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Genetic and molecular analysis of the Enhancer (En) transposable element system of *Zea mays*

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A newly isolated, unstable mutation *wx-844*::En-1 of *Zea mays* was proven to be caused by the insertion of the autonomous transposable element En into the *Waxy (Wx)* gene. Molecular analysis revealed that En-1 is 8.4 kb long, has a 13-bp long perfect inverted repeat at its termini and generates a 3-bp target site duplication. En-1 is integrated into an intron located approximately in the middle of the transcribed region of the *Wx* gene. Structural evidence is presented indicating that a receptor component (Inhibitor) can arise by internal deletion of an autonomous En element.

Key words: autonomous element/receptor element/*waxy* locus/genetic analysis/sequence analysis

Introduction

The Enhancer-Inhibitor (En-I) transposable element system in maize was reported originally by Peterson (1953) to be responsible for controlling the instability of a pale green mutable allele (Peterson, 1960). The Suppressor-Mutator (Spm) system isolated by McClintock (1954) was shown to control the mutability of the *a-m1* allele (McClintock, 1953). Further studies (Peterson, 1965) revealed that the two systems are homologous since the En element could act at the receptor of Spm and, similarly, Spm could act at Inhibitor, the receptor of En. Previously we demonstrated this interaction also at the molecular level by showing that the receptor of Spm in the *wx-m8* allele (Spm-I8) responds to En (Schwarz-Sommer *et al.*, 1984).

The loci known to be under the control of an autonomous En element encode for as yet unidentified functions (for a recent review, see Nevers *et al.*, 1984). Thus the 'classical' strategy (protein-antibody-cDNA-genomic cloning) for molecular cloning of the wild-type gene and its allele containing the controlling element could not be used to isolate the element itself. For this reason we set up a transposon tagging experiment to move En, located somewhere in the genome, into the *Wx* gene that we have previously cloned and studied (Schwarz-Sommer *et al.*, 1984).

Here we report the isolation of an unstable mutation and show by genetic analysis that this mutation is due to the insertion of an autonomous En element at the *Wx* locus. Molecular cloning and sequence analysis reveals the presence of the element within the transcribed region of the *Wx* gene. We also present evidence for the structural relationship between the autonomous En element in the *wx-844* allele and the receptor component of Spm in the *wx-m8* allele (Schwarz-Sommer *et al.*, 1984).

Results

Genetic evidence for an autonomous En element integrated in the Wx gene

Isolation of the En-induced unstable wx-844 mutation. An isolation plot was established in the summer of 1983 at Ames, Iowa, to search for new En-induced mutants at the *Wx* locus. The crossing scheme (cross A) involved an En-containing line as the female parent (which was de-tasseled before flowering) and a *C sh bz wx* tester as the male line. The dominant alleles of all color-determining loci except those shown in cross A were present in the two lines.

$$\text{Cross A}\quad \frac{\textit{C-I Sh Bz Wx}}{\textit{C-I Sh Bz Wx}}\,;\frac{\textit{a1-m(r)}}{\textit{A1}}\,;\frac{\text{En}}{\quad}\ \times\ \frac{\textit{C sh bz wx}}{\textit{C sh bz wx}}\,;\frac{\textit{A1}}{\textit{A1}}$$

Individually isolated mutant *wx* kernels were selected visually and planted in the greenhouse at Cologne, FRG. Detailed genetic analysis will be given elsewhere. Instability of the mutant designated as *wx-844* was observed in one of the initially isolated kernels as variegated sectors of *Wx* over *wx* background on the basis of several tests. The heterozygous kernel containing the *wx-844* allele had the genotype

$$\frac{\textit{C-I Sh Bz wx-844}}{\textit{C sh bz wx}}\,;\frac{\textit{a1-m(r)}}{\textit{A1}}$$

The mutability of the *wx-844* allele was later confirmed by staining the pollen (Figure 1A) from the growing plant with a standard iodine reagent (KI/I_2, Nelson, 1968).

Linkage analysis from an outcross

The heterozygous mutant isolate was used as a male parent on En tester lines (*a1-m(r)/a1-m1*) which test for the presence of En (see cross B in Table I). In this cross, the mutability of *wx-844* cannot be observed due to the presence of the wild-type *Wx* allele in the homozygous female tester. Also, mutability caused by En in the *wx-844* allele on receptor alleles at the *A1* locus (*a1-m(r). a1-m1*) cannot be observed because of the linkage of the dominant color inhibitor allele *C-I* to *wx-844* (see cross A). However, a cross-over between the *C* allele and the *wx-844* allele (cross B in Table II) will result in a *C-wx-844* linkage and the spotted phenotypes can be detected.

The assumption that there is only one autonomous En in the genome and that this En is located at the *Wx* locus in the *wx-844* allele can be verified as follows: the cross-over distance between the *C* and the *Wx* locus on chromosome 9 is known to be 33 map units (Rhoades, 1950). If the single En is linked to the *wx-844* allele the frequency of spotted phenotypes will correspond to the expected frequency of crossing-overs between *C* and *Wx*. As shown in Table I the expected frequency of spotted phenotypes is 8.2%, which is in agreement with the observed frequencies of 7.2 and 7.7% in the two ears obtained.

Linkage analysis on the F2 progeny ear. Independent evidence of linkage for an autonomous En at the *Wx* locus in

Table I.

Results of the outcross (cross B)

C Sh Bz Wx ; *a1-m(r)* x *C-I Sh Bz wx-844* (En) ● *a1-m(r)*
C Sh Bz Wx *a1-m1* *C sh bz wx* ● *A1*

Observed frequencies of phenotypes tested against a hypothesis of En linked to the Wx locus (wx::En) and the alternative hypothesis of En not lined to Wx (wx;En).

