**HAWAIIAN SKIRT: An F-Box Gene That Regulates Organ Fusion and Growth in Arabidopsis**

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A fast neutron-mutagenized population of Arabidopsis (Arabidopsis thaliana) Columbia-0 wild-type plants was screened for floral phenotypes and a novel mutant, termed hawaiian skirt (hws), was identified that failed to shed its reproductive organs. The mutation is the consequence of a 28 bp deletion that introduces a premature amber termination codon into the open reading frame of a putative F-box protein (At3g61590). The most striking anatomical characteristic of hws plants is seen in flowers where individual sepals are fused along the lower part of their margins. Crossing of the abscission marker, ProPGAZAT\(\beta\)-glucuronidase, into the mutant reveals that while floral organs are retained it is not the consequence of a failure of abscission zone cells to differentiate. Anatomical analysis indicates that the fusion of sepal margins precludes shedding even though abscission, albeit delayed, does occur. Spatial and temporal characterization, using ProHWS\(\beta\)-glucuronidase or ProHWS\(\beta\)-green fluorescent protein fusions, has identified HWS expression to be restricted to the stele and lateral root cap, cotyledonary margins, tip of the stigma, pollen, abscission zones, and developing seeds. Comparative phenotypic analyses performed on the hws mutant, Columbia-0 wild type, and ProHWS HWS ectopically expressing lines has revealed that loss of HWS results in greater growth of both aerial and below-ground organs while overexpressing the gene brings about a converse effect. These observations are consistent with HWS playing an important role in regulating plant growth and development.

Abscission involves the detachment of an organ from the body of a plant and takes place at a site that is predestined for the purpose (Sexton and Roberts, 1982; González-Carranza et al., 1998). The phenomenon may be triggered by a range of environmental stresses including drought, waterlogging, nutrient deficiency, or pathogen attack (Addicott, 1982; Taylor and Whitelaw, 2001), but is also programmed to occur at discrete stages during plant development such as after leaf senescence, flower fertilization, or fruit ripening have taken place. Although the precise events that bring about shedding are unclear the process is preceded by an increase in the activity of several wall-loosening enzymes including \(\beta\)-1,4 glucanase and polygalacturonase precisely at the site of abscission (Roberts et al., 2002). It is proposed that the action of these enzymes, coupled with an increase in expansin activity (Belfield et al., 2005; Sampedro and Cosgrove, 2005), may lead to the dissolution of the middle lamella that brings about cell separation (González-Carranza et al., 2002; Roberts et al., 2002).

To dissect further the mechanisms responsible for regulating the abscission process, forward genetic strategies have been employed to identify non- or delayed-shedding mutants followed by the mapping and characterization of the mutated genes (Patterson, 2001; Roberts et al., 2002). While a number of such mutants have been documented only a few of the genes responsible have been cloned and characterized. These include jointless, which is the consequence of a mutation in a MADS-box gene involved in the differentiation of abscission cells in the pedicel of tomato (Solanum lycopersicum) flowers (Mao et al., 2000), and inflorescence deficient in abscission where the mutated gene has been shown to encode a novel class of ligand that seems to play a key role both in defining the site of cell separation (Stenvik et al., 2006) and in regulating the final step of floral organ shedding in Arabidopsis (Arabidopsis thaliana; Butenko et al., 2003). Recently,

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quantitative trait loci analysis has led to the identification of a quantitative trait locus (sh4) that has played a critical role in the domestication of rice (Oryza sativa) by bringing about a reduced shattering phenotype (Li et al., 2006). Although the function of the gene is unknown its characteristics suggest that it acts as a transcription factor. Other genes that have been identified to contribute to the abscission process in Arabidopsis include: HAESA that encodes a Leu-rich receptor-like kinase (Jinn et al., 2000); AGAMOUS-LIKE15, a MADS-domain transcription factor that if nonfunctional results in a delay in the time course of abscission (Fernandez et al., 2000; Harding et al., 2003; Lehti-Shiu et al., 2005); and the redundant BLADE ON PETIOLE1 (BOP1) and BOP2 genes encoding two BTB/POZ domain proteins that when silenced result in a disruption in leaf patterning and floral organ abscission (Hepworth et al., 2005; Norberg et al., 2005).

During the screening of a fast neutron-mutagenized population of Arabidopsis Columbia-0 (Col-0) wild-type plants a mutant, termed hawaiian skirt (hws), was isolated that retained its floral organs even after silique desiccation had taken place. In addition to exhibiting a nonshedding phenotype hws also exhibited sepal bases that are fused for some distance along their margins. Other mutants from Arabidopsis that show varying degrees of sepal fusion include unusual floral organs (ufo) where the mutated gene has been shown to encode an F-box protein (Levin and Meyerowitz, 1995; Samach et al., 1999); leafy, which is the result of a mutation in a floral meristem identity gene (Schultz and Haughn, 1991; Weigel and Meyerowitz, 1993); and the cup shaped cotyledon (cuc) mutants (cuc1, cuc2, cuc3) that arise as a consequence of mutations in putative NAC-transcription factor genes (Aida et al., 1997; Takada et al., 2001; Vroemen et al., 2003). Interestingly, ectopic expression of the microRNA miR164, which has been shown to induce posttranscriptional down-regulation of CUC1 and CUC2 (Laufs et al., 2004), leads to the generation of flowers with fused sepals that fail to undergo the normal shedding process (Mallory et al., 2004). A number of other genes have been shown to play an important role in the specification of lateral organ boundaries in leaves or flowers including LATERAL ORGAN BOUNDARIES (Shuai et al., 2002), LATERAL ORGAN JUNCTIONS (Prasad et al., 2005), PETAL LOSS (Brewer et al., 2004), the FUSED FLORAL ORGANS (FFO) loci (FFO1, FFO2, FFO3; Levin et al., 1998), HANABA TARANU (Zhao et al., 2004), and RABBIT EARS (Krizek et al., 2006). In this article, the mapping and identification of a mutation in a putative F-box gene (At3g61590), which is responsible for bringing about the hws-1 phenotype, is described. Although the transcript of HWS accumulates throughout the plant, reporter gene analysis reveals that expression is restricted to only certain tissues. By comparing and contrasting the phenotypic features of hws-1, an overexpressing HWS line driven by the 35S cauliflower mosaic virus (CaMV) promoter wild-type plants, a role for HWS in regulating plant growth and development has been highlighted.

