An Ac-like Transposable Element Family With Transcriptionally Active Y-Linked Copies in the White Campion, *Silene latifolia*

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ABSTRACT

An RFLP genomic subtraction was used to isolate male-specific sequences in the species *Silene latifolia*. One isolated fragment, SLP2, shares similarity to a portion of the *Activator (Ac)* transposase from *Zea mays* and to related proteins from other plant species. Southern blot analysis of male and female *S. latifolia* genomic DNA shows that SLP2 belongs to a low-copy-number repeat family with two Y-linked copies. Screening of a *S. latifolia* male genomic library using SLP2 as a probe led to the isolation of five clones, which were partially sequenced. One clone contains two large open reading frames that can be joined into a sequence encoding a putative protein of 682 amino acids by removing a short intron. Database searches and phylogenetic analysis show that this protein belongs to the hAT superfamily of transposases, closest to Tag2 (*Arabidopsis thaliana*), and contains all of the defined domains critical for the activity of these transposases. PCR with genomic and cDNA templates from *S. latifolia* male, female, and hermaphroditic individuals revealed that one of the Y-linked copies is transcriptionally active and alternatively spliced. This is the first report of a transcriptionally active transposable element (TE) family in *S. latifolia* and the first DNA transposon residing on a plant Y chromosome. The potential activity and regulation of this TE family and its use for Y chromosome gene discovery is discussed.

The white campion, *Silene latifolia* (previously *Melandrium album*), is a common eudicot weed found in both North America and Europe. There are ~700 species in the genus Silene, the majority of which are outcrossing and hermaphroditic. Breeding systems range, however, from nearly obligate selfing (cleistogamy) to obligate outcrossing (dioecy). Dioecy (separate male and female individuals) likely evolved at least twice from a hermaphroditic state within the last 20–30 million years (Desfeux et al. 1996).

*S. latifolia* is dioecious with an XY-chromosome-based sex determination system analogous to the mammalian system. Dioecy is stable, although hermaphroditic mutants (XY) occur at extremely low frequency in natural populations (our personal observations) and can be induced through the exogenous application of demethylating agents such as 5-azacytidine and with gamma irradiation (Donnison et al. 1996; Janousek et al. 1996). Dioecy is a relatively rare breeding system in plants and is not necessarily coupled with sexually dimorphic chromosomes. The recent evolution of dioecy in *S. latifolia* from a hermaphroditic ancestor distinguishes this system from sex determination in animal systems where chromosome-based dioecy has existed for 200–300 million years (Lahn and Page 1999). This provides the unique opportunity to isolate the genetic loci necessary for the development of separate sexes and to trace some of the chromosomal changes that have occurred through comparisons with hermaphroditic species. Therefore, *S. latifolia* is a model for studying the processes underlying the separation of gender and the evolution of dimorphic sex chromosomes in plants (for review see Charlesworth and Guttmann 1999; Moneger 2001; Charlesworth 2002).

The Y chromosome of *S. latifolia* is the largest chromosome (~925 Mb) in the genome (Westergaard 1946) and is ~50% larger than the X chromosome, in contrast to the mammalian system, where the Y chromosome is typically dramatically smaller. This difference in chromosome size has not yet been explained, although transposable element movement and accumulation is one potential cause. The lack of recombination over the majority of the Y chromosome is thought to reduce the efficacy of selection (for review see Charlesworth 2002) and thus allow transposable elements with potentially detrimental fitness impacts to accumulate in these nonrecombining chromosomal regions (Steinemann and Steinemann 1991, 1992, 1993, 1997, 1998, 2000; Steinemann et al. 1993; Hochstenbach et al. 1994; Abe et al. 1998; Esposito et al. 1999; Underwood and Bianco 1999; Erlandsson et al. 2000). Retrotransposon-like se-
quences have been isolated previously during screens for Y-linked sequences in *S. latifolia* (Donnison et al. 1996; Nakao et al. 2002) and in other dioecious plant species (Sakamoto et al. 2000; Shibata et al. 2000).

