomenon in plants. Bonhomme et al. (1998) observed a low recovery of gametophytic mutations from tDNA insertional lines. It was expected that such mutations would be frequent given the prediction that 60–80% of all plant genes are expressed in the male gametophyte (Willing et al. 1988; Mascarenhas 1990). Bonhomme et al. (1998) postulated that both gene redundancy and “metabolic supplementation” could account for the paucity of gametophytic lethals. One recent example of maternal supplementation was observed in studies with the proliferating (PRL) gene of Arabidopsis (Springer et al. 2000). The PRL gene encodes an MCM-like protein, known in yeast and animal systems to be involved in DNA replication. Loss of prl was embryo lethal, but not completely lethal for

Figure 9.—Calculating the transmission rate of the sec spy chromosome from a + + + + + + + + female parent. The third column shows the frequency for each of the possible genotypes resulting from the cross shown in Figure 8G. See Figure 7 for a description of the equations and calculations used. The calculated values for \( \alpha \) and \( F \) were 0.96 and 0.73, respectively.
the female gametophyte. Maternal supplementation was suggested as one possible explanation for the incompletely penetrant gametophytic lethality of prl. An alternative hypothesis is that maternal or paternal effects are caused by imprinting of the respective alleles; however, imprinting gives a differential effect on either maternal or paternal gametes (Kinoshita et al. 1999; Luo et al. 2000). Because both gametes are affected by loss of SEC and SPY, imprinting is not a likely explanation for the parental affects observed in these experiments.

The carryover of parental SEC, SPY, or O-GlcNAcylated substrates may have also contributed to the phenotypes observed for double-mutant embryos. Double-mutant embryos aborted at various stages of development with none completing embryogenesis and producing viable seeds, suggesting that parental supplementation can partially support embryo development.

The synthetic interaction between sec and spy suggests that OGT activity and protein O-GlcNAcylation are essential for gamete and seed development. Deletion of the mouse OGT gene is lethal (Shafi et al. 2000). Therefore, protein O-GlcNAcylation is likely to be an essential modification in both plants and animals. Interestingly and in contrast to animals, which have one OGT, searches of GenBank have identified both SEC- and SPY-like genes in petunia, soybean, tomato, cotton, M. truncatula, maize, barley, and wheat, suggesting that all angiosperms have two OGTs. These searches have not identified any other candidate OGTs, suggesting that plants have only two OGTs.

The presence of two OGTs in plants raises the possibility that each has a specialized function(s). This hypothesis is supported by the observation that spy plants exhibit phenotypes while sec plants have no obvious phenotypes. While multiple spy alleles have been recovered in independent screens for suppressors of GA deficiency or...
reduced GA response (Jacobsen and Olszewski 1993; Wilson and Somerville 1995; Silverstone et al. 1997), see mutants were not recovered in these screens. These observations do not rule out the possibility that see plays a minor role in GA signaling. Therefore, characterization of see mutants to determine if they have subtle defects in GA signaling are ongoing. This work also suggests that SPY has additional functions in plant development that have not been revealed in previous studies involving the analysis of spy plants. Future experiments are aimed at determining the possible unique functions of SEC as well as the essential functions carried out by both SEC and SPY proteins.

The authors thank Michael Simmons and George Weiblen for helpful advice and discussions and also David Marks, John Ward, Tongseng Tseng, Tina M. Thornton, Steve M. Swain, and Manjula Gopalraj for advice and assistance with laboratory techniques. L.M.H. was supported, in part, by a postdoctoral fellowship awarded by the University of Minnesota Plant Molecular Genetics Institute. This research was supported by Research Grant No. IS-2837-97 from BARD, The United-Isreal Binational Agricultural Research and Development Fund, and grants from the National Science Foundation (MCB-9604126, MCB-9983583, and MCB-0112826) to N.E.O.

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Communicating editor: C. S. Gasser