**RESULTS**

**Mutant Isolation and Characterization**

The hws-1 mutant was isolated, as a result of an inability to shed its floral organs, during a screen of M2 progeny grown from a fast neutron-mutagenized population (dose: 55 Gy; Lehle seeds) of M1 Arabidopsis seeds in the Col-0 (wild type) background. Sepals, petals, and anther filaments were retained throughout reproductive development and remained in situ even after silique desiccation and dehiscence had taken place. To study flower development in more detail material was harvested from positions throughout the primary inflorescence with the first being where petals were visible. From that position all subsequent flowers were numbered. A scanning electron microscope (SEM) study of hws-1 flowers revealed that sepals of the mutant were fused, for a distance along the lower part of their margins, and that characteristically the sepal whorl was broader than that seen in wild-type plants (Fig. 1, A–F). While the shedding of petals and anther filaments did not take place in the mutant, fine structural analysis of these tissues indicated that abscission of these organs could be detected in both wild-type and hws-1 flowers (Fig. 1, G–J). Cell separation at the sepal bases was also apparent in hws-1 flowers, however, the timing of this was delayed in comparison to wild-type plants and the sepal whorl was retained even though abscessed (Fig. 1, H and J).

Further SEM analysis revealed that no distinction could be made between the wild-type and hws-1 plants during early bud development (Fig. 2, A, B, E, and F). However, by the time the buds had reached stage 10 (Smyth et al., 1990) the line of separation between individual sepals had become demarcated to the bud base in wild-type but not in hws-1 material (Fig. 2, C and G). Initially the region of sepal confluence in hws-1 is restricted to just a few cells; however, further division must take place so that by the time buds have reached stage 12 sepals are fused for nearly 25% of their distance (Fig. 2, D and H).

The hws-1 mutant was crossed with a wild-type plant and a 3:1 segregation of shedding;nonshedding individuals in the F2 population showed that the phenotype is due to a recessive mutation in a single gene of nuclear origin.

**Crossing of an Abscission Zone-Specific Gene Marker into the hws-1 Mutant**

To determine whether the nonshedding phenotype of hws-1 was due to a failure of abscission zone (AZ) differentiation a cross was made between the mutant and a transgenic marker line (ProPGAZAT::GUS) that expresses the reporter gene GUS specifically at the site of floral organ separation. PGAZAT (At1g41850) encodes a polygalacturonase that is transcribed immediately prior to organ abscission in Arabidopsis and is thought to contribute to cell wall degradation (González-Carranza et al., 2002). Several homozygous
ProHWS comparison, expression of detectable at the site of floral organ abscission. For plants and by position 20, in contrast to wild-type ProPGAZAT and H) and sections of flower AZs stained with Toluidine blue in Col-0 wild type (G 6 (B and E), and 14 (C and F). Light microscopy of 5

Figure 1. SEM and light microscope analyses of wild-type and hws-1 flowers. Scanning electron microscopy of flower development stages in Col-0 wild type (A–C) and hws-1 plants (D–F) at positions 2 (A and D), 6 (B and E), and 14 (C and F). Light microscopy of 5 μm longitudinal sections of flower AZs stained with Toluidine blue in Col-0 wild type (G and H) and hws-1 plants (I and J) at position 4 (G and I), when petals (Pe) and sepals (Se) are still attached, and position 12 (H and J), when only sepals are attached to the hws-1 mutant and siliques (Si) are visible at all stages. Sites of sepal (Se-AZ), petal (Pe-AZ), and anther filament (Fil-AZ) abscission are indicated. Bars = 500 μm (A–F) and 50 μm (G–J). [See online article for color version of this figure.]

hws-1 lines containing the GUS gene were isolated. Figure 3, A and B, shows that both wild-type and mutant plants express the ProPGAZAT:GUS gene at the base of the sepals, petals, and anther filaments, indicating that the hws mutation does not seem to impede AZ differentiation. However, although ProPGAZAT:GUS expression is apparent in position 8 flowers of wild-type plants (Fig. 3A), it cannot be detected until position 10 in hws-1 flowers (Fig. 3B). In addition, ProPGAZAT:GUS expression is less pronounced in hws-1 plants and by position 20, in contrast to wild-type plants, expression of the reporter gene is no longer detectable at the site of floral organ abscission. For comparison, expression of ProHWS:GUS is shown at different positions throughout floral development (Fig. 3C). However, these observations will not be described in detail until a later part of the ‘Results’ section of this article.

Other Phenotypic Features of the hws Mutant

The identification of another allele of HWS (hws-2) from the SALK collection (Alonso et al., 2003), during the course of mapping the gene, lead to the confirmation of additional phenotypic characteristics associated with the hws-1 mutation.

A detailed analysis revealed that 28% of flowers in the hws-1 mutant had fused anther filaments to differing extents along their length (Fig. 4A), compared with 2% in the wild type. Occasionally, in hws-1 plants, these filaments were fused to the side of siliques (Fig. 4B). The top of the hws-1 siliques was consistently broader than wild type, and the length of the AZ region, exposed by manually removing the floral organs, was longer in the mutant (Fig. 4, C and D). Some hws-1 siliques comprised more than two valves (Fig. 4E) and dissection of aberrant pods revealed that this was associated with abnormal development of the septum (Fig. 4F). The lamina tissue of the primary cauline leaves of hws-1 plants routinely exhibited fusion to the inflorescence stems (Fig. 4G).

