Running title:

*hawaiian skirt* and Arabidopsis development

Corresponding author:

Jeremy A. Roberts

Plant Sciences Division, School of Biosciences, University of Nottingham, Sutton Bonington Campus, Loughborough, Leicestershire, UK. LE12 5RD

Tel: 0044 1159 516339

Fax: 0044 1159 516334

E mail: jeremy.roberts@nottingham.ac.uk

Development and hormone action
**HAWAIIAN SKIRT** – an F-box gene that regulates organ fusion and growth in *Arabidopsis*

Zinnia H. González-Carranza, Unchalee Rompa, Janny L. Peters, Anuj M. Bhatt, Carol Wagstaff, Anthony D. Stead, and Jeremy A. Roberts

Plant Sciences Division, School of Biosciences, University of Nottingham, Sutton Bonington Campus, Loughborough, Leicestershire, UK. LE12 5RD (Z.H. G-C., U.R., J.R.);
Section Plant Genetics, Institute for Wetland and Water Research, Radboud University Nijmegen, Toernooiveld 1, 6525 ED Nijmegen, The Netherlands (J.L.P);
Department of Plant Sciences, University of Oxford, South Parks Road, Oxford, UK. OX1 3RB (A.M.B.);
School of Biological Sciences, University of Southampton, Bassett Crescent East, Southampton, UK. S016 7PX (C.W.);
School of Biological Sciences, Royal Holloway, University of London, Egham, Surrey, UK. TW20 0EX (A.D.S.)
The work was supported by a grant from the Biotechnology and Biological Sciences Research Council and a studentship funded by the Thailand government.

Corresponding author: Jeremy A. Roberts
e-mail jeremy.roberts@nottingham.ac.uk; fax 00 44 1159-516334.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantphysiol.org) is: Jeremy A. Roberts (Jeremy.roberts@nottingham.ac.uk).
ABSTRACT

A fast neutron-mutagenised population of Arabidopsis thaliana Col-0 WT 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 28bp deletion that introduces a premature amber termination codon into the ORF 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:GUS into the mutant reveals that whilst 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 characterisation, using ProHWS:GUS or ProHWS:GFP 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, Col-0 WT, and Pro35S:HWS ectopically expressing lines has revealed that loss of HWS results in greater growth of both aerial and below-ground organs whilst over-expressing the gene brings about a converse effect. These observations are consistent with HWS playing an important role in regulating plant growth and development.
INTRODUCTION

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 fertilisation, 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 β 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; Sampredo and Cosgrove, 2005), may lead to the dissolution of the middle lamella that brings about cell separation (Roberts et al., 2002; González-Carranza 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). Whilst a number of such mutants have been documented only a few of the genes responsible have been cloned and characterized. These include jointless (j) that is the consequence of a mutation in a MADS box gene involved in the differentiation of abscission cells in the pedicel of tomato flowers (Mao et al., 2000), and inflorescence deficient in abscission (ida) 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 (Butenko et al., 2003). Recently QTL analysis has led to the identification of a gene (sh4) that has played a critical role in the domestication of rice 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 leucine-rich receptor-like kinase (Jinn et al., 2000); AGAMOUS-LIKE15 (AGL15) a MADS domain transcription factor that if non-functional 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** and 2 (**BOP1 & 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 mutagenised population of *Arabidopsis thaliana* Col-0 WT 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 non-shedding phenotype **hws** also exhibited sepals 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 (lfy)** 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** mutants (**cuc1, cuc2, cuc3**) that arise as a consequence of mutations in putative NAC-transcription factors 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 post-transcriptional 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 (LOB)** (Shuai et al., 2002); **LATERAL ORGAN JUNCTIONS (LOJ)** (Prasad et al., 2005); **PETAL LOSS** (Brewer et al., 2004), the **FUSED FLORAL ORGANS** loci (**FFO1, FFO2, FFO3**) (Levin et al., 1998); **HANABA TARANU** (Zhao *et al*., 2004); and **RABBIT EARS (RBE)** (Krizek *et al*., 2006).

In this report, 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 over-expressing **HWS** line driven by the **35SCaMV** promoter, and wild type plants a role for HWS in regulating plant growth and development has been highlighted.
RESULTS

Mutant isolation and characterization

The *hawaiian skirt-1* (*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 mutagenised population (dose: 55Gy; Lehle seeds) of M1 *Arabidopsis* seeds in the Col-0 (Wild-type - WT) 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 WT plants (Figures 1A-F). Whilst 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 WT and *hws-1* flowers (Figures 1G-J). Cell separation at the sepal bases was also apparent in *hws-1* flowers, however, the timing of this was delayed in comparison to WT plants and the sepal whorl was retained even though abscised (Figures 1H and J).

Further SEM analysis revealed that no distinction could be made between the WT and *hws-1* plants during early bud development (Figures 2A, B, E and F). However, by the time the buds have reached stage 10 (Smyth et al., 1990) the line of separation between individual sepals had become demarcated to the bud base in WT but not in *hws-1* material (Figure 2C 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 (Figures 2D and H).

The *hws-1* mutant was crossed with a WT plant and a 3:1 segregation of shedding:non-shedding 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 non-shedding phenotype of *hws-1* was due to a failure of abscission zone differentiation a cross was made between the mutant and a transgenic
marker line (ProPGAZAT:GUS) that expresses the reporter gene β-glucuronidase (GUS) specifically at the site of floral organ separation. PGAZAT (At2g41850) 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 hws-1 lines containing the GUS gene were isolated. Figures 3A and B show that both WT 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 abscission zone differentiation. However, although ProPGAZAT:GUS expression is apparent in Position 8 flowers of WT plants (Figure 3A) it cannot be detected until Position 10 in hws-1 flowers (Figure 3B). In addition, ProPGAZAT:GUS expression is less pronounced in hws-1 plants and by Position 20, in contrast to WT 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 (Figure 3C). However, these observations will not be described in detail until a later part of the results section of this paper.

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 (Figure 4A), compared with 2% in the WT. Occasionally, in hws-1 plants, these filaments were fused to the side of siliques (Figures 4B). The top of the hws-1 silique was consistently broader than WT and the length of the abscission zone region, exposed by manually removing the floral organs, was longer in the mutant (Figures 4C and D). Some hws-1 siliques comprised more than two valves (Figure 4E) and dissection of aberrant pods revealed that this was associated with abnormal development of the septum (Figure 4F). The lamina tissue of the primary cauline leaves of hws-1 plants routinely exhibited fusion to the inflorescence stems (Figure 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 (SSLP), and insertion/deletion (InDel) markers this interval was reduced to a region of 56kb containing 18 annotated genes (Figure 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 475bp and 491bp from the ATG (Figure 5B).