Here we have used restriction fragment length polymorphism (RFLP) genomic subtraction to isolate male-specific sequences from the progeny of an intraspecific cross of *S. latifolia*. This technique was used to isolate low-copy sequences linked to the Y chromosome. One of the fragments, SLP2, was similar to the maize Activator (Ac) transposase and belonged to a low-copy-number repeat family with two Y-linked copies. We found that an intact transposase gene still resides in the genome of *S. latifolia*, but probably not on the Y chromosome. We provide evidence, however, that one of the Y-linked transposase pseudogenes is transcribed and alternatively spliced in both male and hermaphrodite leaf and bud tissues. These results are discussed in the context of Y chromosome evolution in *S. latifolia*, with an emphasis on the potential activity of this transposable element family and its use as a tool for Y-linked gene discovery.

**MATERIALS AND METHODS**

**Plant material:** The F1 population was generated using an individual from an Italian population as the female parent and an individual from a western Massachusetts population as the male parent. The hermaphroditic plant (chromosomally XY) was derived from a single hermaphroditic mutant found in a wild population at the University of Massachusetts, Boston, and maintained by crossing wild-type female plants with hermaphroditic pollen or selfing the hermaphroditic individual. Because Y chromosome deletions can yield hermaphrodites (Westergaard 1946), this individual was used here in comparative screens with normal males. All plant materials were maintained in the greenhouse at the University of Massachusetts, Boston.

**RFLP subtraction and Southern hybridization:** This procedure is a modification of the RFLP subtraction described in Rosenberg et al. (1994). Female DNA sequences were subtracted from male DNAs, allowing the isolation of male-specific sequences located on the nonrecombinating portion of the Y chromosome. The “tester” DNA was extracted from an F1, male individual (Italy female × western Massachusetts male) and the “driver” DNA was extracted from a pool of 10 female individuals from the same cross. Oligonucleotides were purchased from Amplitf Biotech (Boston). The male-specific bands were gel purified with a QIA gel extraction kit (QIAGEN, Chatsworth, CA). The product was ligated to HindIII-digested dephosphorylated pUC 18 vector. The insert DNAs from 24 white clones were prepared individually by PCR using primers purchased from Amplitf Biotech (Boston). The male-specific primers were designed using the sequencing from SLP2 in an attempt to retrieve sequences having a high degree of homology to the transposase pseudogenes from SLP2. The PCR products were sequenced and aligned using the software package ClustalW (Thompson et al. 1994). Pairwise comparisons were made with the Blast2 Sequence function at NCBI (Altschul et al. 1997). The phylogenies were constructed using parsimony or distance methods with 1000 bootstrap replicates of the consensus and the programs provided in the PAUP* package. Branches from parsimony or distance trees were labeled with bootstrap values on the well-supported branches of interest. Well-supported branches from the *S. latifolia* phylogeny, which are composed of genomic-derived sequences from multiple individuals, are considered to represent unique genomic locations.

**RT-PCR:** RNA was extracted using tissue collected from plants provided by Paige Dennis (University of Massachusetts, Boston). Tissue was ground in liquid nitrogen and the RNA was extracted using the RNeasy Mini RNA extraction kit available from QIAGEN. Total RNA was treated with DNase I purchased from GIBCO BRL (Gaithersburg, MD). 1 unit of DNase I per 1 μg RNA, incubated at room temperature for 15 min and EDTA inactivated. Reverse transcription was completed using 3’ rapid amplification of hybridization followed (Bernatzky and Tanksley 1986).

**Library construction and screening:** The genomic DNA was extracted from a pool of five male F1 individuals from the previously described cross and partially digested with Sau3A. The digested DNAs were separated on a 1% agarose gel and the 9- to 23-kb DNA fragment was purified with QIAEXII gel extraction kit. The purified DNA was ligated to λ-arms from the Stratagene (La Jolla, CA) Lambda DASH II/BamHI kit. The cloning and screening were performed according to the library’s protocol. The library was screened with SLP2 (previously described) from this study. Positive clones were picked and DNA was extracted.