Mapping the hws Locus

Homozygous hws-1 (Col-0 ecotype) plants were crossed with the Landsberg erecta (Ler) ecotype and the F2 progeny used as a mapping population. An amplified fragment length polymorphism (AFLP)-based genome-wide approach (Peters et al., 2004) was adopted to map the hws mutation to a 3.28 Mb domain at the bottom of chromosome 3. Using cleaved amplified polymorphic sequences (CAPS), simple sequence length polymorphisms (SSLPs), and insertion/deletion (InDel) markers this interval was reduced to a region of 56 kb containing 18 annotated genes (Fig. 5A).

Insertional mutant lines from the SALK collection of these 18 candidate genes were examined and two individuals in a population of 50 plants from line SALK_088349 (located in the gene At3g61590) exhibited phenotypic characteristics reminiscent of hws-1. Further analysis of this line revealed that it contained two T-DNA insertions, arranged in opposing configurations, downstream from the ATG of the At3g61590 gene. These insertions were located 475 and 491 bp from the ATG (Fig. 5B).

PCR amplification of the At3g61590 genomic region of wild type and hws-1 DNA and restriction analyses of the amplified products with the high frequency cutting enzymes AluI, Rsal, and TaqI revealed subtle differences in banding patterns between the two genotypes (Fig. 5C). Sequencing of this region from hws-1 revealed that it contained a 28 bp deletion located 966 bp downstream of the translation start of the open reading frame (ORF) of At3g61590. The consequence of this deletion is to introduce a frame shift resulting in the introduction of a premature termination amber codon in place of an Ile residue and the predicted production of a truncated version of the At3g61590 protein (Fig. 5B).
To determine whether the hws-1 phenotype was a consequence of a mutation in the At3g61590 gene, a cross between the mutant and the SALK_088349 line (knockout [KO]) was performed. All progeny of this cross displayed hws-1 characteristics. To test that these had not arisen by a self-pollination event two plants were analyzed by PCR and reverse transcription (RT)-PCR. The PCR demonstrated that both a gene-specific product (originating from the hws-1 mutant) and an insertion product (originating from the KO) were amplified in each individual but only the former was present in the wild-type control (Fig. 5D). RT-PCR analysis of RNA extracted from various tissues of the SALK_088349 line and from two F1 plants (hws-1 × KO) showed no At3g61590 expression in the KO but the presence of a transcript in both progeny (Fig. 5E).

Proof that HWS is encoded by the At3g61590 gene was obtained by complementing the hws-1 mutant with a 3.513 kb fragment amplified from wild-type DNA containing 1,291 bp upstream of the promoter, plus the 5′ and 3′ untranslated regions (UTRs), and intron and exon of the At3g61590 gene. This segment proved to be of sufficient length to rescue fully the hws-1 mutant (Fig. 5F).

**Figure 2.** SEM analysis of wild-type and hws-1 buds at developmental stage 6 (A and E), stage 8 (B and F), stage 10 (C and G), and stage 12 (D and H). Buds dissected from Col-0 wild type (A–D) and hws-1 (E–H) plants. Bars = 100 μm.

**Figure 3.** Flower and silique expression from ProPGAZAT::GUS, ProPGAZAT::GUS crossed into hws-1, and ProHWS::GUS. Time course of expression from different developmental positions of flower and siliques of GUS-stained tissue after 12 h incubation in GUS substrate. Flowers are identified from positions 2 to 20. Position 1 is the first flower where petals are visible to the eye. A, ProPGAZAT::GUS. B, ProPGAZAT::GUS crossed into hws-1. C, ProHWS::GUS. Bars = 1 mm.
HWS Encodes a Putative F-Box Protein

The likely function of the HWS gene (At3g61590) is that it encodes an F-box protein. These proteins, as part of a SCF complex, are proposed to interact with a substrate leading to their degradation by the 26S proteasome (Ni et al., 2004). HWS encodes a protein of 412 amino acids. It has no introns within the ORF but has an intron of 532 bp within the 5′ UTR of the gene (Fig. 5B). The truncated version of the mutant protein, predicted to be generated in hws-1 plants, contains the intact F-box region. Information retrieved from the Web site PlantsUBQ, which is a functional genomics database for the Ubiquitin/26S proteasome proteolytic pathway in plants (http://plantsubq.genomics.purdue.edu/plantsubq/cgi-bin/detail.cgi?db=plantsubq&id=163137), indicates that the predicted F-box domain of HWS is located between amino acids 40 and 85. The software also proposes the existence of a transmembrane-spanning region between amino acids 120 and 140 and a low similarity (29.3%) to a Kelch_2 motif in the region between amino acids 290 and 338.

Outside the F-box region, the ORF of HWS gene (At3g61590) is that it encodes an F-box protein. These proteins, as part of a SCF complex, are proposed to interact with a substrate leading to their degradation by the 26S proteasome (Ni et al., 2004). HWS encodes a protein of 412 amino acids. It has no introns within the ORF but has an intron of 532 bp within the 5′ UTR of the gene (Fig. 5B). The truncated version of the mutant protein, predicted to be generated in hws-1 plants, contains the intact F-box region. Information retrieved from the Web site PlantsUBQ, which is a functional genomics database for the Ubiquitin/26S proteasome proteolytic pathway in plants (http://plantsubq.genomics.purdue.edu/plantsubq/cgi-bin/detail.cgi?db=plantsubq&id=163137), indicates that the predicted F-box domain of HWS is located between amino acids 40 and 85. The software also proposes the existence of a transmembrane-spanning region between amino acids 120 and 140 and a low similarity (29.3%) to a Kelch_2 motif in the region between amino acids 290 and 338.

Outside the F-box region, the ORF of HWS shows only a low level of sequence similarity with other putative F-box proteins that have been annotated within the Arabidopsis genome. The Arabidopsis gene encoding a protein with highest sequence similarity (approximately 30%) to HWS is UFO. UFO is a protein that has been shown to be required for normal patterning and growth of the floral meristem (Samach et al., 1999).