Cross No.	Hypothesis tested	Number	Colored	Colorless	Spotted	(%)	Total
527–1/517		Observed	131	268	31	(7.2)	430
	wx::En	Expected	144	251	35	(8.2)	
	wx;En	Expected	134	242	54	(12.5)	
527–2/517		Observed	119	263	32	(7.7)	414
	wx::En	Expected	138	242	34	(8.2)	
	wx;En	Expected	129	233	52	(12.5)	

Table II.

(A) Chromosome constitution of the F1 mutant isolate.

	Chromosome 9	Chromosome 3
	C-I Sh Bz wx-844 (En) ●	*a1-m(r)*
Map distance	3 2 28	
	C sh bz wx ●	*A1*

Genetic distances are given in map units between the chormosomes.

(B) Frequencies of phenotypes observed against the frequencies expected in the F2 on the assumption of linkage exemplified above (A).

	Number of kernels			
	Colored	Coloreless	Spotted	Total
Expected	62	257	11	330
Observed	56	263	11	330

(C) Phenotypes of spotted kernels (from B)

Phenotypes	Genotypes	Number of kernels	
		Expected	Observed
Purple, spotted, round	*CC Sh_ Bz_ wx*(En)_	1	3
Purple, spotted, shrunken	*CC shsh Bz_ wx*(En)	1	1
Bronze, spotted, shrunken	*CC shsh bzbz wx*(En)	9	7

the *wx-844* allele can be obtained from the F2 progeny ear of the selfed original mutant isolate. On the basis of the linkage group on chromosome 9 and the independently segregating *a1-m(r)* alleles on chromosome 3 (outlined in Table IIA), the frequency of individual phenotypes can be calculated. Because the observed frequency of the three expected phenotypes corresponds to the evaluations based on linkage (Table IIB and C), En is indeed located at *Wx*. In addition, if the En causing instability at the *a1-m(r) and a1-m1* alleles segregated independently of the *wx-844* allele, the spotted phenotype class should contain some stable *wx* phenotype kernels (not mutable at the *wx* locus). By iodine staining of the available 11 spotted kernels we could prove that each of these also show instability at the *wx* locus (see Figure 2). Hence this En is co-segregating with the *wx-844* allele.

Molecular properties of the wx-844 allele

Southern blot analysis of genomic DNA. To identify the molecular structure of the *wx-844* allele, genomic Southern blots were prepared using plant DNA originating from the following genotypes: the heterozygous *wx-844/wx* plant, the stable recessive (*wx/wx*) parent and a plant carrying both progenitor alleles (*Wx/wx*). Since the physical map of the wild-type *Wx* gene in line C plants is known (Schwarz-Sommer *et al.*, 1984, see also scheme in Figure 3) the insertion site of any foreign DNA into the gene can readily be determined by the proper choice of the restriction enzyme to digest genomic plant DNA and by the use of a suitable probe.

In the hybridization experiment shown in Figure 3A *Bam*HI-digested genomic DNAs were hybridized with the wild-type 0.6-kb *Bam*HI probe. The progenitor *Wx* and the stable recessive *wx* allele contain the expected 0.6-kb fragment. The *wx-844* allele, however, contains two new *Bam*HI fragments of 3 and 6 kb in size indicating an insertion into the 0.6-kb (wild-type) fragment that at least contains one *Bam*HI restriction site. The minimum size of the insertion is $3+6-0.6=8.4$ kb. Since it is the autonomous En element we term it En-1. Due to the presence of the stable recessive *wx* allele in this heterozygous plant the 0.6-kb *Bam*HI fragment also lights up. One should note that if somatic reversion events take place induced by the autonomous En present at the locus, the size of the restored *Bam*HI fragment after excision of En would also be 0.6 kb in size.

To prove whether En-induced somatic reversion events in fact can restore the wild-type fragment size, we used *Eco*RI to digest the genomic DNAs. Since the entire *Wx* gene is contained on one *Eco*RI fragment (Shure *et al.*, 1983; Schwarz-Sommer *et al.*, 1984) the same band will hybridize irrespective of whether the 5′ (*Bam* 4.5 see Figure 3B) or the 3′ region (*Sal* 0.75 see Figure 3C) of the *Wx* gene is used as a hybridization probe. Depending on polymorphisms outside the *Wx* gene (Shure *et al.*, 1983; Schwarz-Sommer *et al.*, 1984) the size of the *Eco*RI-generated fragment is 21 kb in the stable recessive *wx* allele and 14 kb in the wild-type *Wx* progenitor allele (see middle and right lanes in Figure 3B and C).

As shown in the left lanes of Figure 3B and C in the *Eco*RI-digested DNA of the heterozygous *wx-844* plant, several bands are present when hybridized with the wild-type *Bam* 4.5 or *Sal* 0.75 probes. The 21-kb band hybridizing to both probes clearly comes from the male recessive allele. The less intensive 14-kb band, which also hybridizes to both probes, can be interpreted to result from frequent somatic reversion events induced by En-1. Restoration of wild-type-sized fragments was also seen previously due to En-induced reversion