To determine the insert size of positive clones, field inversion gel electrophoresis was performed using the PC500 switchback pulse controller system (Pharman, Piscataway, NJ). Gel electrophoresis was performed in 1% Seakem LE agarose gel in 0.5× TBE with the following pulse conditions: 150 V, F/R = 3:1, pulse time = 0.6–2.0 sec for 24 hr.

**Primer design and PCR walking:** The primers used in PCR walking were designed using a computer program Oligo 3.0 and synthesized by Operon Technologies (Alameda, CA). PCR walking followed the principle previously described (Parker et al. 1991). For each PCR, a specific primer (18–24 bases) and a random primer (18–24 bases) were used. DNA prepared from positive clones screened by SLP2 served as the template in PCR walking. Specific primers were designed using the sequence from SLP2 in an attempt to retrieve sequences allowing the reconstruction of the coding region. The PCR mixture was 2.5 μl of 10× buffer (100 mm Tris-HCl, pH 8.3, 500 mm KCl, 15 mm MgCl2, 0.1% gelatin), 2.0 μl of dNTP (1.25 mm each of dATP, dCTP, dGTP, and dTTP), 0.5 μl of specific and random primer (10 μM), 2.0 μl of MgCl2 (10 mm), 0.2 μl of Taq DNA polymerase (5 units/μl), 2.5 μl of DNA (5 ng/μl), and 14.8 μl of H2O. The cycling parameters were 1 cycle 94° 1 min; 30 cycles 94° 1 min; 50° 1 min; 72° 2 min; 1 cycle 72° 5 min, 4° hold.

**Sequencing, database searches, and mining:** Clones and PCR products were sequenced using d-rhodamine dye-terminator or Big Dye chemistry with an ABI prism 377 from Applied Biosystems (Foster City, CA). Homology searches were completed using BLASTX and the nonredundant databases at the National Center for Biotechnology Information (NCBI) website (Gish and States 1993). Intron prediction was completed using the NetGene2 server (Hedegaard et al. 1996). Closely related transposase sequences were gathered from the database through iterative searches with the partial sequence of one λ-cloned and closely related accessions using the search algorithm BLASTX (Gish and States 1993).

**Sequence comparison and phylogeny construction:** DNA or protein sequences were aligned using the software packages ClustalW (Thompson et al. 1994). Pairwise comparisons were made with the Blast2 Sequence function at NCBI (Altschul et al. 1997). The phylogenies were constructed using parsimony or distance methods with 1000 bootstrap replicates of the consensus and the programs provided in the PAUP* package, Version 4 (Swofford 2002). Branches from parsimony or distance trees were labeled with bootstrap values on the well-supported branches of interest. Well-supported branches from the *S. latifolia* phylogeny, which are composed of genomic-derived sequences from multiple individuals, are considered to represent unique genomic locations.
cycles to 2; step 6, 1 min 95

1

min 95

mRNA was incubated with 50 μM of RT Anchor primer at 70° for 10 min. The reaction was completed according to the manufacturer’s protocol. Two rounds of PCR were completed using a nested primer strategy. The following primers were used: RT Amp, 5'-CCGACAGCAGCTAGACA-3'; intron F, 5'-CATGGCT GACAGATGAGATC-3'; intron R, 5'-AAGAGTGTTGTCCT TCCATTTCATC-3'. The first round of PCR was completed using the primers RT Amp and intron F. The second round of PCR utilized the primers intron F and intron R. The primers were used at a final concentration of 1 μM. AmpliTaq Gold brand Tag from Applied Biosystems was used at a final concentration of 0.25 units at the suggestion of the manufacturer. Taq brand of PCR utilized the primers intron F and intron R. The primers using the primers RT Amp and intron F. The second round

TCCATTTCATC-3

GACAGAATGAGGATC-3

into the PGEM-T vector (Promega, Madison, WI) as directed

PCR purification kit (QIAGEN). PCR products were ligated

above. PCR products were purified directly using a QIAquick

intron R primers and PCR conditions were used as described marker (M) is

were the same as those used for the RT-PCR. The intron F and arrows indicate the two male-specific fragments. The size

FIGURE 1.—Southern blot of F1 male and female genomic DNA digested with HindIII and hybridized with SLP2. The arrows indicate the two male-specific fragments. The size marker (M) is λ cut with HindIII.