Spatial and Temporal Expression of the HWS Gene

RT-PCR analysis of RNA isolated from wild-type plants revealed that the HWS transcript is expressed in many different tissues of the plant. Levels of expression were highest in buds and flowers, however, expression could also be detected in roots, leaves, stem, and siliques (Fig. 6). A more detailed analysis of the spatial and temporal pattern of expression, with the aid of the GUS or green fluorescent protein (GFP) reporter genes fused to the HWS promoter, showed that the gene is expressed at discrete sites within a range of tissues including the outer margins of cotyledons (Fig. 7A), the sepals of young buds and flowers (Fig. 7, B–D), the stigmatic papillae and tip of the elongating silique (Fig. 7, E and G), the base and vascular tissue of petals and sepals (Fig. 7F), the anther filaments and pollen (Fig. 7G), the floral and cauline leaf AZs (Fig. 7, H and I), the testa of developing seeds (Fig. 7J), the vascular tissue of the primary root and emerging laterals (Fig. 7K), and lateral root cap (Fig. 7L). A detailed time course of GUS accumulation during flower and silique development revealed that intense HWS expression could be detected at the site of floral organ abscission in position 8 flowers continuing up to the stage when desiccation of the pods took place (Fig. 3C).

Ectopic Expression of the HWS Gene Produces Smaller Seedlings

Transgenic plants were generated where the ORF from the HWS gene was expressed ectopically using a double 35S CaMV promoter. Homozygous plants from two lines (8.3, A23.3) were examined in detail as RT-PCR analysis had indicated that these lines most strongly expressed both HWS and the transgene (Supplemental Fig. S1) and contained only a single insertion (data not shown).

Line 8.3 exhibited a more severe phenotype than line A23.3 in all the experiments that were undertaken. A growth study, carried out 2 weeks after emergence, revealed that both ectopic expressing lines were substantially smaller than wild type (Fig. 8A). However, hws-1 plants at the same stage were larger than the control (Fig. 8A). Compared to the wild type, the rosette leaves of the overexpressing lines had shorter petioles, narrower and more rounded lamina with serrated borders, and displayed a greater degree of hyponastic bending (Fig. 8B).

Impact of Ectopic Expression of HWS on Flower Development

A comparison of flower development in wild type, hws-1, and Pro35S:HWS line 8.3 demonstrated that...
Figure 5. Mapping and identification of the HWS gene. A, Mapping strategy used to identify the HWS locus using an AFLP approach to analyze the DNA from 33 F2 hws-1 mutant plants. The initial location of the locus was identified to a region of 3.28 MB at the bottom of chromosome 3 between the markers SM148,6 and SM239,119,5. Further use of CAPS and SSLP on DNA from 156 F2 hws-1 mutant plants allowed the reduction of the region to a segment of 0.4 MB. With the use of InDels on DNA from 18 Candidate genes were tested by:

a) Growing of SALK lines to observe if phenotype of the hws could be identified
b) PCR and Frequent cutter restriction enzymes

B

C

D

E

F

hawaiian skirt and Arabidopsis Development


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organ shedding took place at an earlier developmental stage in the overexpressing line (position 10) compared to the wild type (position 12). Flowers from line 8.3 also underwent sepal senescence prematurely, as evidenced by a visual decline in chlorophyll, compared to the wild type (Fig. 9; Supplemental Fig. S2). Figure 9 shows that hws-1 had the longest, while the overexpressing line had the shortest, stigmatic papil- lae. A close-up view of flowers confirmed these observations (Supplemental Fig. S2).

Measurements of dissected sepals and petals from flowers at position 3 revealed that hws-1 had significantly longer and wider sepals and petals compared to the wild type (Fig. 9; Supplemental Fig. S2). Figure 9 shows that hws-1 had the longest, while the overexpressing line had the shortest, stigmatic papil- lae. A close-up view of flowers confirmed these observations (Supplemental Fig. S2).

Manipulation of HWS Expression Affects Root Growth and Seed Size

Seeds from hws-1, wild type, and the two overexpressing lines were germinated in germination medium (GM) or Murashige and Skoog media and root length was measured after a period of 2 weeks. The mutant exhibited the longest roots while both Pro35S:HWS lines 8.3 and A23.3 had significantly shorter roots than the wild type (Fig. 10A).

The dimensions of mature seeds from the three different genotypes were analyzed. The hws-1 mutant was found to have statistically larger (both in length and width) seeds compared to the wild type while the overexpressing lines produced the smallest seeds (Fig. 10B).

DISCUSSION

We have described the isolation and characterization of a novel Arabidopsis mutant termed hws that fails to shed its floral organs. The mutated gene responsible for bringing about this phenotype (At3g61590) encodes a putative F-box protein. HWS is expressed throughout the plant but reporter gene analysis indicates that expression is restricted to specific tissues. Loss of HWS function results in an elevation of plant size while overexpression of the gene, using the 35S CaMV promotor, generates plants exhibiting a significant reduction in root and vegetative shoot growth. These observations are consistent with HWS playing a role in the regulation of organ development in Arabidopsis.

Phenotype of the hws Mutant

The hws-1 mutant was originally isolated as a consequence of an inability to shed its sepals, petals, and anther filaments. Indeed, these organs are retained throughout silique growth and development and remain at the base of the siliques even after desiccation and dehiscence is complete. A close examination of the flowers has revealed that the mutation results in the fusion of the sepals along their basal margins. Ectopic expression of the microRNA miR164 results in the generation of flowers with similar characteristics (Mallory et al., 2004). This miRNA is thought to act by targeting for degradation the mRNA of the genes CUC1 and CUC2 (Laufs et al., 2004). In addition to exhibiting fused sepals, hws-1 has a prevalence to generate fused anthers, multi-valved first-formed siliques, and fusion of the calyxeal leaf lamina to the inflorescence stem. These observations suggest that, like CUC1 and CUC2, the HWS gene might act to regulate lateral boundary development and that the degree of penetrance of some of its phenotypic characteristics could be due, in part, to redundancy between HWS and other peptides.