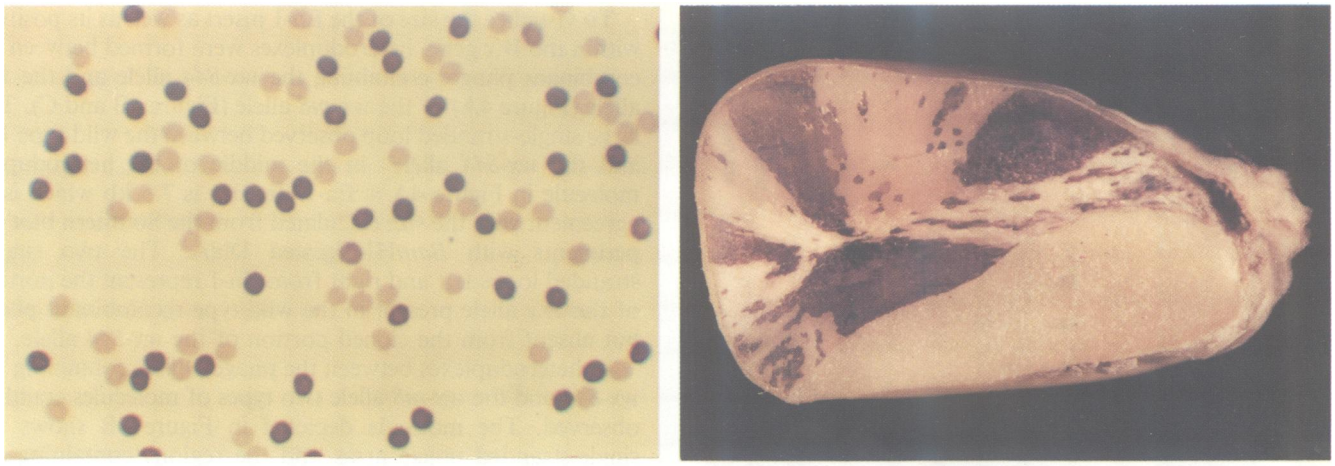

Fig.1A

Fig.1B

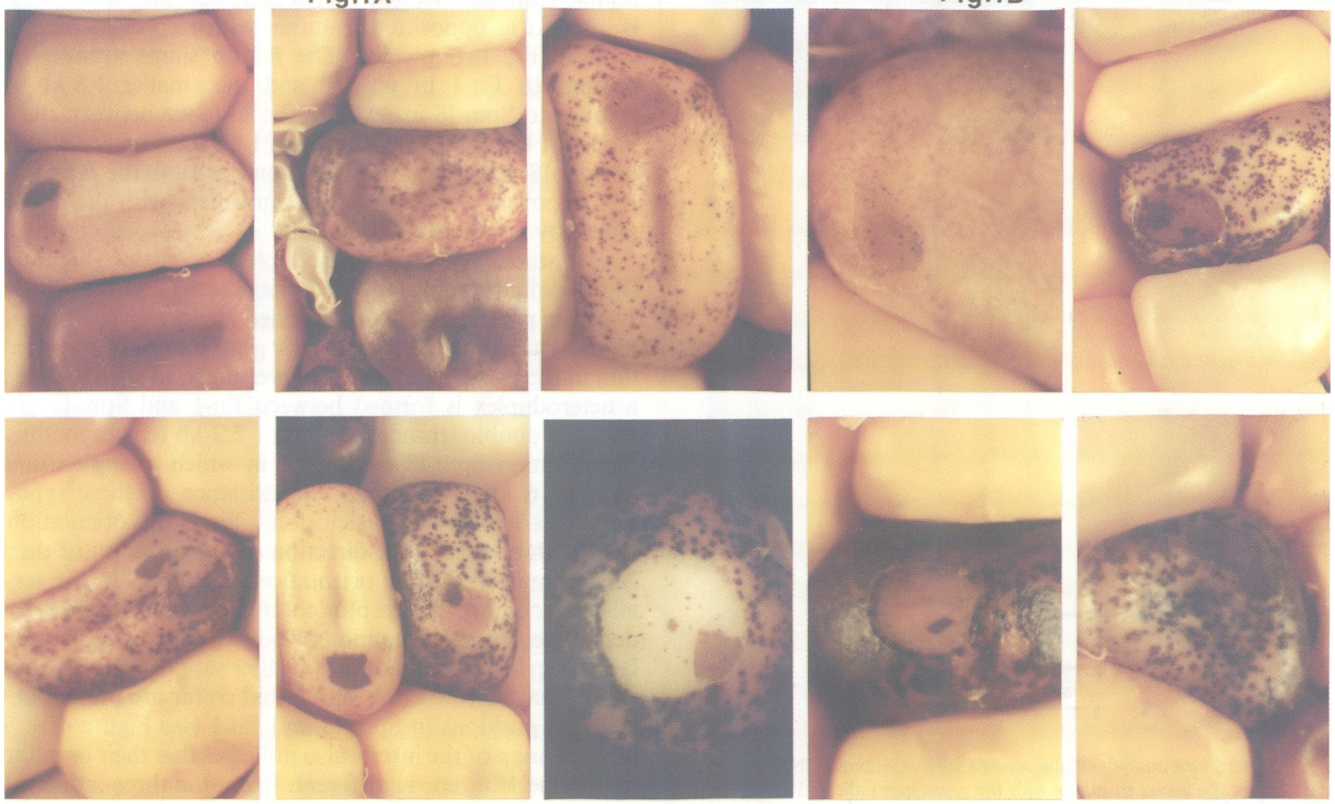

Fig.2

Fig. 1. Mutability at the *wx-844* allele as revealed by iodine staining of the pollen **(A)** of the heterozygous F1 plant (genotype 1, see Results) and of the endosperm **(B)** of one kernel taken from the F2 progeny ear of the selfed F1 plant. Dark staining pollen **(A)** or endosperm sectors **(B)** indicate reversion events.