PCR amplification of the At3g61590 genomic region of WT and *hws-1* DNA and restriction analyses of the amplified products with the high frequency cutting enzymes, *AluI*, *RsaI* and *TaqI* revealed subtle differences in banding patterns between the two genotypes (Figure 5C). Sequencing of this region from *hws-1* revealed that it contained a 28bp deletion located 966 bp downstream of the translation start of the 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 isoleucine residue and the predicted production of a truncated version of the At3g61590 protein (Figure 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 (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 analysed by PCR and 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 WT control (Figure 5D). RT-PCR analysis of RNA extracted from various tissues of the SALK_088349 line and from two F₁ plants (*hws-1* x KO) showed no At3g61590 expression in the KO but the presence of a transcript in both progeny (Figure 5E).
Proof that \textit{HWS} is encoded by the \textit{At3g61590} gene was obtained by complementing the \textit{hws-1} mutant with a 3.513kb fragment amplified from WT DNA containing 1291bp upstream of the promoter, plus the 5’ and 3’ UTRs, and intron and exon of the \textit{At3g61590} gene. This segment proved to be of sufficient length to rescue fully the \textit{hws-1} mutant (Figure 5F).

**\textit{HWS encodes a putative F box protein}**

The likely function of the \textit{HWS} gene (\textit{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). \textit{HWS} encodes a protein of 412 amino acids. It has no introns within the open reading frame (ORF) but has an intron of 532bp within the 5’ UTR of the gene (Figure 5B). The truncated version of the mutant protein, predicted to be generated in \textit{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 \textit{HWS} is located between amino acids 40 to 85. The software also proposes the existence of a transmembrane spanning region between amino acids 120-140 and a low similarity (29.3%) to a Kelch_2 motif in the region between amino acids 290-338.

Outside the F-box region, the ORF of \textit{HWS} shows only a low level of sequence similarity with other putative F-box proteins that have been annotated within the \textit{Arabidopsis} genome. The \textit{Arabidopsis} gene encoding a protein with highest sequence similarity (approximately 30%) to \textit{HWS} is \textit{UNUSUAL FLORAL ORGANS (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 \textit{HWS} gene**

RT-PCR analysis of RNA isolated from wild type plants revealed that the \textit{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 (Figure 6). A more detailed analysis of the spatial and temporal pattern of expression, with the aid of the β-glucuronidase (\textit{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 (Figure 7A), the sepals of young buds and flowers (Figure 7B, C and D), the stigmatic papillae and tip of the elongating siliques (Figure 7E and G), the base and vascular tissue of petals and sepals (Figure 7F), the anther filaments and pollen (Figure 7G), the floral and cauline leaf abscission zones (Figures 7H and I), the testa of developing seeds (Figure 7J), the vascular tissue of the primary root and emerging laterals (Figure 7K) and lateral root cap (Figure 7L). A detailed time-course of GUS accumulation during flower and siliques 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 (Figure 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 35SCaMV (Cauliflower Mosaic Virus) 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 (Figure 1S) 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 two weeks after emergence, revealed that both ectopic expressing lines were substantially smaller than WT (Figures 8A). However, hws-1 plants at the same stage were larger than the control (Figure 8A). Compared to the WT, the rosette leaves of the over-expressing lines had shorter petioles, narrower and more rounded lamina with serrated borders and displayed a greater degree of hyponastic bending (Figure 8B).

Impact of ectopic expression of HWS on flower development
A comparison of flower development in WT, hws-1 and Pro35S:HWS line 8.3 demonstrated that organ shedding took place at an earlier developmental stage in the over-expressing line (Position 10) compared to the WT (Position 12). Flowers from line 8.3 also underwent sepal senescence prematurely, as evidenced by a visual decline in chlorophyll, compared to the WT (Figures 9 and 2S). Figure 9 shows that
hws-1 had the longest, while the over-expressing line had the shortest, stigmatic papillae. A close-up view of flowers confirmed these observations (Figure 2S).

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 WT or Pro35S:HWS A23.3 line. The floral bases of the two over-expressing lines were the narrowest (Figures 9 and 3S), whilst hws-1 was the widest compared to the WT (see also Figure 1B and E).

**Manipulation of HWS expression affects root growth and seed size**

Seeds from hws-1, WT and the two over-expressing lines, were germinated in GM or MS media and root length was measured after a period of two weeks. The mutant exhibited the longest roots while both Pro35S:HWS lines 8.3 and A23.3 had significantly shorter roots than the WT (Figure 10A).

The dimensions of mature seeds from the three different genotypes were analysed. The hws-1 mutant was found to have statistically larger (both in length and width) seeds compared to the WT while the over-expressing lines produced the smallest seeds (Figure 10B).

**DISCUSSION**

We have described the isolation and characterization of a novel Arabidopsis mutant termed *hawaiian skirt* 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 whilst over-expression of the gene, using the 35SCaMV promoter, 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 silique 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 \textit{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 \textit{CUP-SHAPED COTYLEDON} genes \textit{CUC1} and \textit{CUC2} (Laufs et al., 2004). In addition to exhibiting fused sepals, \textit{hws-1} has a prevalence to generate fused anthers, multi-valved first-formed siliques and fusion of the cauline leaf lamina to the inflorescence stem. These observations suggest that, like \textit{CUC1} and \textit{CUC2}, the \textit{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 WT and \textit{hws-1} plants indicates that floral morphology is initially indistinguishable, however, whilst sepal separation proceeds to the base of the bud in WT this is terminated prematurely in \textit{hws-1} material. Thus the \textit{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 abscission zones was taking place in \textit{hws-1} we crossed the gene marker \textit{ProPGAZAT:GUS} into the mutant. \textit{PGAZAT} (\textit{At2g41850}) encodes a polygalacturonase that is expressed specifically within the abscission zone cells of Arabidopsis and has been proposed to play a role in middle lamella degradation (González-Carranza et al., 2002). The results show that \textit{hws-1} is not compromised in terms of abscission zone (AZ) differentiation, however, the onset and duration of \textit{ProPGAZAT:GUS} expression is delayed and reduced respectively in the mutant. These revelations, together with our SEM and LM observations, indicate that the non-shedding phenotype of \textit{hws-1} is not the consequence of a failure of cell separation to take place at the base of petals, anther filaments, and sepals. Whilst the fusion of the sepal margins, encircling the separated petals and anthers, provides a structural barrier to preclude organ shedding in \textit{hws-1} our data indicate that the timing of cell separation in the mutant is delayed.