An examination of the early stages of development in wild-type and hws-1 plants indicates that floral morphology is initially indistinguishable, however, while sepal separation proceeds to the base of the bud in wild type this is terminated prematurely in hws-1 material. Thus the hws phenotype is not the consequence of postgenital fusion of the sepals but due to their failure to undergo complete separation.
To test whether differentiation of the floral AZs was taking place in hws-1 we crossed the gene marker ProPGAZAT::GUS into the mutant. PGAZAT (At2g41850) encodes a polygalacturonase that is expressed specifically within the AZ cells of Arabidopsis and has been proposed to play a role in middle lamella degradation (González-Carranza et al., 2002). The results show that hws-1 is not compromised in terms of AZ differentiation, however, the onset and duration of ProPGAZAT::GUS expression is delayed and reduced, respectively, in the mutant. These revelations, together with our SEM and light microscope observations, indicate that the non-shedding phenotype of hws-1 is not the consequence of a failure of cell separation to take place at the base of petals, anther filaments, and sepals. While the fusion of the sepal margins, encircling the separated petals and anthers, provides a structural barrier to preclude organ shedding in hws-1 our data indicate that the timing of cell separation in the mutant is delayed.

**HWS Gene Encodes an F-Box Protein**

Mapping and characterization of the hws-1 locus has revealed that the mutant phenotype is a consequence of a 28 bp deletion in the ORF of a gene (At3g61590) encoding a putative F-box protein. The 28 bp deletion in hws-1 introduces a premature translation termination codon, predicted to truncate HWS. The phenotype of hws-1 is indistinguishable from that of a null mutant (SALK_088349; hws-2) caused by the insertion of two T-DNAs into At3g61590, suggesting that the shortened HWS protein, if synthesized, is nonfunctional. Evidence to support the proposal that HWS functions as an F-box protein has come from the demonstration by Takahashi et al. (2004) that in a targeted yeast (Saccharomyces cerevisiae) two-hybrid screen At3g61590 strongly associates with a number of Arabidopsis Skp1-related proteins (ASKs). We have confirmed that HWS interacts with both ASK1 and ASK4 in an anther-specific yeast two-hybrid library (X. Zhang, Z.H. González-Carranza, and J.A. Roberts, unpublished data).

HWS contains a single intron located within the 5′ UTR region of the gene. A bioinformatic analysis of HWS indicates that in addition to having an F-box domain, the protein contains a predicted transmembrane sequence, and a Kelch-like repeat region. The truncated protein produced by the hws-1 mutant would lack an important element of the Kelch_2 motif and it has been proposed that this region, in other F-box proteins, might play a key role in the recognition of the substrate (Jarillo et al., 2001). It is possible therefore that the abbreviated version of HWS might have the capacity to bind ASKs but not the peptide targeted for degradation. Over 700 annotated genes within the genome of Arabidopsis have been classified,
on the basis of sequence similarity, to be members of the F-box super family. The ORF of HWS shows only a low level of sequence similarity to other family members with the highest (approximately 30%) being to that encoded by the UFO gene. UFO has been shown to have a role in several aspects of flower development. Mutations in the UFO gene can bring about inappropriately fused floral organs (Levin and Meyerowitz, 1995) through its role in regulating patterning of primordial initiation. In addition, UFO is required for the specification of organ identity through its ability to promote the function of the type B class of floral homeotic genes (Durfee et al., 2003) and, by conditionally restoring UFO function, an additional role for the protein in regulating petal outgrowth has been identified (Laufs et al., 2003). There is no evidence that HWS plays a role in specifying organ identity as neither alleles of the hws mutant, that we have examined, show consistent evidence of abnormalities in floral organ identity. In addition, it is evident that HWS functions within vegetative tissues and that, if silenced, results in fusions of tissues such as the cauline leaf lamina to the stem of the inflorescence.

Expression of HWS

As the phenotypic characteristics of the hws-1 mutant were primarily restricted to the shoot and reproductive tissues it was a surprise to discover that the transcript of the HWS gene could be detected by RT-PCR at high levels throughout the plant. Reporter gene analysis, using either GUS or GFP, revealed that although expression is evident in many tissues HWS promoter activity is restricted to specific cell types or regions of an organ. In roots, the pattern of expression is limited to the vascular tissues and the cells that comprise the lateral root cap. In leaves and stems expression is rarely detected in the vasculature but is associated with the margins of cotyledons. Floral tissues strongly express HWS with GUS activity being detected throughout development in sepal, the distal

Figure 8. Comparative phenotypic analysis of young plants. Two-week-old plants were analyzed and compared from: (1) Pro_{35S}:HWS homozygous line 8.3, (2) hws-1, (3) Col-0 wild type, and (4) Pro_{35S}:HWS homozygous line A23.3. A, A comparison of 2-week-old plants. B, Plants from A dissected to show size, shape, and number of leaves and cotyledons. Images of the remaining plantlets are on the far right. Bars = 5 mm. [See online article for color version of this figure.]

Figure 9. Comparative phenotypic analysis of flowers. Flowers of 4-week-old plants were analyzed and compared. The flowers are identified from position 1 to 15, considering position 1 as the first flower where petals are visible to the eye, and in the hws-1 mutant a dry silique was included to show the presence of floral organs at that late stage of development. A, Col-0 wild type. B, hws-1 mutant; note the bigger stigmatic papillae in the flowers and wider floral bases. C, Pro_{35S}:HWS line 8.3; note that abscission takes place earlier (position 10) than in the Col-0 wild type (position 12). Bars = 1 mm. [See online article for color version of this figure.]
end of the stigma, anther filaments, and pollen. Expression is also evident in the AZs of caulline leaves and floral organs. Aspects of the root and floral organ expression pattern are reminiscent of that exhibited by the F-box protein TIR1 (Gray et al., 1999) that has been shown to encode a receptor for indole-3-acetic acid (Kepinski and Leyser, 2005). Mutations in the TIR1 gene confer a reduced sensitivity to indole-3-acetic acid, however, the responses of hws-1 to auxin are indistinguishable from wild-type plants (Z.H. González-Carranza and J.A. Roberts, unpublished data) and while ectopic expressing lines of HWS exhibit reduced root growth, elongation of laterals root is not promoted.