Fig. 2. En-induced instability at the *a1-m(r)* and *a1-m1* alleles in the kernels obtained on ears of the selfed F2 progeny plant carrying the *wx-844* allele (see Table II). The colored spots arise by En-induced reversion events at the receptor alleles *a1-m(r)* and *a1-m1*. Dark spots are seen if *Bz* and bronze spots if *bz/bz* is present in the genome of the kernel (see Table II). The 11 spotted kernels were cut at their top to stain with the iodine reagent. Mutability at the *Wx* locus is seen as dark sectors *(Wx)* over a non-staining background *(wx)*. Differences in the spotting patterns among the kernels indicate frequent changes in the state of the En element.

events of the *wx-m8* allele (Schwarz-Sommer *et al.*, 1984).

In the mutant *wx-844* two new bands appear, which are 10 kb (Figure 3B, left lane) and 7.8 kb (Figure 3C, left lane) in size and represent the 5′ and 3′ junction fragments, respectively, between En-1 and the *Wx* gene. Since the size estimate of 3.8 kb for En-1 from the *Eco*RI junction fragments (10+7.8−4) is considerably smaller than the estimate from the *Bam*HI digestion, En-1 seems to have more than one internal *Eco*RI site. We have no explanation for the origin of the 7-kb *Eco*RI fragment in the *wx-844* allele hybridizing to the *Bam* 4.5 probe.

Cloning and isolation of En-1 at wx-844

DNA isolated from leaves of the growing heterozygous *wx-844* plant with the genotype *C-I Sh Bz wx-844 / C sh bz wx* was used for partial digestion with *Mbo*I. DNA fragments in the size range of 14−25 kb were taken to construct a library

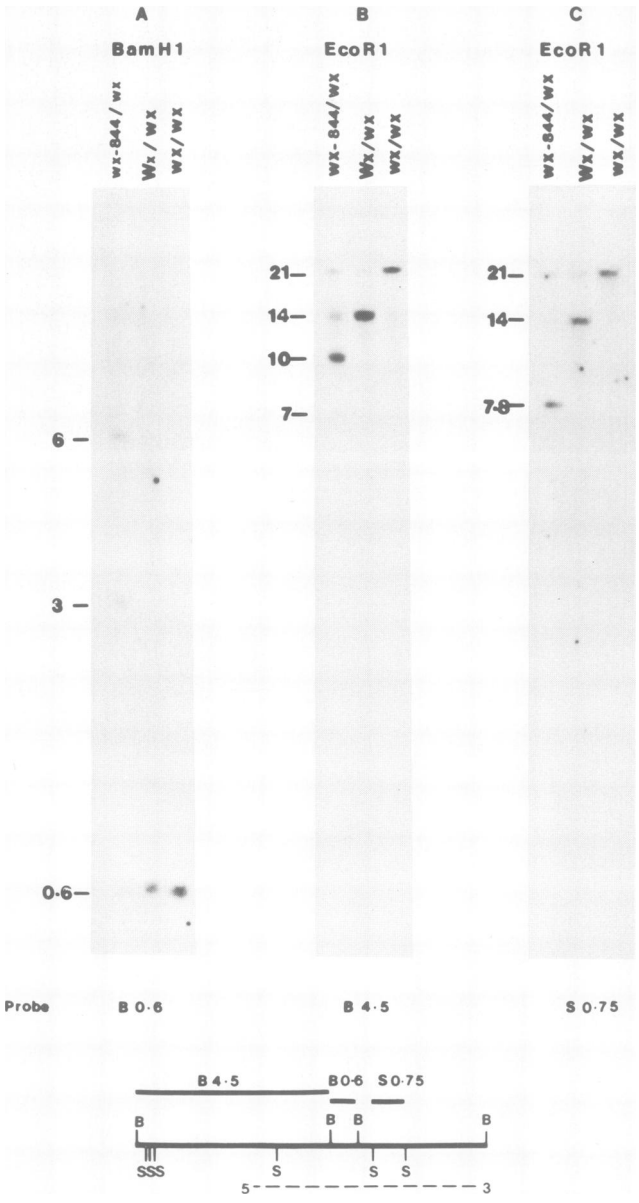

Fig. 3. Southern blot analysis of the *wx-844* allele. Genomic DNA was prepared from the leaves of plants with the genotypes indicated above each lane. After digestion with the restriction enzymes *Bam*HI **(A)** and *Eco*RI **(B,C)** the DNA was loaded onto an 0.7% agarose gel, followed by electrophoresis and transfer onto nitrocellulose filters. Hybridization with the nick-translated probes at 65°C was performed as described in Materials and methods. The probes used for the experiments are indicated below each set and the size of the hybridising DNA fragments is given in kb. The origin of the radioactive probes with respect to their position within the wild-type *Wx* gene is shown in the physical map at the bottom of the Figure. The staggered line below the physical map indicates the transcribed region of the *Wx* gene (Schwarz-Sommer *et al.*, 1984).

in λEMBL4 (see Materials and methods). A library of ~8 x 10^5 p.f.u. was screened with a probe containing a mixture of fragments originating from the 5′ and 3′ region of the cloned wild-type *Wx* gene (see scheme in Figure 3). The recombinant phages identified to contain both regions of the *Wx* gene were then screened using an Spm-I8 probe corresponding to the 2.2-kb insert in the cloned *wx-m8* allele of the *wx* gene (Schwarz-Sommer *et al.*, 1984). One clone, λ wx 844-148, hybridizing to all three probes, was characterized further.

To visualize the size of the En-1 insert as well as its position within the *Wx* gene, heteroduplexes were formed between recombinant phages containing the *wx-844* allele and the *Wx* allele (Figure 4A) or the *wx-m8* allele (Figure 4B and C). The large single-stranded loop observed between the wild-type *Wx* and the *wx-844* allele in the middle of the heteroduplex molecule in Figure 4A is En-1. Its size is 7.5 kb which is in agreement with the size calculated from the Southern blot experiments with *Bam*HI-digested DNA. The two single-stranded loops left and right from En-1 represent the portion of the *Wx* allele present in the wild-type recombinant phage but absent from the cloned portion of the *wx-844* allele.