\textbf{HWS gene encodes an F-box protein}

Mapping and characterization of the \textit{hws-1} locus has revealed that the mutant phenotype is a consequence of a 28bp deletion in the ORF of a gene (\textit{At3g61590}) encoding a putative F-box protein. The 28 bp deletion in \textit{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 non-functional. 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 targetted yeast 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 results).

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 UNUSUAL FLORAL ORGANS gene (UFO). 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, shows 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 \textit{hws-1} mutant were primarily restricted to the shoot and reproductive tissues it was a surprise to discover that the transcript of the \textit{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 \textit{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 sepals, the distal end of the stigma, anther filaments and pollen. Expression is also evident in the abscission zones of cauline 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 IAA (Kepinski and Leyser, 2005). Mutations in the \textit{TIR1} gene confer a reduced sensitivity to IAA, however, the responses of \textit{hws-1} to auxin are indistinguishable from WT plants (Z.H. González-Carranza and J.A. Roberts, unpublished results) and whilst ectopic expressing lines of \textit{HWS} exhibit reduced root growth, elongation of laterals root is not promoted.

GUS expression in \textit{ProHWS:GUS} plants is intense at both the site of abscission of floral organs and cauline 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 sepals in \textit{hws} plants, rather than as a consequence of a failure to undergo cell separation, a comparison between the mutant, over-expressing lines and WT 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 \textit{ProPGAZAT:GUS} gene also supports the assertion that HWS is necessary for cell separation to proceed at the ‘normal’ rate.

\textbf{Role of HWS in plant development}

Although the principal characteristic of \textit{hws-1} plants is their non-shedding phenotype the isolation of a null allele (\textit{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 WT 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 over expressing 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 (ARF2), 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 (Schruff et al., 2006). Further work is on going to identify the mechanism by which HWS exerts its effect on growth and ascertain whether this may be mediated through an auxin-signalling pathway.

METHODS

Plant material and growth conditions

Arabidopsis thaliana ecotypes Col-0 (Col) and Landsberg erecta (Ler), the hws-I mutant, SALK lines for the 18 genes in the 56kb segment which were obtained from the NASC stock centre, diverse crosses and the mapping population were grown in a glasshouse with a temperature of 23±2°C in plastic pots containing Levington M3 (The Scotts Company Ltd., Ipswich, Suffolk, UK) compost and Vermiculite (2.0-5.0mm, Sinclair, Lincoln UK,) in a ratio 3:1 respectively. To grow plants under sterile conditions; Petri plates were prepared with 4.33g L⁻¹ of Murashige and Skoog basal salt mixture (MS) pH 5.9 (Sigma; Murashige and Skoog, 1962) medium and 0.8% agar or GM (germination medium; 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 5 times in sterile water before being placed, using a pipette tip, on top of the MS agar Petri dishes. Plates were sealed with micropore tape (3M) and maintained at 4°C for 72h to stratify them and to facilitate uniform germination. Plates were then transferred to a growth room with a temperature of 23±1°C under 16h light 8h 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 Columbia-0 (WT) 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 four hours, 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 primorida. They were sputter-coated with gold palladium before viewing. Flowers were viewed immediately without fixation or sputter coating at 20kV accelerating voltage in variable pressure mode under low vacuum (70Pa). All samples were viewed under a Hitachi S-3000 scanning electron microscope (Tokyo, Japan). A minimum of five buds/flowers were examined from each position.

**Light Microscopy**

Unopened and open flowers were collected from WT 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: PBS buffer [1.3 mM NaCl, 0.03M Na₂HPO₄, 0.03M NaH₂PO₄ pH7.2, 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 1h, then brought to room temperature and washed with PBS for 1.5 h. The tissues were post-fixed with 2% (w/v) OsO₄ in PBS at room temperature for 3h, then rinsed twice in PBS for 15 min and dehydrated with an ethanol series [30%, 50%, 70%, 90% and 100% (v/v), twice, for 1h in each]. Specimens were processed with 100% ethanol/propylene oxide (1:1) for 20 min, washed 3 times with propylene oxide for 10
infiltrated with pure Spurrs resin at 4°C for 10-12 h. Individual samples were placed in appropriate flat labelled silicone embedding moulds filled with Spurrs resin and placed at 72°C for 10-12 h.

Longitudinal semi-thin sections (0.5 µm) were cut on a Reichert-Jung Ultracut ultramicrotome using glass knives. The glass knives were made from a glass bar (TAAB) on a Leica EM KMR2 knife maker machine. Sections were stained with Toluidine Blue (TBO) 0.25-1% (w/v), mounted with DePex (Sigma), and observed under a Nikon Optiphot-2 microscope equipped with a Leica DFC320 camera using the IM50 Leica software (Leica Microsystems Imaging Solutions Ltd, Cambridge, UK).

**Phenotypic and statistical analysis**

Six plants from the hws-1 mutant, WT, and 35SCaMV ectopic expression lines 8.3 or A23.3 were grown under the same conditions and 25 flowers were dissected and analysed under a Zeiss Stemi SV6 Stereo microscope. Organ fusion and other phenotypic characteristics were recorded either with a Kodak MDS290 digital camera attached to the microscope or with a Fujifilm FinePix A205S. Images were analysed with Adobe photoshop software.

Seedlings of two weeks old were grown in MS media or in soil. Rosette leaves were dissected, and pictures taken with a Fujifilm FinePix A205S. For measurements of roots (26 plants grown in MS media or GM media), sepals, petals (12 flowers from different plants at Position 3) and seeds (70 seeds from 5 plants) pictures were taken and measurements were performed with the image processor and analyser in Java software ImageJ Version 1.37h (Abramoff et al., 2004). Values were then used to perform statistical analysis and create graphics in Microsoft Excel 2002. Statistical analysis of variance (one-way ANOVA) using LSD and Duncan Analysis were performed using the software SPSS 14.0.1.