GUS expression in ProHWS::GUS plants is intense at both the site of abscission of floral organs and caulline leaves. Expression precedes sepal, petal, and anther filament shedding and is maintained throughout silique development and maturation. This spatial and temporal pattern of expression is similar to that observed by genes that have been proposed to contribute to the process of cell separation (González-Carranza et al., 2002). Although anatomical analysis indicates that floral organ shedding is precluded by fusion of the sepal in hws plants, rather than as a consequence of a failure to undergo cell separation, a comparison between the mutant, overexpressing lines and wild-type plants demonstrates that HWS does, in addition, have a direct influence on the timing of abscission. A study of the mutant material crossed with the ProPGAZAT::GUS gene also supports the assertion that HWS is necessary for cell separation to proceed at the normal rate.

Role of HWS in Plant Development

Although the principal characteristic of hws-1 plants is their nonshedding phenotype, the isolation of a null allele (hws-2) of the gene has enabled us to dissect other consistent phenotypic features. A key difference from wild-type plants is that hws-1 plants grown under the same environmental conditions have more elongated leaves and larger seeds. Plants ectopically expressing HWS exhibit a reduced overall stature compared to wild type with the degree of reduction being associated with the intensity of the up-regulation of the gene. These observations indicate that the target for HWS degradation plays a key role in the maintenance of organ growth. Indeed the impact of losing HWS can be seen in individual cells such as the stigmatic papillae where elongation is substantially elevated in the mutant and reduced in the overexpressing lines. A role for UFO in regulating organ growth has been previously identified (Laufs et al., 2003) and a number of F-box proteins have been linked to the regulation of the cell cycle (Taylor et al., 2001). Like UFO, HWS may have more than one direct role or perhaps may target more than one protein for degradation, alternatively, the consequence of HWS on sepal fusion might be mediated through its effects on the lateral expansion of sepal primordia, resulting in the generation of overlapping margins and a failure in organ separation. Intriguingly, silencing of AUXIN RESPONSE FACTOR 2, a transcription factor that mediates gene expression in response to auxin, also results in delayed floral organ abscission (Ellis et al., 2005) and increased growth of aerial organs and seeds (Schruft et al., 2006). Further work is ongoing to identify the mechanism by which HWS exerts its effect on growth and ascertain whether this may be mediated through an auxin-signaling pathway.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Arabidopsis (Arabidopsis thaliana) ecotypes Col-0 and Ler, the hws-1 mutant, SALK lines for the 18 genes in the 56 kb segment that were obtained from the Nottingham Arabidopsis Stock Centre, diverse crosses, and the mapping population were grown in a glasshouse with a temperature of 23°C ± 2°C in plastic pots containing Levington M3 (The Scotts Company) compost and Vermiculite (2.0:5.0 mm, Sinclair) in a 3:1 ratio, respectively. To grow plants under sterile conditions, petri plates were prepared with 4.33 g L−1 of Murashige and Skoog basal salt mixture, pH 5.9 (Sigma; Murashige and 

Figure 10. Comparative phenotypic analysis of root length and seed size. A, Twenty-six roots from 2-week-old seedlings grown in GM were photographed and their roots measured and statistically analyzed. Root length comparisons among ProHWS line 8.3, hws-1 mutant, Col-0 wild type, and ProHWS line A23.3. The significance was analyzed by ANOVA test. *, Different characters (x, y) indicate a significant difference in the mean at P < 0.05, n = 26. B, Seventy dry seeds from five independent plants were photographed, measured, and statistically analyzed. Seed length and width comparisons among ProHWS line 8.3, hws-1 mutant, Col-0 wild type, and ProHWS line A23.3. The significance of the differences was analyzed by ANOVA test. *, Different characters (p, q, r and x, y, z) indicate a significant difference in the mean at P < 0.05, n = 70.
Skog, 1962), and 0.8% agar or GM (Sedbrook et al., 2002); seeds were sterilized in a solution containing 50% (v/v) sodium hypochlorite then rinsed with a solution containing 0.01% (v/v) Triton X-100 for 5 min followed by ethanol (70% v/v). Seeds were then rinsed five times in sterile water before being placed, using a pipette tip, on top of the Murashige and Skog plant agar petri dishes. Plates were sealed with micropore tape (3 M) and maintained at 4°C for 72 h to stratify them and to facilitate uniform germination. Plates were then transferred to a growth room with a temperature of 23°C ± 1°C under 16 h light/8 h dark.

The position of the flower was determined from the first site where petals were visible. From that position all subsequent flowers were numbered.

**Scanning Electron Microscopy**

Inflorescence apices and flowers were taken from Col-0 (wild type) and hws-1 plants. Buds were staged in accord with Smyth et al. (1990) and flowers were harvested at inflorescence position 2, position 4, and position 14. The apex of the floral inflorescence was excised and placed directly into fixative (4% glutaraldehyde [w/v] in 0.5M potassium phosphate buffer, pH 7.5) for 4 h prior to dehydration through a graded ethanol series and critical point drying in liquid CO2. Specimens were then mounted on steel stubs with double-sided tape and dissected to reveal the floral primordia. They were sputter coated with gold palladium before viewing. Flowers were viewed immediately without additional fixation or sputter coating at 20 kV accelerating voltage in variable pressure mode under low vacuum (70 Pa). All samples were viewed under a Hitachi S-3000 SEM. A minimum of five buds/flower were examined from each position.