In heteroduplexes between the phage DNAs containing the *wx-844* and the *wx-m8* allele two types of molecules could be observed. The molecule depicted in Figure 4B shows the single-stranded phage arms and the regions containing the double-stranded *Wx* gene. This indicates that the *Wx* region is inserted in opposite orientation with respect to the phage arms in the two phages. The 8.2-kb single-stranded loop belongs to En-1 in *wx-844* and the smaller 1.8-kb loop belongs to Spm-I8 in *wx-m8*. The distance between the two loops is 1 kb. In fact, a distance of 1 kb from the insertion site of Spm-I8 in *wx-m8* would position En-1 into the *Bam* 0.6-kb *Wx* fragment. This again is in agreement with the data obtained by Southern blot analysis.

The second type of heteroduplex molecules (Figure 4C) shows paired double-stranded phage arms and the wild-type *Wx* gene portions of the two cloned alleles as single-stranded loops. This confirms that the *Wx* gene is in opposite orientation in the two phages. Most interestingly, in such a molecule a heteroduplex is formed between En-1 and Spm-I8 giving rise to a double-stranded region of 2.2 kb (corresponding to the common Spm-I8 sequences) in which a single-stranded loop of 6 kb is inserted. The existence of these types of molecules allows two conclusions. First, the orientation of En-1 with respect to the direction of transcription of the *Wx* gene is opposite to the orientation of Spm-I8 in the *wx-m8* allele. Secondly, the receptor component Spm-I8 is a deletion derivative of the autonomous En-1. The strong structural relationship between I and En is further supported by the observation that all restriction sites tested so far are present at unchanged positions in Spm-I8 and En-1 (see Figure 4D). The physical map of the inserts also indicates that their orientation within the *Wx* gene is different. From the three independent heteroduplexes studied the size of En-1 seems to be ~8 kb, which is in good agreement with the size estimate from the genomic Southern blots. This also proves that the size of the En-1 element has not been altered during the cloning process.

DNA sequence analysis of the termini of the integrated En-1 element

The subcloned 0.6-kb *Bam*HI fragment of the wild-type *Wx* gene and the two *Bam*HI fragments containing the left (5′) and right (3′) junction fragments of En-1 in the *wx-844* allele were sequenced. As presented in Figure 5 the integration of En-1 into the *Wx* gene generates a 3-bp target site duplication. Comparison of *Wx* wild-type genomic with *Wx* cDNA sequences (cDNA sequence to be published elsewhere) reveals that En-1 has integrated into an intron of the *Wx* gene (Figure 5). The termini of the En-1 element have regions of inverted sequence repetition identical to the previously described ends of the receptive Spm-I8 element. Only a few differences were observed which all occur within the loops of the compared region of the stem and loop structure shown in Figure 5.