**Mapping of the hws gene**

A mapping population was generated by crossing the hws-1 mutant in Col-0 background with the Landsberg erecta (Ler) ecotype and the F1 progeny was allowed to self. DNA was extracted, following manufacturer instructions (Qiagen, DNAeasy Plant Mini kit), from a small population of 33 F2 hws-1 mutant plants and used to map
the HWS locus to a region of 3.28MB between markers SM148.6 and SM239_119.5 at the bottom of Chromosome 3 using an Amplified Fragment Length Polymorphism (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 Cleaved Amplified Polymorphic Sequences (CAPS) and Simple Sequence Length Polymorphisms (SSLP), the region was reduced to a 0.404 MB between the markers FUS6.2 and NGA6. From 600 F2 hws-1 mutant plants, and the use of Insertion/Deletions (InDels) that were identified from the Cereon Arabidopsis Polymorphism collection (Cereon Genomics, Cambridge, MA, USA) (http://www.arabidopsis.org/Cereon/index.html), the region was further reduced to a segment of 56Kb between markers 470113 and 469675. This region contained 18 annotated genes (Figure 5A). The InDel flanking primers designed for fine mapping hws-1 are summarized in Supplementary Table 1. PCR reactions were performed in a reaction of 20µl following red taq (ABgene) manufacturer instructions; the PCR conditions for amplification were: 3 min at 94ºC, 30 cycles of 94ºC for 30sec, 55-60ºC for 30 sec (depending on primer combination), 72ºC for 30 sec, and 7 min 72ºC, 4ºC ∞. PCR products were run in a 3% low melting point agarose gel (MetaPhor® Agarose, Cambrex, Wokingham, UK).

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 At3g61590, proved to exhibit a similar phenotype to the hws-1 mutant.

Primers from the region corresponding to the At3g61590 gene which amplified a genomic PCR segment of 1271bp are At361590forcDNA: 5’GCTCTTGAGAATGGAAGCAGAAAC 3’ and At3g61590Rev: 5’CAGACCCATTTGCTTCTTCATTGC 3’. PCR reactions were performed in a 50µl reaction following red taq (ABgene) manufacturer instructions. Conditions for amplification were: 3 min at 94ºC, 30 cycles of 94ºC for 15sec, 61ºC for 1 min, 72ºC for 2 min, 7 min 72ºC, 4ºC ∞ 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, 10X Reaction Buffer, 2µg of BSA, and 0.5 µl of each enzyme, incubated at 37ºC (RsaI and AluI) or 65ºC (TaqI) and run in a 3% agarose gel. The
expected restriction sizes for the amplified PCR segment corresponding to the 
*At3g61590* ORF genomic region are shown in Supplementary Table 2.

A plant from the Salk_088349 KO line was used to cross with the *hws-l* line and PCR analyses were performed to identify the presence of the two T-DNA insertions and the genomic fragment from the *hws-l* mutant from the two parental lines; information about primers is described previously. To test the T-DNA insertion the LBb1 of pBIN-pROK2 for SALK lines primer was used 5’ GCGTGGACCGCTTGCTGCAACT 3’ (http://signal.salk.edu/tdnaprimers.2.html; Alonso et al., 2003) PCR conditions were the same as for the PCR amplification of *At3g61590* segment described earlier.

**Reverse Transcriptase PCR analysis of expression**

Total RNA from roots, buds, flowers, rosette leaves, stem, young siliques and old siliques from WT; and a mix of tissues from progeny plants 1 and 2 from the cross between the *hws-l* mutant with Salk_088349 line (*hws-2*) and from a mix of flowers from several developmental positions from F1 over-expressing 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 4M LiCl at 20ºC for 1h instead of using the CTAB buffer. RNA was quantified with a spectrophotometer and its quality was analysed 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. First strand 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 taq (ABgene) manufacturer instructions; the PCR conditions for amplification were: 3 min at 95ºC, 30 cycles of 95ºC for 1 min, 50-58ºC for 1-2 min (depending on primer combination and size of expected band), 72ºC for 1-2 min, and 7 min 72ºC, 4ºC. 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 over expressing lines, were 590 5’UTR: 5’ CTTCTCTCATCCTCGCGCTTGCAACT 3’ and
At3g61590Rev (previously described) which give a genomic band of 2,213bp and a cDNA of 1668bp. Use of these primers allows the identification of genomic contamination.

To amplify the transgene of over-expressing lines the primer pKT735Sprom 5’ GAGGAGCATCGTGGAAAAAG 3’ from the 35S promoter and At3g61590Rev were used. The amplified band from this primer combination is 1,677bp.

Ubiquitin (At4g05320) primers UBQ10For, 5’ TAAAAACTTTCTCTCAATTCTCTCT-3’ and UBQ10Rev, 5’ AAGCTCCGACACCATGGACAA-3’ were used to evaluate the amounts of RNA levels in all tissues; these primers amplify a 1,555bp from genomic DNA and 1251bp 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 WT genomic DNA extracted (Qiagen, DNAeasy Plant Mini kit) and amplified with the proof reading pfx DNA polymerase (Invitrogen) following the manufacturers instructions. All the PCR products were sub-cloned 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 *At3g61590* containing 1,291bp of the promoter region, 419bp from the 5’ UTR, 532bp from the intron, 1,236bp from the ORF and 181bp from the 3’ UTR using the primers: 590compfor 5’ CCTCCAGTTTCAGAATCCGACC 3’ and 590comprev 5’ CCTCCAGTTTCAGAATCCGACC 3’ was amplified from DNA of Columbia-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’ GCAGTCGACGGCACAAGGAGCAATGTTG 3’ 590CompBamRev 5’ GCCGGATCCTCCAGTTTCAGAATCCGAC 3’. The PCR parameters used were: 94°C for 5 min, followed by 35 cycles of 94°C 15 sec and 68°C 3.5 min, and a final elongation step at 68°C for 7 min.
To generate the β-glucuronidase reporter lines, a segment containing the promoter of the *HWS* (*At3g61590*), the 5’UTR and the intron (2,242bp), was also amplified by PCR. The primers used to amplify this genomic segment were the 590CompSalFor described previously and the 590PrBamRev 5’ GCCGGATCCCTCTCAAGAGCCTCTGAAAC 3’.