**Light Microscopy**

Unopened and open flowers were collected from wild type and the hws-1 mutant when the plants were 1 month old. Young buds, mature buds, and flowers from positions 4, 6, 8, 12, 16, or 20 were fixed in 4% (v/v) paraformaldehyde: phosphate-buffered saline (PBS) buffer (1.3 mM NaCl, 0.03 mM NaHPO4, 0.03 mM Na2HPO4, pH 7.2, 0.1% [v/v] Triton X-100, and 0.1% [v/v] Tween 20) overnight at 4°C. Tissues were washed with PBS at 4°C for 1 h, then brought to room temperature and washed with PBS for 1.5 h. The tissues were postfixed with 2% (w/v) O2O4 in PBS at room temperature for 3 h, then rinsed twice in PBS for 15 min and dehydrated with ascending series (30%, 50%, 70%, 90%, and 100% [v/v], twice, for 1 h in each). Specimens were processed with 100% ethanol/propane oxide (1:1) for 20 min, washed three times with propane oxide for 10 min, and propylene oxide/Suwrps resin (1:1; TAAB) for 1.5 h. Specimens were infiltrated with pure Spurrs resin at 4°C for 10 to 12 h. Individual samples were placed in appropriate flat-labeled silicone embedment molds filled with Spurrs resin and placed at 72°C for 10 to 12 h.

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**Phenotypic and Statistical Analysis**

Six plants from the hws-1 mutant, wild type, and 355 CaMV ectopic expression lines 8.3 or A23.3 were grown under the same conditions and 25 flowers were dissected and analyzed under a Zeiss Stemi SV6 Stereo microscope. Organ fusion and other phenotypic characteristics were recorded either to map the hws locus to a region of 3.28 MB between markers SM146.6 and SM219.1195 at the bottom of chromosome 3 using an AFLP strategy as described by Peters et al. (2004).

With a further analysis of 156 hws-1 mutant plants from the original F2 population, using a combination of CAPS and SSLP, the region was reduced to a 0.404 MB between the markers FUS6.2 and NGAl6. From 600 F2 hws-1 mutant plants, and the use of InDels that were identified from the Cereon Arabidopsis Polymorphism collection (Cereon Genomics; http://www.arabidopsis.org/Cereon/index.html), the region was further reduced to a segment of 56 kb between markers 470113 and 469675. This region contained 18 annotated genes (Fig. 5A). The InDel flanking primers designed for fine mapping hws-1 were summarized in Supplemental Table S1. PCR were performed in a reaction of 20 µL following red tag (ABgene) manufacturer instructions; the PCR conditions for amplification were: 3 min at 94°C, 30 cycles of 94°C for 30 s, 55°C to 60°C for 30 s (depending on primer combination), 72°C for 30 s, and 7 min at 72°C, 4°C ∞. PCR products were run in a 3% low molten agarose gel (MetaPhor Agarose,Cambrex).

Salk KO lines were identified for the 18 annotated genes within this region and grown in the glasshouse. Individuals from one of these KO lines (Salk, 088349), documented to contain a T-DNA insertion in At3g1590, was used to prove the similarity to the hws-1 mutant.

Primers from the region corresponding to the At3g1590 gene that amplified a genomic PCR segment of 1.271 bp are At3g1590for: 5’-GCTTCTGA-GAATGGAGGCAAGAAC-3’ and At3g1590REV: 5’-CAGACCATTTGCCCTTCATGTC-3’. PCR reactions were performed in a 50 µL reaction following red tag (ABgene) manufacturer instructions. Conditions for amplification were: 3 min at 94°C, 30 cycles of 94°C for 15 s, 61°C for 1 min, 72°C for 2 min, 7 min at 72°C, 4°C, and the PCR products were run in a 1% agarose gel. The digestion of PCR products was performed in 20 µL reactions containing 500 ng of PCR product, 10× reaction buffer, 2 µg of bovine serum albumin, and 0.5 µL of each enzyme, incubated at 37°C (Rsa I and Alu I) or 65°C (Taq I) and run in a 3% agarose gel. The expected restriction sizes for the amplified PCR segment corresponding to the At3g1590 ORF genomic region are shown in Supplemental Table S2.

A plant from the Salk, 088349 KO line was used to cross with the hws-1 line and PCR analyses were performed to identify the presence of the two T-DNA insertions and the genomic fragment from the hws-1 mutant from the two parental lines; information about primers is described previously. To test the T-DNA insertion the LbII of pBin-PR02 for SALK lines primer was used: 5’-GGTCGAGCCTCGTTCTGCAACT-3’ (http://signal.salk.edu/tdnaprimer2.html; Alonso et al., 2003). PCR conditions were the same as for the PCR amplification of the At3g1590 segment described earlier.

**RT-PCR Analysis of Expression**

Total RNA from roots, buds, flowers, rosette leaves, stem, young siliques, and old siliques from wild type, and a mix of tissues from progeny plants 1 and 2 from the cross between the hws-1 mutant with Salk, 088349 line (hws-2) and from a mix of flowers from several developmental positions from F2 overexpressing lines, was extracted using a modified method described by Han and Grierson (2002) where the removal of carbohydrates was performed by differential precipitation of RNA using 4 µL LiCl at 20°C for 1 h instead of using the cetyl-trimethyl-ammnonium bromide buffer. RNA was quantified with a spectrophotometer and its quality was analyzed by visualization in a 1% (w/v) agarose gel.

Expression analyses were determined using the SuperScript II Reverse Transcriptase kit from Invitrogen according to the manufacturer’s instructions. cDNA synthesis was performed in a 20 µL reaction containing 2 µg of total RNA, 1 µL of 500 µg mL−1 oligo (dT), and 2 µL of 5 mM dNTPs and 13 µL of water. PCR reactions were performed in 25 µL following red tag (ABgene) manufacturer instructions; the PCR conditions for amplification were: 3 min at 95°C, 30 cycles of 95°C for 1 min, 50°C to 58°C for 1 to 2 min (depending on primer combination and size of amplified band), 72°C for 1 to 2 min, and 7 min at 72°C, 4°C. PCR products were run in a 1% agarose gel. The forward and reverse primers used in tissues from wild type, the progeny from crosses, and endogenous gene for overexpressing lines were 590 5’ UTR: 5’-CCCTCTCTCCTCCTCCTCCTCCTC3’ and At3g1590Rev (previously described)
that gave a genomic band of 2,213 bp and a cDNA of 1,668 bp. Use of these primers allows the identification of genomic contamination.

