Visual Detection of Transposition of the Maize Element *Activator* (*Ac*) in Tobacco Seedlings

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A bacterial streptomycin resistance gene (SPT) was engineered to make it possible to detect visually the transposition of the maize transposon *Activator* (*Ac*) in tobacco. In the presence of streptomycin, transgenic seedlings carrying the SPT gene appear green, whereas those carrying an SPT:: *Ac* construct display clones of green cells on a white background. Fully green seedlings arise in the progeny of SPT:: *Ac* transformants as a result of excision of *Ac* before fertilization. About half of these germinal revertants carry a transposed *Ac* element. Therefore, SPT:: *Ac* constitutes an efficient marker for selecting plants that have undergone transposition. In maize, there is a negative effect of increasing *Ac* dosage on the frequency and timing of *Ac* transposition. This negative effect is not observed in tobacco with the streptomycin variegation assay.

THE RECENT INTEREST IN PLANT transposable elements is due largely to the successful use of transposon tagging in the isolation of important plant genes that had been previously characterized solely on the basis of their genetic properties (*1*). This topic has received added attention now that transposable elements from maize have been introduced into other species that lack a characterized transposon. The autonomous (transposase-encoding) maize element *Activator* (*Ac*) can transpose in tobacco, carrot, and *Arabidopsis* (*2*). The element is well characterized molecularly; its nucleotide sequence (*3*) and that of the spliced mRNA encoding the presumed transposase (*4*) have been determined.

A major limitation to transposon tagging as a means to isolate genes in plants is the low frequency of transposition. Typically, on the order of 1% of seed progeny may show evidence of transposition. Moreover, the chance of *Ac* inserting in a particular gene is low because of the large number of DNA sequences into which tranposition could take place and the nonrandom pattern of *Ac* reinsertion (*5*). The overall mutation frequency from *Ac* insertion is generally less than 1×10^{-4} for genes closely linked to the *Ac* donor site and considerably less for unlinked genes. This means that well in excess of 10,000, and perhaps on the order of a million individuals, may need to be screened for a high probability of obtaining a mutation.

To improve the efficiency of transposon tagging, we developed an assay for transposition in seedlings of tobacco and other dicots based on excision of *Ac* from a chimeric streptomycin resistance gene (SPT:: *Ac*). The effectiveness of selection with a kanamycin resistance gene has been demonstrated in callus tissue (*2*). In our assay, somatic excision events are detected as green sectors on the white cotyledons of tobacco seedlings grown on streptomycin, and germinal excision events are detected as fully green seedlings. We used this assay to determine whether germinal excision events are accompanied by reinsertion of *Ac* and to measure the effect of varying the dosage of the introduced SPT:: *Ac* gene on the incidence of green sectors.

We developed several chimeric streptomycin phosphotransferase (SPT) genes, which confer streptomycin resistance on tobacco cells (*6*). The binary vector plasmid 2668 (Fig. 1) contains a chimeric kanamycin resistance gene (NPT; neomycin phosphotransferase) and a streptomycin resistance gene with a single Cla I site upstream from the SPT gene translational initiation site. An Sst I–Stu I fragment carrying a derived *Ac* element was cloned in at this site to produce plasmid 2853. This plasmid was mobilized into *Agrobacterium tumefaciens* LBA4404 (*7*), and the resulting strain was used to transform *Nicotiana tabacum* cv. Petite Havana (*8*). These plants were self-pollinated and backcrossed, and progeny seed were analyzed on streptomycin- and kanamycin-containing media.

An array of variegated phenotypes of seedlings that were plated on streptomycin media are displayed in Fig. 2. Some seedlings showed intense variegation, with many green spots per seedling (Fig. 2, A and C), whereas others had one or few spots per seedling (Fig. 2E). In some individuals large sectors were observed (Fig. 2D), presumably derived from an early *Ac* excision event. Several individuals were observed in which a sector extended through both cotyledons (Fig. 2F). Fully green individuals were observed with a consistent frequency in each batch of seed (Fig. 2B).