With a *SalI* and a *BamHI* site at the forward and reverse primers respectively (highlighted in bold). The PCR parameters used were: 94°C for 5 min, followed by 35 cycles of 94°C 15 sec 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 β-glucuronidase gene with the *GFP* ORF also digested from the MOG vector used previously for the *PGAZAT* gene (González-Carranza et al., 2002). The enzymes used were *BamHI* and *SacI* (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 visualised in a 1% agarose gel and the linearized/dephosphorylated vector and the *GFP* segment was gel-extracted using a phenol: chloroform extraction method. The two segments were ligated using T4 DNA ligase from Promega following the manufacturer’s instructions.

The over expressor construct was generated by amplifying the ORF from the *HWS* gene using the primers: 59035SBamHATGfor 5’ GCCGGATCCCTCTGAGAATGGAAGCAG 3’ and 59035SsacIrev 5’ CGTGAAGCTCCCCAGTCTTCAGAAATCCGACC 3’ with a *BamHI* and a *SacI* site at the forward and reverse primers respectively (highlighted in bold). The PCR parameters used were the same as for the *ProHWS:GUS* construct. This segment was sub-cloned in a MOG402 engineered vector containing two copies of the *35SCaMV* promoter.

*Escherichia coli* DH5α cells were transformed and positive colonies were selected by PCR and the integrity of all plasmids generated was confirmed by sequencing, these plasmids were electroporated into *Agrobacterium tumefaciens* C58 strain and grown to an OD600 of 0.5-0.8. WT *Arabidopsis* plants were transformed using the “floral dip” method described by Clough and Bent (1998).

Selection of transformants was carried out in a growth room at 22°C using Petri dishes containing MS media at pH 5.9, 0.8% (w/v) agar and Kanamycin 40µg ml⁻¹. Transformation was confirmed by PCR using the correct set of primers per construct. For complementation tests, primary transformants were screened for
kanamycin resistance and plants were grown and analysed for rescue of the hws phenotype. T2 seeds were collected from individual lines for the GUS and GFP reporter lines and the over-expressor lines, and screened for kanamycin resistance to identify at least six homozygous lines to check for consistency of expression.

**Histochemical analysis of β-glucuronidase (GUS) activity**

GUS staining, washing and mounting for the different tissues analysed was performed as described in (González-Carranza et al., 2002). The material was analysed and photoghws 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 Ltd, Cambridge, UK).

**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, Bannockburn, IL). GFP was excited at 488 nm with the argon ion laser. Images were recorded using the Leica CONFOCAL software.

**ACKNOWLEDGMENTS**

We thank Rick Noteboom for technical help in the map-based cloning experiments and Patricia Goggin of the Electron Microscopy Unit at Royal Holloway.
FIGURE LEGENDS

Figure 1. Scanning electron microscope (SEM) and light microscope (LM) analyses of WT and hws-I flowers.

Scanning electron microscopy of flower development stages in Columbia-0 WT ([A] to [C]), and hws-I plants ([D] to [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 abscission zones (AZ) stained with Toluidine blue in Columbia-0 WT ([G] and [H]), and hws-I plants ([I] and [J]) at Positions 4 ([G] and [I]) when petals (Pe), sepals (Se) are still attached, and Position 12 ([H] and [J]), when only sepals are attached to the hws-I mutant, siliques (Si) are visible at all stages. Sites of sepal (Se-AZ), petal (Pe-AZ), and anther filament (Fil-AZ) abscission are identified. Bars = 500 μm ([A] to [F]), and 50 μm ([G] to [J]).

Figure 2. Scanning electron microscope (SEM) analysis of WT and hws-I 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 Columbia-0 WT ([A], [B], [C] and [D]) and hws-I ([E], [F], [G] and [H]) plants. Bars = 100 μm.

Figure 3. Flower and silique expression from ProPGAZAT:GUS, ProPGAZAT:GUS crossed into hws-I, and ProHWS:GUS.

Time course of expression from different developmental positions of flower and siliques of GUS-stained tissue after 12h 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-I, (C) ProHWS:GUS. Bars = 1 mm.

Figure 4. Phenotypic characteristics of hws-I. Characteristics shown in (C), (D), and (G) appeared consistently.

(A) Fused anthers (arrow).
(B) Anther fused to the silique (arrow).
(C) Top of mature siliques from Columbia-0 WT (left) or hws-1 (right). Note the difference in width of siliques and length of residual papillae.

(D) Bottom of mature siliques from Columbia-0 WT (left) or hws-1 (right). Note the difference in longitudinal dimensions of the abscission zone (AZ) region.

(E) Siliques from hws-1 with more than two valves.

(F) Dissected siliques from Columbia-0 WT (left & centre) and hws-1 (right) with aberrant septum development.

(G) First cauline leaves from hws-1 fused to the stem of the inflorescence (indicated by an arrow). Bars = 500 µm ([A] to [F], and 5 mm (G).

**Figure 5.** Mapping and identification of the HWS gene.

(A) Mapping strategy used to identify the HWS locus using an AFLP approach to analyse 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 0.4MB. With the use of InDels on DNA from 600 F2 hws-1 plants, the region was reduced to a 56Kb segment between InDel 470113 and 469675. This region contained 18 candidate genes. Insertional SALK lines of these 18 genes were analysed for hws-1 characteristics and the DNA from region At3g61590 was amplified by PCR and products were subject to digestion by restriction enzymes.

(B) Structure of the HWS gene. The gene has a 5’UTR of 419bp interrupted by an intron of 532bp, an ORF of 1236bp and a 3’UTR of 181bp. The hws-1 mutation is a consequence of a 28bp deletion 966bp downstream from the ATG that introduces a premature amber nonsense codon. Individuals from the SALK_088349 line (hws-2) that phenocopies the hws-1 mutation, have two T-DNA insertions inserted in opposite directions 475bp and 491bp downstream from the ATG (shown).

(C) Patterns of digestion with frequent cutters AluI, RsaI and TaqI from the region corresponding to the position of gene At3g61590. Arrows indicate different patterns of digestion between the hws-1 mutant and the Columbia-0 WT.

(D) PCR in two progeny plants from the hws-1 mutant and the SALK_088349 line (hws-2) and the Columbia-0 WT with insert primers and specific primers, note that
two bands were amplified with vector border primer and each one of the specific primers, indicating that this line has two insertions.

(E) RT-PCR in root, bud/flower and rosette tissues from the SALK_088349 line (hws-2) and a mix of the same tissues in two progeny plants from the hws-1 mutant and SALK_088349 (hws-2). Ubiquitin (At4g05320) primers were used to demonstrate equal amounts of mRNA amplified.