To amplify the transgene of overexpressing lines the primer pKT735Sprom 5'-GGAGGAGCATGCGAAGAAAAGC-3' from the 3S promoter and A3g61590C for were used. The amplified band from this primer combination is 1,677 bp.

Ubiquitin (AH1p63520) primers UBQ10For, 5'-TTCTCTCT-3' and UBQ10Rev, 5'-AACGCTTGACACATTGACA-3' were used to evaluate the amounts of RNA levels in all tissues; these primers amplify a 1,555 bp from genomic DNA and 1,251 bp from cDNA; in addition, control reactions without reverse transcriptase were performed using the same conditions to confirm absence of genomic contamination in all RT-PCR reactions performed.

**Plasmid Construction and Plant Transformation**

All the constructs generated originated from wild-type genomic DNA extracted (Qagen, DNAeasy Plant Mini kit) and amplified with the proof reading pfx DNA polymerase (Invitrogen) following the manufacturer’s instructions. All the PCR products were subcloned in P-GEM T-Easy from Promega, unless otherwise specified, digested, and fused to the binary vectors at the multiple cloning site or at the site of digestion, as described below.

To perform the complementation test of the hws-1 mutant, a genomic segment from A3g61590 containing a 1,291 bp of the promoter region, 419 bp from the 5' UTR, 532 bp from the intron, 1,236 bp from the ORF, and 181 bp from the 3' UTR using the primers 590Compfor 5'-CCTCCAGTTTCA-GATCCGAC-3' and 590comprev 5'-CCTCCAGTTTCA-GATCCGAC-3' was amplified from DNA of Col-0 wild type. Using this PCR product as template, the following primers containing a SalI site and a BamHI site in the forward and reverse primer, respectively (highlighted in bold), were used: 590CompSalFor, 5'-CCAGTCGACGCGCATTGACGCGAGATG-3' and 590CompBamRev, 5'-GCCGGATCCCTCA-GATCCGAC-3'. The PCR parameters used were: 94°C for 5 min, followed by 35 cycles of 94°C for 15 s and 68°C for 5.5 min, and a final elongation step at 68°C for 7 min.

To generate the GUS reporter lines, a segment containing the promoter of the HWS (A3g61590), the 5' UTR and the intron (2,242 bp), was also amplified by PCR. The primers used to amplify this genomic segment were the 590CompSalFor described previously and the 590PrBamRev 5'-AAGCTCCGACACCATTGACAA-3' were used: 590CompSalFor, 5'-CCAGTCGACGCGCATTGACGCGAGATG-3' and 590PrBamRev, 5'-GCCGGATCCCTCA-GATCCGAC-3'. The PCR parameters used were: 94°C for 5 min, followed by 35 cycles of 94°C for 15 s and 68°C 2.5 min, and a final elongation step at 68°C for 7 min.

To generate the GFP lines, the ProHWS::GUS construct was modified by digesting and replacing the GUS gene with the GFP ORF also digested from the pOG vector used previously for the PGAZAT gene (González-Carranza et al., 2002). The enzymes used were BamHI and Sall (Fermentas) following manufacturer’s instructions. Once the HWS::GUS construct was digested, the reaction was dephosphorylated with alkaline phosphatase (Promega) following the manufacturer’s instructions. The digestions were visualized in a 1% agarose gel and the linearized/dephosphorylated vector and the linearized/dephosphorylated vector were used for transformation of Escherichia coli DH5α cells. DNA from the T4 DNA ligase from Promega following the manufacturer’s instructions. The digestions were visualized in a 1% agarose gel and the linearized/dephosphorylated vector and the linearized/dephosphorylated vector were used for transformation of E. coli DH5α cells. DNA from the T4 DNA ligase from Promega was used for transformation of Escherichia coli DH5α cells. DNA from the T4 DNA ligase from Promega was used for transformation of E. coli DH5α cells.

**Confocal Analyses of GFP Expression**

GFP fluorescence in the transgenic lines was examined using a Leica (TCS SP2) laser-scanning confocal microscope equipped with argon krypton and green HeNe lasers and an AOBS scan head system (Leica Microsystems). GFP was excited at 488 nm with the argon ion laser. Images were recorded using the Leica CONFOCAL software.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession numbers BT000054/BX823146/BX824604 (A3g61590) and AAC02763 (A2g41850).

**Supplemental Data**

The following materials are available in the online version of this article.

**Supplemental Figure S1.** Analysis of expression from ProHWS:HWS lines by RT-PCR.

**Supplemental Figure S2.** Comparative phenotypic analysis of flowers at position 9.

**Supplemental Figure S3.** Comparative phenotypic analysis of sepal and petals.

**Supplemental Table S1.** InDel primers used for mapping the HWS locus through the identification of Col/Ler polymorphisms.

**Supplemental Table S2.** Restriction enzymes and expected sizes for the digestion of the PCR product corresponding to the A3g61590 ORF genomic region.

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**LITERATURE CITED**


GUS staining, washing, and mounting for the different tissues analyzed was performed as described in González-Carranza et al. (2002). The material was analyzed and photographs taken using either a Zeiss Stemi SV6 Stereo microscope with a Kodak MDS290 digital camera or a Nikon microscope with a Leica DFC320 camera using the IM50 Leica software (Leica Microsystems Imaging Solutions).

**Histochemical Analysis of GUS Activity**

GUS staining, washing, and mounting for the different tissues analyzed was performed as described in González-Carranza et al. (2002). The material was analyzed and photographs taken using either a Zeiss Stemi SV6 Stereo microscope with a Kodak MDS290 digital camera or a Nikon microscope with a Leica DFC320 camera using the IM50 Leica software (Leica Microsystems Imaging Solutions).