SLP2 has similarity to the Ac transposase: The 827-bp SLP2 insert was sequenced and used as a query to search the nonredundant database at NCBI using BLASTX with default settings. This search revealed that the entire sequence of SLP2 shares significant similarity to the transposases from the hAT (hobo/Ac/Tam3) superfamily (E values for the first 45 matches ranged from $1 \times 10^{-44}$ to $8 \times 10^{-44}$), including those encoded by the Ac element from Zea mays (E value = $5 \times 10^{-44}$). This fragment encodes the first three of the six conserved hAT superfamily domains (Rubin et al. 2001).

Isolation of SLP2-related sequences from a male S. latifolia genomic library and sequence analysis: A genomic λ-library was constructed from a pool of five male full siblings. Screening of the library (one million or single-copy DNA fragments (data not shown).

From a F1 population, genomic DNAs from five males and five females were digested with HindIII and probed with the plasmid inserts isolated in the RFLP subtraction. Fragments present in males but absent from females are likely to be linked to the nonrecombining portion of the Y chromosome. One clone, SLP2, hybridized to 4–8 bands in both males and females, including two unique male-specific fragments (Figure 1). The male-specific fragments are ~3.0 and 0.9 kb in size. In addition, a band of ~0.7 kb is twice as bright in all female individuals when compared to all males, indicating that these fragments are likely X-linked, although other explanations are possible. Additional fragments of ~16 and 7 kb in the SLP2 hybridization show 1:1 segregation patterns in males and females, indicating that they are located to either maternal X chromosomes or autosomes.

RESULTS

Isolation and identification of male-specific sequences: Fifty-eight recombinant clones obtained from one transformation of subtraction products were transferred to a nylon membrane and probed with pooled female genomic DNA. On the basis of the intensity of the hybridization signals, 24 clones appeared to contain only low- or single-copy DNA fragments (data not shown).

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The regions sequenced (boxed) of the λ-clones flanking the original male-specific plasmid SLP2. SLP2 was used as a probe to a male λ genomic library and five clones, Thelma13, -7, -2, -6-2, and -6-1 (12–22 kb), were recovered. Primers (SF and SR are indicated by arrowheads) based on the sequence of SLP2 were used to amplify the corresponding region from each of the λ-clones. The 3482-bp Thelma13 fragment containing the complete transposase coding region (coding region indicated by solid box) was sequenced by primer walking using primers designed to SLP2 and random primers. The region flanking the putative intron was amplified using primers (IF and IR indicated by arrowheads) from Thelma7, -13, and -2. The 5174-bp region of Thelma7 was sequenced (regions that are not homologous to Thelma13 are indicated by diagonal lines). Multiple stop codons (*) and deletions (d) resulting in frameshifts indicate that it is a likely pseudogene.
Thelma, a Tag2-like Transposase With Y-Linked Copies

Figure 3.—Comparative phylogenetic analysis of hAT superfamily proteins with the putative Thelma13 transposase. The sequences are labeled with their GenBank accession numbers. The first two letters are the genus species abbreviations (Fo, Fusarium oxysporum; At, A. thaliana; Ol, Oryza latipes; Nt, Nicotiana tabacum; Zm, Z. mays; Pg, Pennisetum glaucum; Os, Oryza sativa; Sl, S. latifolia; Am, Antirrhinum majus; Bd, Bactrocera dorsalis; Ag, Anopheles gambiæ; Md, Musca domestica; Dm, Drosophila melanogaster; Lc, Lucilia cuprina; Ti, Toxoplasma inflatum; Ai, Ascolobus immersus; Ip, Ipomoea purpurea). The transposable elements that have been described are named after the accession number. The well-supported clades are indicated by brackets and given the name of the first described element in that clade.

from the male and hermaphrodite (both XY), but not from the female. The appropriate controls eliminated the likelihood of genomic contamination.