(F) Complementation of hws-1 using a 3.5Kb genomic fragment including the promoter, UTRs, intron and exon of At3g61590. Siliques from position 16: (1) Columbia-0 WT, (2) F1 complementation plants, (3) hws-1 mutant, (4) SALK_088349 KO line (hws-2).

**Figure 6.** Analysis of expression of the HWS gene by RT-PCR.

RT-PCR of RNA extracted from Arabidopsis Columbia-0 WT tissues using HAWAIIAN SKIRT (HWS) or ubiquitin (At4g05320) primers.

**Figure 7.** Analysis of expression from ProHWS: GUS and ProHWS:GFP transgenic lines.

Cleared whole-mount GUS-stained tissue samples after 12 h from ProHWS:GUS plants at different stages of development ([A] to [I]), and confocal images from plants ProHWS:GFP ([J] to [L]).

(A) Two day-old seedling.

(B) Young flowers (note the GUS expression in sepals including their vascular tissue).

(C) Close view of youngest floral buds.

(D) Young buds (note GUS expression becoming more discrete at the top of the silique as the flowers become older).

(E) Close view of the silique from a flower where petals have not emerged (note that GUS expression is spread along approximately 1/3 of the top of the silique).

(F) Separated petal (left) and sepal (right) material from a Position 12 flower.

(G) A flower from Position 9 (note how the GUS expression from the top of the silique becomes more localized at the stigma)

(H) An abscission zone from a flower of Position 10.

(I) An abscission zone from a cauline leaf.
(J) A confocal image of developing seeds isolated from a mature silique.
(K) A confocal image of an emerging lateral root.
(L) A confocal image of a root cap.
Bars = 300 µm ([A], [B] and [D] to [K]), 75 µm ([C] and [L]).

Figure 8. Comparative phenotypic analysis of young plants.

Plants of two weeks old were analysed and compared from: (1) Pro35S:HWS homozygous line 8.3, (2) hws-1, (3) Columbia-0 WT and (4) Pro35S:HWS homozygous line A23.3.
(A) A comparison of two weeks 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 = 5mm

Figure 9. Comparative phenotypic analysis of flowers.

Flowers of four weeks old plants were analysed 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) Columbia-0 WT.
(B) hws-1 mutant, note the bigger stigmatic papillae in the flowers and wider floral bases.
(C) Pro35S:HWS line 8.3, note that abscission takes place earlier (Position 10) than in the Columbia-0 WT (Position 12). Bars = 1mm.

Figure 10. Comparative phenotypic analysis of root length and seed size.

(A) Twenty six roots from two weeks old seedlings grown in GM were photographed and their roots measured and statistically analysed. Root length comparisons among, Pro35S:HWS line 8.3, hws-1 mutant, Columbia-0 WT, Pro35S:HWS 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 analysed. Seed length and width comparisons among, Pro35S:HWS line 8.3, hws-1 mutant, Columbia-0 WT, Pro35S:HWS line A23.3. The significance of the differences was analyzed by ANOVA test. *Different characters (p,q,r & x,y,z) indicate a significant difference in the mean at P<0.05, n=70.
Supplementary Figures

Supplementary Figure 1. Analysis of expression from Pro35S:HWS lines by RT-PCR.

RT-PCR of RNA extracted from a mix of floral tissues from fourteen over-expressing Pro35S:HWS F1 lines.

(A) RT-PCR with specific primers to amplify the HWS cDNA.
(B) RT-PCR with a specific primer from the CaMV 35S promoter and a specific primer from the HWS ORF to amplify the transgene encoded cDNA. (Note the intensity of both bands in lines 8 and A23).
(C) RT-PCR with ubiquitin (At4g05320) primers.

Supplementary Figure 2. Comparative phenotypic analysis of flowers at Position 9.
A close-up view of flowers from (A) Columbia-0 WT, (B) hws-1 and (C) Pro35S:HWS line 23.3. Note the difference in length of stigmatic papillae, bases of flowers and senescence of sepals. Bars = 500 µm.

Supplementary Figure 3. Comparative phenotypic analysis of sepals and petals.

Twelve flowers from Position 3 were dissected and photographed, and petals and sepals measured and analysed statistically.

(A) Flowers from (1) hws-1 mutant, (2) Columbia-0 WT, and (3) Pro35S:HWS line A23.3 showing how measurements were taken.
(B) Comparative visual analysis of petal and sepal sizes from flowers of (A).
(C) Comparison of petal length and width, and sepal length and width of hws-1 mutant, Columbia-0 WT, and Pro35S:HWS line A23.3. The significance of the differences between hws-1, Columbia-0 WT, and Pro35S:HWS line A23.3 was analyzed by ANOVA test. *Different characters (x,y,z) indicate a significant difference in the mean at P<0.05, n=48. Bars = 1mm [(A) and (B)].
Supplementary Tables

**Supplementary Table 1.** InDel primers used for mapping the *HWS* locus through the identification of Col/Ler polymorphisms. InDels were taken from the Cereon *Arabidopsis* Polymorphism collection.

| SNP name | BAC name | Forward primer (5’ → 3’) | Reverse Primer (5’ → 3’) | Fragment size (bp) Col Ler | InDel size (bp) Col Ler |
|----------|----------|---------------------------|---------------------------|----------------------------|--------------------------|
| 470109   | F2A19    | GATTCTATGTGG CATCAGTGGAGG | CACACACACCTTCT CTCGTTTCTC | 396/363                    | 33/-33                   |
| 470113   | F2A19    | GTGTCTGACAGT AACTCTCTG    | CATCCCCTAGAG AAGGATAAC   | 107/104                    | 3/-3                     |
| 469675   | F15G16   | GAAGCATGAGTC ACTCTCATTC  | GACACGCATTTT AGATACCGTG  | 295/283                    | 12/-12                   |
| 469678   | F15G16   | GGGATGTAGCTCA TATGGTAGACG | GACGTGATCTCCT CTCGTTTTAA  | 192/174                    | 18/-18                   |
| 469682   | F15G16   | GATGCTAATCATG GGAGACATCTCA | GAGGTTCAAGGT TATGATATG | 250/231                    | 9/-19                    |
Supplementary Table 2. Restriction enzymes and expected sizes for the digestion of the PCR product corresponding to the At3g61590 ORF genomic region.