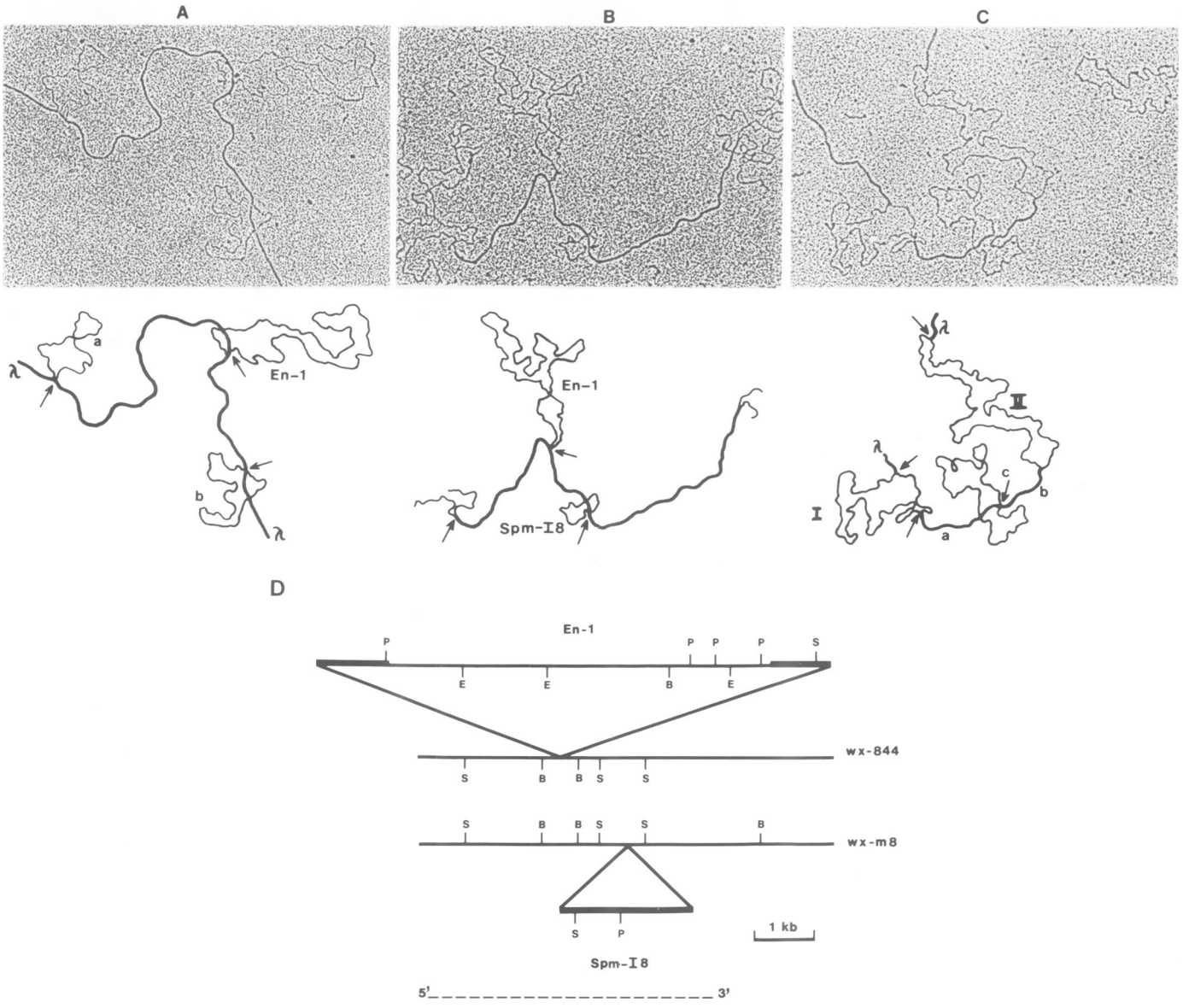

Fig. 4. Comparison of the *wx-844* allele with the wild-type *Wx* **(A)** and with the *wx-m8* mutant allels **(B,C)** by heteroduplex and restriction analysis **(D)**. **(A)** The heteroduplex shown was formed between recombinant phages carrying the wild-type *Wx* (λWx-45, see Schwarz-Sommer *et al.*, 1984) and the *wx-844* (λwx844-148, see Results) alleles. The single-stranded loops a and b (narrow lines) belong to the portion of the *Wx* gene which is present in the λWx-45 phage but absent from the λwx844-148 phage. The 7.5-kb long single-stranded loop emerging from the double-stranded DNA region between the flanking loops is En-1. The size estimate of the insert is based on measurements of five independent molecules. **(B,C)** Heteroduplexes are formed between recombinant phages carrying the *wx-8* (λwxm8-2, see Schwarz-Sommer *et al.*, 1984) and the *wx-844* allele (λwx844-148). The orientation of the *Wx* gene with respect to the EMBL4 phage arms is opposite in these two clones. The molecules shown in the middle panel **(B)** represents heteroduplexes in which the *Wx* portions of the two phages are double-stranded (bold lines) and the phage arms are single-stranded. The single-stranded loops indicated by arrows belong to En-1 (8.2 kb) within the *wx-844* allele and to Spm-I8 (1.8 kb) within the *wx-m8* allele, respectively. The distance separating the two loops is 1 kb. These data are based on four molecules measured. In the heteroduplex shown in panel **(C)** DNA of the λEMBL4 phage arms hybridized. The displacement loops I and II belong to the *Wx* portions within the two phages. The 2.2-kb long double-stranded region (a,b) corresponds to the Spm-I8 insert of *wx-m8* which is homologous to parts of the En-1 insert in *wx-844*. The single-stranded loop (6-kb) emerging from that double-stranded region is En-1 material not present in the receptor I. Five heteroduplexes were measured. **(D)** Physical map of En-1 within the cloned segment of the *wx-844* allele as compared with the map of Spm-I8 within *wx-m8*. The bold lines indicate Spm-I8 sequences present in En-1. The restriction enzymes used are abbreviated as follows: B, *Bam*HI; E, *Eco*RI; P, *Pst*I; S, *Sal*I. The scheme also gives the insertion site of En-1 and Spm-I8 within the wild-type *Wx* regions present in the recombinant phages from both mutants. The direction of transcription and the size of the *Wx* transcription unit is depicted by staggered lines at the bottom.

Discussion

Usually it takes several years of genetic analysis to confirm that a mutable allele contains an autonomous element at a given locus. However, simultaneous molecular and genetic analysis of the *wx-844* allele has allowed the identification of the autonomous En element one generation following the observation of mutability. We used several lines of evidence to prove that En-1 is an autonomous element at the *wx* locus. (i) In the genetic analysis of co-segregation we obtained evidence for the linkage between the mutable *wx-844* allele and En. (ii) Molecular cloning of the allele allowed us to identify an 8.4-kb insert within the transcribed region of the *Wx* gene. The insert is homologous to its receptor (I) and the heterduplex analysis showed that I is a deletion produce of an autonomous En. (iii) Knowledge of the molecular properties of En-1 allowed the isolation of another autonomous En ele-