The primers flanking the intron site were also used to amplify genomic DNA from the male, female, and hermaphroditic individuals. A single sharp band of ~600 bp was obtained from each individual (data not shown). This band was directly cloned and 20–25 clones were sequenced for each individual, with the goal of identifying most of the amplifiable copies in each of these genomes and the specific copy being expressed. Six of 25 copies from the male, 3 of 20 copies from the female, and 2 of 20 copies from the hermaphrodite were unique. We constructed a phylogeny of the unique amplified sequences (per individual), excluding the intron (Figure 5b), to understand the relationship of these sequences and to estimate copy number in the genome. The phylogeny includes the cDNA fragments from male and hermaphrodite; the unique genomic clones from the male, female, and hermaphrodite; and the corresponding regions of the genomic clones Thelma7, -13, and 2. The deduced phylogeny reveals three major clades supported by high bootstrap values. The phylogeny is further broken down into five subdivisions labeled A–E, each likely representing a separate locus with males having copies in all five branches. Branches A and E represent the two putative Y-linked copies first identified by Southern blots (Figure 1). Branch A consists solely of the Y-linked λ-clone Thelma7 fragment. Branch E contains one genomic clone from the male (gDNAm13) and one from the hermaphrodite (gDNAh16). The clade also contains the two alternatively spliced transcripts, RNA1m21 and RNA2m9, isolated from the male and identical to the male genomic clone, and the two alternatively spliced transcripts isolated from the hermaphrodite and identical to its genomic clone. The genomic sequence gDNAm13 is 99.2% identical to the genomic copy gDNAh16, suggesting that they are allelic. Branch E is a strongly supported clade that lacks female sequences, but includes copies with gender-specific expression. Together, this suggests that branch E is a male-specific Y-linked grouping.

DISCUSSION

We report a novel gene family in the genome of S. latifolia, with homology to a large superfamily of class 2 transposase proteins. Class 2 transposons are mobile
Figure 4.—Boxshade Thelma13, Tag2, and Ac (ORFα). The solid boxes indicate identical or similar residues shared between all sequences. The shaded boxes indicate identical or similar residues shared by two of the sequences. The first underlined region is involved in DNA localization. The second underlined region represents the BED zinc finger DNA-binding domain (Aravind 2000) conserved between Ac and Thelma13, predicted by RPS-BLAST at NCBI, and includes the DNA-binding domain biochemically determined for Ac. Conserved hAT superfamily protein blocks are indicated by boxes and represented by letters A–F (Rubin et al. 2001). The regions marked D–F are located in the region involved in dimerization (Essers et al. 2000). An open arrow identifies the position of the single alternatively spliced intron shared by Thelma and Tag2. Ac transposase mutants truncated at the regions marked by the solid arrows upregulate transposition when cotransfected with wild-type TPase (Kunze et al. 1993).

Genetic units capable of changing chromosomal location, via a DNA-mediated, cut-and-paste mechanism (for review see Feschotte et al. 2002). An intact putative transposase coding region in S. latifolia, Thelma13, is likely to be linked to the X or autosomes. Thelma13 belongs to the hAT superfamily of DNA transposons, branching with the Ac/Tam3 clade, most closely related to Tag2 of Arabidopsis (Figure 3). Thelma13 possesses all six described domains found in Ac-like transposases. Thus, Thelma transposase genes are likely dispersed as part of a class 2 TE family, although we currently have no evidence for their mobility. Two copies in S. latifolia are Y-linked, but both appear to be pseudogenes, although one is transcribed in leaf and bud tissue. Thelma7, a Y-linked copy, has acquired several mutations, including a large insertion or deletion (indel) in the 5′ end, which interrupts the coding region. The degree to which Thelma7 is degraded is consistent with it being an ancient component of the Y chromosome, although no orthologous copy was identified in our survey of the XY hermaphrodite’s genome. This suggests that in the hermaphrodite, the region may have been deleted and therefore includes the female suppression loci (Westergaard 1946; Lebel-Hardenack et al. 2002).