| Name | Sequence | Site Length | Overhang | Frequency | Cut Positions |
|------|----------|-------------|-----------|-----------|---------------|
| AluI | AGCT     | 4           | blunt     | 6         | 48, 71, 197, 663, 1104, 1198 |
|      |          |             |           |           | Fragments: 48, 23, 126, 466, 441, 94, 73 |
|      |          |             |           |           | In order: 23, 48, 73, 94, 126, 441, 466 |
| RsaI | GTAC     | 4           | blunt     | 4         | 44, 202, 290, 356 |
|      |          |             |           |           | Fragments: 44, 158, 88, 66, 915 |
|      |          |             |           |           | In order: 44, 66, 88, 158, 915 |
| TaqI | TCGA     | 4           | 5’        | 13        | 111, 363, 378, 515, 596, 608, 809, 821, 949, 1053, 1131, 1218, 1230 |
|      |          |             |           |           | Fragments: 111, 252, 15, 137, 81, 12, 201, 12, 128, 104, 78, 87, 12, 41 |
|      |          |             |           |           | In order: 12, 12, 12, 15, 41, 78, 81, 87, 104, 111, 128, 137, 201, 252 |
LITERATURE CITED

Abramoff MD, Magelhaes PJ, Ram SJ (2004) Image processing with ImageJ. J Biophotonics Int 11: 36-42

Addicott FT (1982) Abscission. (Berkeley: University of California Press)

Aida M, Ishida T, Fukaki H, Fujishawa H, Tasaka M (1997) Genes involved in organ separation in Arabidopsis: An analysis of the cuc-shaped cotyledon mutant. Plant Cell 9: 841-857

Alonso JM, Stepanova AN, Leisse TJ, Kim CJ, Chen H, Shinn P, Stevenson DK, Zimmerman J, Barajas P, Cheuk R, Gadrinab C, Heller C, Jeske A, Koesema E, Meyers CC, Parker H, Prednis L, Ansari Y, Choy N, Deen H, Geral M, Hazari N, Hom E, Karnes M, Mulholland C, Ndubaku R, Schmidt I, Guzman P, Aguilar-Henonin L, Schmid M, Weigel D, Carter, DE, Marchand T, Risseeuw E, Brogden D, Zeko A, Crosby WL, Berry CC, Ecker JR (2003) Genome-wide insertional mutagenesis of Arabidopsis thaliana. Science 301: 653-657

Belfield EJ, Ruperti B, Roberts JA, McQueen-Mason S (2005) Changes in expansin activity and gene expression during ethylene-promoted leaflet abscission in Sambucus nigra. J Exp Bot 56: 817-823

Brewer P, Howles P, Dorian K, Griffith M, Ishida T, Kaplan-Levy R, Kilinc A, Smyth D (2004) Petal Loss, a trihelix transcription factor gene, regulates perianth architecture in the Arabidopsis flower. Development 131: 4035-4045

Butenko MA, Patterson SE, Grini PE, Stenvik G-E, Amundsen SS, Mandal A, Aalen RB (2003) INFLORESCENCE DEFICIENT IN ABSCISSION controls floral organ abscission in Arabidopsis and identifies a novel family of putative ligands in plants. Plant Cell 15: 2296-2307.

Clough SJ, Bent AF (1998) Floral dip: a simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana. Plant J 16: 735-743
Durfee T, Roe JL, Sessions RA, Inouye C, Serikawa K, Feldmann KA, Weigel D, Zambrayski PC (2003) The F-box-containing protein UFO and AGAMOUS participate in antagonistic pathways governing early petal development in Arabidopsis. Proc Natl Acad Sci USA 100: 8571-8576

Ellis CM, Nagpal P, Young JC, Hagen G, Guilfoyle TJ, Reed JW (2005) AUXIN RESPONSE FACTOR1 and AUXIN RESPONSE FACTOR2 regulate senescence and floral organ abscission in Arabidopsis. Development 132: 4563-4574

Fernandez DE, Heck GR, Perry SE, Patterson SE, Bleecker AB, Fang S-C (2000) The embryo MADS domain factor AGL15 acts postembryonically. Inhibition of perianth senescence and abscission via constitutive expression. Plant Cell 12: 183-198

González-Carranza ZH, Lozoya-Gloria E, Roberts JA (1998) Recent developments in abscission: shedding light on the shedding process. Trends Plant Sci 3: 10–14

González-Carranza ZH, Whitelaw CA, Swarup R, Roberts JA (2002) Temporal and spatial expression of a polygalacturonase during leaf and flower abscission in oilseed rape and Arabidopsis. Plant Physiol 128: 534-543

Gray WM, del Pozo CJ, Walker L, Hobbie L, Risseeuw E, Banks T, Crosby WL, Yang M, Ma H, Estelle M (1999) Identification of an SCF ubiquitin-ligase complex required for auxin response in Arabidopsis thaliana. Genes Dev 13: 1678-1691

Han Y, Grierson D (2002) Relationship between small antisense RNAs and aberrant RNAs associated with sense transgene mediated gene silencing in tomato. Plant J 29: 509-519

Harding EW, Tang W, Nichols KW, Fernandez DE, Perry SE (2003) Expression and maintenance of embryogenic potential is enhanced through constitutive expression of AGAMOUS-Like 15. Plant Physiol 133: 653-663
Hepworth SR, Zhang Y, McKim S, Li X, Haughn GW (2005) BLADE-ON-PETIOLE1 dependent signaling controls leaf and floral patterning in Arabidopsis. Plant Cell 17: 1434-1448

Jarillo JA, Capel J, Tang RH, Yang HQ, Alonso JM, Ecker JR, Cashmore AR (2001) An Arabidopsis circadian clock component interacts with both CRY1 and phyB. Nature 410: 487–490

Jinn T-L, Stone JM, Walker JC (2000) HAESA, an Arabidopsis leucine-rich repeat receptor kinase, controls floral organ abscission. Genes Dev 14: 108-117

Kepinski S, Leyser O (2005) The Arabidopisis F-box protein TIR1 is an auxin receptor. Nature 435: 446-451

Krizek BA, Lewis MW, Fletcher JC (2006) RABBIT EARS is a second-world repressor of AGAMOUS that maintains spatial boundaries in Arabidopsis flowers. Plant J 45: 369-383