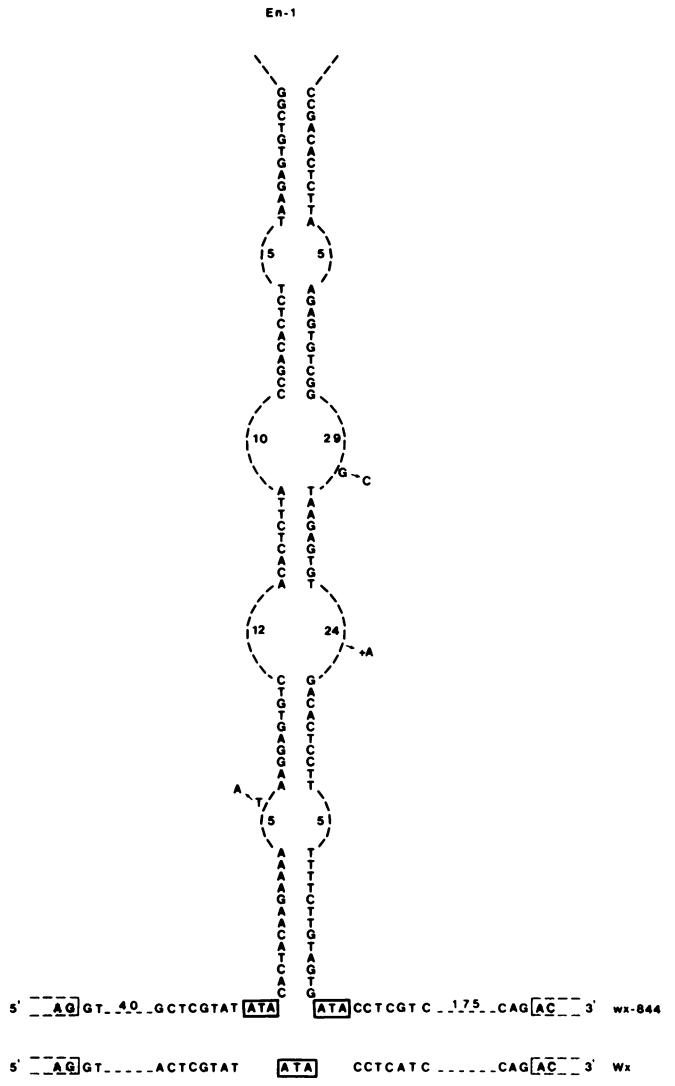

Fig. 5. Nucleotide sequence of the 5′ and 3′ junctions of En-1 and its integration site within the wild-type *Wx* gene. Exon-intron junctions are indicated by shadowing the exon sequences which were determined by comparison of genomic and cDNA sequences from the corresponding region of the *Wx* gene (Klösgen *et al.*, unpublished results). The target site of En-1 within the *Wx* gene (lower sequence) and the target site duplication within the *wx-844* allele (upper sequence) is shown in boxes. The left and right termini of En-1 are folded to produce the longest possible double-stranded structure. Sequence deviations between Spm-I8 (Schwarz-Sommer *et al.*, 1984) and En-1 are indicated by arrows. We should note that the wild-type sequence given here does not correspond to the sequence present in the progenitor allele of *wx-844*. Sequencing several alleles of the *Wx* gene we realized that although exon sequences are conserved, there are sequence deviations within the introns.

ment (papu) at the *A1* locus (Peterson, 1970). The isolation of the *a1-m(papu)* allele of maize will be described elsewhere. Heteroduplex molecules formed between the two Ens show a perfect double-stranded 8.2-kb long structure. No differences in the position of restriction sites studied so far were found between the two elements.

Genetic properties of the autonomous En element present at the wx-844 allele

In genetic crosses we obtained strong evidence that there is only one autonomous En element in the genome of the heterozygous mutable kernel carrying the *wx-844* allele and that this element is linked to the *Wx* locus. The most convincing evidence comes from the observation that the action of the autonomous element on a receptor component located at the *A1* locus *(a1-m1)* always co-segregates with the mutability at the *Wx* locus. Since any action of En at the *a1-m1* allele can only be observed in the absence of the *C-I* allele which was present within the *wx-844* allele-containing chromosome, the spotted and *wx* mutable kernels must contain En and the *C* allele. This recombinant chromosome, carrying En and *C* can arise by crossing-over during meiotic division and since the observed frequency of spotted kernels (containing En) corresponds to the theoretical frequency of cross-overs between the *C* and *Wx* alleles the En must be located at the *wx-844* allele.

The element has some interesting properties that are already evident from this preliminary analysis. The frequency of somatic reversion events induced by En-1 at the *wx-844* allele is very high (see iodine-stained pollen and kernel in Figure 1). The excision of plant transposable elements is known to be imprecise (Sachs *et al.*, 1983; Pohlman *et al.*, 1984; Bonas *et al.*, 1984; Weck *et al.*, 1984). Since En-1 occurs within an intron of the *Wx* gene (see below), small sequence deviations caused by the excision of the element might not alter the splicing process. Thus an intact gene product can always be synthesized. In contrast, somatic reversion events induced by En at the receptor component Spm-I8 within the *wx-m8* allele often lead to intermediate expression levels (McClintock, 1962). This can be explained by the fact that Spm-I8 is inserted in an exon and that En-induced excision of Spm-I8 does not always lead to restoration of the wild-type amino acid sequence (Schwarz-Sommer *et al.*, in preparation).

In addition, changes in the state of the element (e.g., timing and frequency of reversion events) have frequently been observed (see spotting patterns on the kernels in Figure 2). This is true for ears on different tillers of the original plant as well as for kernels growing on a single ear. In forthcoming genetic analyses and molecular cloning experiments the molecular basis of these changes in state will be reported.

Molecular structure of the autonomous En-1 element as compared with the receptor component I

Molecular cloning of the *wx-844* allele allowed us to identify En-1 as an 8.4-kb insert occurring within an intron located near the middle of the *Wx* gene (Figure 5). Like Spm-I8 within the *wx-m8* allele (Schwarz-Sommer *et al.*, 1984) En-1 also generates a 3-bp target site duplication upon integration. They also both contain the same 13-bp long perfect inverted repeat sequence at their termini. The sequence homology observed between the autonomous element and its receptor component is striking. Folding the left and right ends to form a double-stranded stem and loop structure leads to the same figure in both cases. The fact that the few differences occur exclusively within the loops may reflect some functional importance of the double-stranded stems. As supported by restriction, heteroduplex and sequence analysis, the receptor component Spm-I8 is a deletion derivative of an autonomous En element. This relationship was already suggested by genetic analysis (Peterson, 1961, 1970) and was proved at the molecular level to be true for some members of the Ac-Ds family (Döring and Starlinger, 1984; Fedoroff *et al.*, 1983).