Transposon regulation: Class 2 transposable element movement is likely regulated at multiple levels both by the host and in an autoregulatory fashion. Alternative splicing via occasional intron retention has been reported for Tag2 (Henk et al. 1999) and the alignment shows that the intron detected in Thelma is in the same position as the single intron in Tag2 (Figure 4). Intron retention, the most common form of alternative splicing (Mironov et al. 1999), frequently functions as a posttranscriptional regulatory mechanism with the short isoform acting as a dominant-negative regulator of the longer one (Boise et al. 1993; Okada et al. 1997; Ross
et al. 1997; Srinivasula et al. 1999). For Tag2 and Thelma, the retention of the intron would introduce a premature stop codon and lead to a C-terminal truncation. Thus, the translation of both transcripts would result in the presence of long and short protein isoforms. The long isoform would contain all the domains necessary for catalyzing transposition and presumably functions as the transposase. In contrast, the short isoform would retain the DNA localization signal, DNA-binding domain, and regions of conserved but unknown function (regions A, B, and C in Figure 4), but would not have the dimerization domain, which likely overlaps the catalytic domain (regions D, E, and F in Figure 4). The short isoform may be capable of nuclear localization and DNA interaction, but not capable of the dimerization mandatory for transposition. The short isoform might compete with the transposase (long isoform) dimers for DNA interaction and therefore prevent transposition in a concentration-dependent manner.

For some elements (Ac and Tag1), post-transcriptional regulation has been implicated because there is little, if any, correlation between transcript levels and excision frequencies (see Kunze and Weil 2002). Dominant-negative mutants have been found for Ac, but result from coexpression of proteins that lack the N terminus DNA-binding domain like those that arise from alternative transcription or translation start site usage (Kunze et al. 1993). Interestingly, proteins that lack regions of the C terminus when coexpressed with wild-type Ac transposase actually increase excision frequencies in transfected petunia protoplasts (Kunze et al. 1993). Further experiments are required to understand the potential role of the two isoforms in S. latifolia.

Y degradation and transposon tagging: The accumulation of transposable elements in chromosomal regions with low recombination has been well documented in animal species (e.g., Steinemann and Steinemann 1998; Erlandsson et al. 2000). Class 1 TE fragments have been isolated in S. latifolia during screens for male-specific sequences (Donnison et al. 1996; Nakao et al. 2002) as well as in two other dioecious plant species, Cannabis sativa and Rumex acetosa (Sakamoto et al. 2000; Shibata et al. 2000). In animals, Y-linked, class 2 TEs have been reported much less frequently and this is the first report of a transposase-related sequence on a plant Y chromosome.

The lack of recombination over the majority of the Y chromosome probably causes the unavoidable decay of linked genes due to population genetic processes such as genetic hitchhiking, selective sweeps, and Muller’s ratchet (for recent review see Charlesworth and Charlesworth 2000). Genes remaining active are most likely either new arrivals awaiting erosion or ancient and selectively advantageous. The Y-linked sequence, Thelma7, is clearly a pseudogene in the process of erosion with no detectable transcription. The other Y-linked Thelma locus recovered from both the male and

![Image](image_url)
The Thelma family is the first class 2 transposable element family isolated in the genus Silene and the first described in plants with copies that are Y-linked. One copy not on the Y chromosome appears to be intact and capable of encoding a complete transposase with all the domains necessary to catalyze its own movement and the movement of other elements in trans. Previous reports of genetic XY males expressing the hermaphroditic phenotype when treated with demethylating agents (Donnison et al. 1996; Janousek et al. 1996) might be explained by the movement of hAT superfamily transposons, since methylation has been shown to be involved in their regulation (e.g., Ac in maize reviewed in Kunze and Weil 2002). Since hAT superfamily transposable elements move both locally and into gene-rich regions (Kunze and Weil 2002), an endogenous hAT transposon tagging system may be a useful tool for gene discovery of Y-linked genes in the S. latifolia genome. The S. latifolia Y chromosome is the largest of the 24 chromosomes present in this species and is largely nonrecombinable, making gene discovery by conventional methods extremely difficult. The possibility of using an endogenous transposable element family for a targeted tagging approach is worthy of further investigation, as it may be possible to bypass the need for generating transformed plants, a technically difficult procedure in Silene.

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