Laufs P, Coen E, Kronenberger J, Traas J, Doonan J (2003) Separable roles of UFO during floral development revealed by conditional restoration of gene function. Development 130: 785-796

Laufs P, Peaucelle A, Morin H, Traas J (2004) MicroRNA regulation of the CUC genes is required for boundary size control in Arabidopsis meristems. Development 131: 4311-4322

Lehti-Shiu MD, Adamczyk BJ, Fernandez DE (2005) Expression of MADS-box genes during the embryonic phase in Arabidopsis. Plant Mol Biol 58: 89-107

Levin J, Fletcher J, Chen X, Meyerowitz E (1998) A genetic screen for modifiers of UFO meristem activity identifies three novel fused floral organs genes required for early flower development in Arabidopsis. Genetics 149: 579-595

Levin JZ, Meyerowitz E M (1995) UFO: An Arabidopsis gene involved in both floral meristem and floral organ development. Plant Cell 7: 529-548
Li C, Zhou A, Sang T (2006) Rice domestication by reducing shattering. Science 311: 1936-1939

Mallory AC, Dugas DV, Bartel DP, Bartel B (2004) MicroRNA regulation of NAC-domain targets is required for proper formation and separation of adjacent embryonic, vegetative, and floral organs. Curr Biol 14: 1035-1046

Mao L, Begum D, Chuang HW, Budiman MA, Szymkowiak EJ, Irish EE, Wing, RA (2000) JOINTLESS is a MADS-box gene controlling tomato flower abscission zone development. Nature 406: 910-913

McGinnis KM, Thomas SG, Soule JD, Strader LC, Zale JM, Sun TP, Steber CM (2003) The Arabidopsis SLEEPY1 gene encodes a putative F-box subunit of an SCF E3 ubiquitin ligase. Plant Cell 15: 1120–1130

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 18: 100-127

Ni W, Xie D, Hobbie L, Feng B, Zhao D, Akkara J, Ma H (2004) Regulation of flower development in Arabidopsis by SCF complexes. Plant Physiol 134: 1574-1585

Norberg M, Holmlund M, Nilsson O (2005) The BLADE ON PETIOLE genes act redundantly to control growth and development of lateral organs. Development 132: 2203-2213

Patterson SE (2001) Cutting loose. Abscission and dehiscence in Arabidopsis. Plant Physiol 126: 494-500

Peters JL, Cnops G, Neyt P, Zethof J, Cornelis K, Van Lijsebettens M, Gerats T (2004) An AFLP-based genome-wide mapping strategy. Theor Appl Genet 108: 321–327

Prasad AM, Sivanandan C, Resminath R, Thakare DR, Bhat SR, Srinivasan (2005) Cloning and characterization of a pentatricopeptide protein encoding
gene (LOJ) that is specifically expressed in lateral organ junctions in *Arabidopsis thaliana*, Gene **353**: 67-79

Roberts JA, Elliott KA, González-Carranza ZH (2002) Abscission, dehiscence, and other cell separation processes, Ann Rev Plant Biol **53**: 131-158

Samach A, Klenz JE, Kohalmi SE, Risseeuw E, Haughn GW, Crosby WL (1999)
The *UNUSUAL FLORAL ORGANS* gene of *Arabidopsis thaliana* is an F-box protein required for normal patterning and growth in the floral meristem. Plant J **20**: 433–445

Sampredro J, Cosgrove DJ (2005) The expansin superfamily, Genome Biology **6**: 242

Schruff MC, Spielman M, Tiwari S, Adams S, Fenby N, Scott RJ (2006) The *AUXIN RESPONSE FACTOR2* gene of *Arabidopsis* links auxin signalling, cell division, and the size of seeds and other organs. Development **133**: 251-261

Schultz EA, Haughn GW (1991) LEAFY, a homeotic gene that regulates inflorescence development in Arabidopsis. Plant Cell **3**: 771-781

Sedbrook JC, Carroll KL, Hing KF, Masson PH, Somerville CR (2002) The *Arabidopsis SKU5* gene encodes an extracellular glycosyl phosphatidylinositol–anchored glycoprotein involved in directional root growth. Plant Cell **14**: 1635–1648

Sexton R, Roberts JA (1982) Cell biology of abscission. Ann Rev Plant Physiol **33**:133–162.

Shuai B, Reynaga-Peña CG, Springer PS (2002) The *LATERAL ORGAN BOUNDARIES* gene defines a novel, plant-specific gene family. Plant Physiol **129**: 747-761

Smyth DR, Bowman JL, Meyerowitz EM (1990) Early flower development in *Arabidopsis*. Plant Cell **2**: 755-767
Stenvik G.-E, Butenko MA, Urbanowicz BR, Rose JKC, Aalen RB (2006) Overexpression of INFLORESCENCE DEFICIENT IN ABSCISSION activates cell separation in vestigial abscission zones in *Arabidopsis*. Plant Cell 18: 1467-1476

Takada S, Hibara K, Ishida T, Tasaka M (2001) The *CUP-SHAPED COTYLEDON1* gene of *Arabidopsis thaliana* regulates shoot apical meristem formation. Development 128: 1127-1135

Takahashi N, Kuroda H, Kuromori T, Hirayama T, Seki M, Shinozaki K, Shimada H, Matsui M (2004) Expression and interaction analysis of *Arabidopsis Skp1*-related genes. Plant Cell Physiol 45: 83-91

Taylor JE, Whitelaw CA (2001) Signals in abscission. New Phytol 151: 323–339

Taylor S, Hofer J, Murfet I (2001) *Stamina pistilloida*, the pea orthologue of *Fim* and *UFO*, is required for normal development of flowers, inflorescences, and leaves. Plant Cell 13: 31-46

Vroemen CW, Mordhorst A P, Albretch C, Kwaaitaal CJ, de Vries SC (2003) The *CUC-SHAPED COTYLEDON3* gene is required for boundary and shoot meristem formation in *Arabidopsis*. Plant Cell 15: 1563-1577

Weigel D, Meyerowitz EM (1993) Activation of floral homeotic genes in *Arabidopsis*. Science 261: 1723-1726

Zhao Y, Medrano L, Ohasho K, Fletcher JC, Yu H, Sakai H, Meyerowitz EM (2004) HANABA TARANU is a GATA transcription factor that regulates shoot apical meristem and flower development in *Arabidopsis*. Plant Cell 16: 2586-2600
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