The use of En-1 in transposon tagging of other loci

In the case of the autonomous element Ac, where internal portions of Ac are of low copy number (Fedoroff *et al.*, 1983) these regions were used for isolation of another Ac-controlled

locus by transposon tagging (Fedoroff *et al.*, 1984). Surprisingly, all regions of En-1 tested by us so far are repetitive in the maize genome (50 and more copies, data not shown). However, assuming that the copy number of intact En-homologous sequences is low we recently succeeded in the isolation of the *A1* locus (O'Reilly *et al.*, in preparation) using the En-tagged *a1-m(papu)* allele (Peterson, 1970; Nowick and Peterson, 1981). Within the limits of resolution of heteroduplex and restriction analysis, the 8.2-kb En (*papu*) element is identical to En-1. Thus the size and alignment of the internal portions of En within a newly isolated En at a different locus are indicative of the functional integrity of this En. The more so, because internal portions of En-1, if taken as probes in Northern hybridization experiments, show homology to at least two poly(A)$^+$ RNAs isolated from plants carrying a functional En element (Gierl *et al.*, in preparation).

Materials and methods

Genetic stocks and mutant identification

An En-containing line of *Zea mays* was developed carrying the dominant color inhibitor allele *C-I* to facilitate identification of *wx* mutants. A *C sh bz wx* tester line, dominant for all other anthocyanin color-determining genes was used as a male parent in a field experiment to identify *wx* mutants in the female, En-containing, *C-I Wx* parent. The *wx* mutants identified (see cross A in Results) were grown in the greenhouse and crosses were made on three different En tester lines A, *a1-m(r)/(a1-m1)*; B, *a2-m(r)/a2-m(r)*; and C, *wx-m8/wx-m8*; *c2-m2/c2-m2*.

In agreement with a previous suggestion for general nomenclature dealing with transposable elements (Bonas *et al.*, 1983; Nevers *et al.*, 1984), the mutable *wx* allele carrying En at the *wx* locus (see Results) is designated *wx-844*::En-1.

Isolation of plant DNA

Leaf material from 4–5 week old plants was frozen in liquid nitrogen and stored at −50°C. DNA from individual plants was isolated from 25 g of leaf material by the procedure reported previously (Schwarz-Sommer *et al.*, 1984).

Isolation of plasmid and phage DNAs

Large scale plasmid preparations were carried out using the procedure described by Maniatis *et al.* (1982). After lysis by the boiling method plasmid DNA was precipitated with polyethylene glycol (Yamamoto *et al.*, 1970), phenol-extracted and purified twice on CsCl-ethidium bromide gradients.

Bacteriophage λ purification and isolation of phage DNA was done using the protocol of Yamamoto *et al.* (1970).

Cloning of plant DNA in λ EMBL4

λ EMBL4 vector arms were prepared by digestion of phage DNA with *Bam*HI and *Sal*I (Frischauf *et al.*, 1983) followed by precipitation with 5% polyethylene glycol (Lis and Schleif, 1975).

*Mbo*I partial digestion was performed according to Maniatis *et al.* (1982). After electrophoresis on an 0.7% agarose gel the 14–20-kb fragment size range was cut out from the gel, electroeluted and purified on a DEAE-cellulose column.

Ligation and *in vitro* packaging was done by the procedure described by Wienand *et al.* (1982). For initial infection with the packaged phage material the *Escherichia coli* strain K803 was used (Fedoroff *et al.*, 1983).

Recombinant phages were screened by plaque hybridization (Benton and Davis, 1977) using nick-translated fragments originating from the 5′ and the 3′ region of the *Wx* gene followed by hybridization with an Spm-I8 probe isolated from the *wx-m8* mutant (Schwarz-Sommer *et al.*, 1984).

Radioactive labelling of DNA

The nick translation of DNA fragments was done using the Amersham nick translation kit. [α-^{32}P]dCTP was purchased from NEN (sp. act., 3000 Ci/mmol). Specific activity of the probes was 10^8 c.p.m./μg.

For 3′ end-labelling (Maxam and Gilbert, 1980) DNA polymerase (Klenow fragment, Boehringer, Mannheim) was used.

Southern blot hybridization

Restriction digests of plant genomic, phage or plasmid DNAs were fractionated on 0.7–2% agarose gels and transferred to nitrocellulose filters (Southern, 1975). The filters were pre-hybridized at 65°C in 6 x SSPE containing 1x Denhardt's solution (without protein), 0.5 μg/ml calf thymus DNA and 0.1% SDS (Wahl *et al.*, 1979). Hybridization was performed overnight at 65°C in 3 x SSPE, 1x Denhardt's solution (without protein), 0.5 μg/ml calf thymus DNA, 0.1% SDS. The filters were then washed in several changes of 2x SSPE, 0.1% SDS at 68°C.

Heteroduplex analysis

The procedure described by Davis *et al.* (1971) and Davis and Hyman (1971) was followed for heteroduplex analysis.

Sequence analysis

For DNA sequence analysis the chemical degradation procedure described by Maxam and Gilbert (1980) was followed. Electrophoresis was carried out on 0.35 mm thick polyacrylamide gels which had concentrations between 5 and 16%.

For sequence analysis of the target in the wild-type DNA the *Bam* 0.6-kb fragment was subcloned into the *Bam*HI site of pUC 9 (Vieira and Messing, 1982) and sequenced using the *Eco*RI site within the polylinker of the vector after 3′ end-labelling. The 5′ and 3′ junctions of En-1 were sequenced following the same strategy using the *Bam*HI sites at the left and at the right junctions of En-1 within the *wx-844* allele (see Figure 4D).

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Note added in proof

En-1 contains an additional *Sal*I restriction site not included in Figure 4D. This site is located on the right hand side of the *Bam*HI cut.