Genetic segregation analysis of kanamycin resistance for the two transformants that have been studied in most detail, 2853.2 and 2853.6, revealed the presence of two independent kanamycin-resistance (Kan^R) loci in 2853.2 and one Kan^R locus in 2853.6. Seedlings were also germinated on streptomycin and classified by the number of green spots per seedling (Table 1). Test cross progeny of 2853.2 and 2853.6

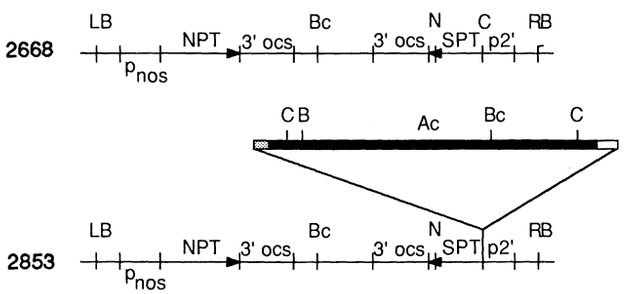

Fig. 1. T-DNA region of the binary vector plasmids used to analyze *Ac* excision from an SPT gene. Plasmid 2668 contains the chimeric kanamycin resistance gene NPT and the streptomycin resistance gene SPT and has a single Cla I site in the untranslated leader of the SPT gene (*6*). A fragment carrying a modified *Ac* was inserted at this Cla I site to make plasmid 2853. The construction of the modified *Ac* was initiated from a clone of the *bronze* allele *bz-m2(Ac)* (*14*). A Cla I site was inserted by oligonucleotide mutagenesis (*15*) 82 bp 3′ to the mapped polyadenylation site (*4*) to enable the future introduction of marker genes that will facilitate the mapping of new *Ac* locations and the cloning of linked plant DNA sequences. The *bz* DNA between the Sma I site and *Ac*, but not between Stu I and *Ac*, contains the sequence ATG on both strands (*16*). These sequences would lead to false translational initiation that would prevent drug resistance gene expression even after *Ac* excision. Therefore, a recombinant *Ac* element was made by recombining the *wx-m7(Ac)* allele with the *bz-m2(Ac)* allele, so that the resulting *Ac* was flanked by sequences that lack ATG codons at either end. Plasmid pJAC1 (kindly made available by John Yoder and Peter Starlinger, Cologne) carried the *wx-m7(Ac)* allele cloned in pBR322. The DNA between the Sma I site in *bz* and the internal Xba I site in *Ac* was replaced by the corresponding DNA from plasmid pJAC1, which had been cut with BssH II (blunt-ended with Klenow) plus Xba I, and the resulting plasmid was designated 2542. An *Ac*-containing fragment was cut from 2542 with Stu I plus Sst I and cloned into the Cla I site of 2668, which lies at the SPT gene translational initiation site ATCG*ATG*. LB, left border of T-DNA; RB, right border of T-DNA; P_{nos}, promoter from the nopaline synthase gene of *Agrobacterium*; 3′ ocs, 3′ end of the octopine synthase gene of *Agrobacterium*; p2′, promoter from the gene encoding the 2′ transcript of *Agrobacterium*; B, Bam HI; C, Cla I; Bc, Bcl I; N, Nco I.

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showed an approximately 1::1 ratio of individuals with no spots to individuals with one to six spots. This is consistent with the presence in each transformant of only one SPT::*Ac* locus that confers a variegated phenotype on streptomycin. Therefore, one of the two T-DNA inserts in transformant 2853.2 does not carry a functional SPT::*Ac* gene. In the self progeny, approximately one-fourth (2853.2) or one-third (2853.6) showed no variegation. We cannot at this time account for the excess of white seedlings in the 2853.6 self progeny, particularly because both paternal and maternal transmission of the SPT::*Ac* locus appear normal. Some self progeny were observed with more variegation than could be seen in any test cross progeny. This could mean that homozygotes exhibit more variegation than heterozygotes. Such an interpretation would imply that *Ac* in tobacco does not exhibit the negative effect of increased *Ac* dosage on transposition frequency that is characteristic of *Ac* in maize. This idea was tested genetically, as described later.

Of 17 kanamycin-selected primary transformant individuals, 9 gave rise to progeny that showed green-white variegation when they were germinated on streptomycin-containing media. The segregation data are consistent with a one-locus segregation of variegated to white progeny in six cases and with a two-locus segregation in the remaining three cases, although several families gave a slight excess of white seedlings (*9*).

Green seedlings occur in 1% to 9% of the self progeny of the primary transformants. To determine the molecular nature of these putative revertants, we carried out experiments to test whether green seedlings arise by excision of the *Ac* element from the SPT::*Ac* gene and whether *Ac* reinserts at new locations in the genome. Seedlings with fully green cotyledons on streptomycin were grown to mature plants. DNA was extracted from them and digested with Nco I (which does not cut in *Ac* but does cut in the SPT gene), and blots were hybridized with an *Ac*-homologous probe (Fig. 3).

Transformant 2853.6, which has only Kan^R locus, shows one *Ac*-homologous band of about 13 kb (lane 7), while all five streptomycin-resistant progeny of 2853.6 (lanes 8 through 10) exhibit a new 8.5-kb *Ac*-homologous band that is 4.5 kb smaller than the progenitor band. We have accumulated substantial DNA hybridization data (*9*) indicating that the 13-kb *Ac*-homologous band in 2853.6 corresponds to an insert consisting of two T-DNAs in an inverted repeat about the right border. Such inverted repeats are not uncommon in T-DNA (*10*). The 8.5-kb *Ac*-homologous band results from excision of an *Ac* from one copy of this inverted repeat, leaving an *Ac*-homologous Nco I fragment which is 4.5 kb smaller. Thus, in this genetic stock, an *Ac* probe visualizes not only the new *Ac* location, if any, but also the empty site.

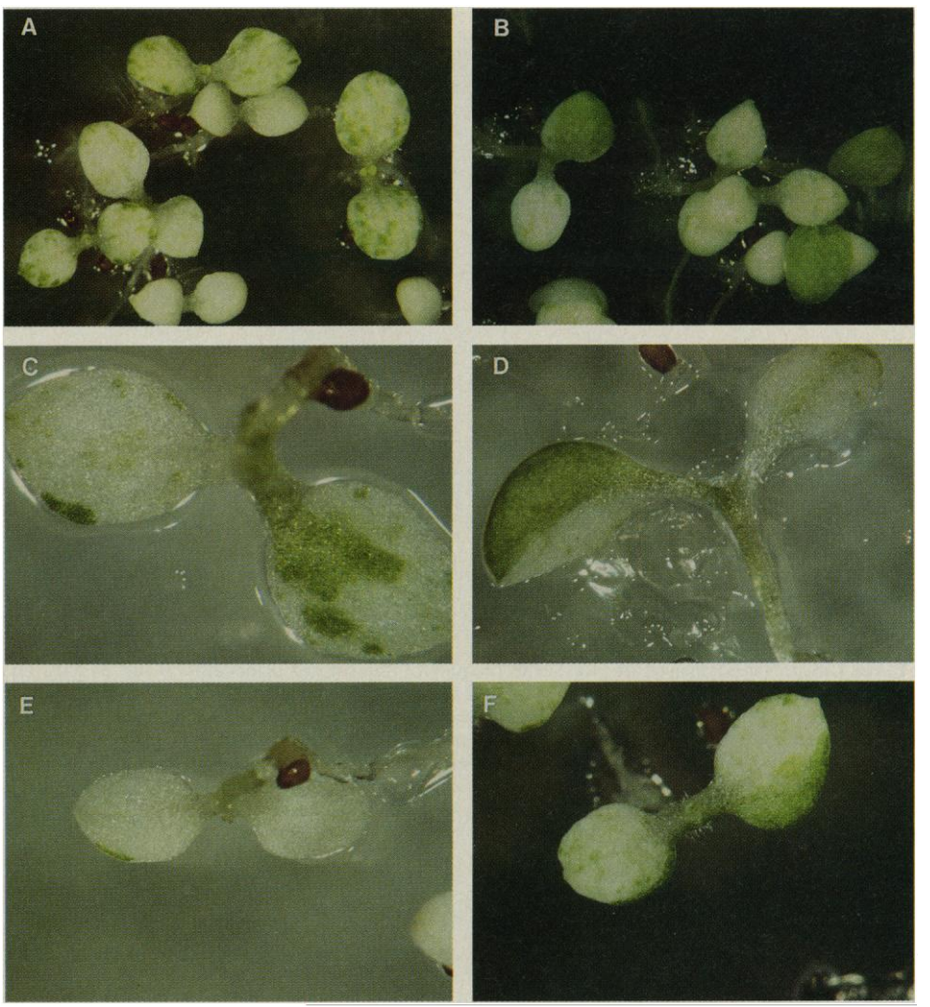

Fig. 2. Phenotypes of seedlings carrying an SPT::*Ac* gene when germinated on media containing Murashige and Skoog salts (*17*), 1% glucose, and streptomycin (200 μg/ml).

Two of the five streptomycin-resistant progeny of transformant 2853.6 (lanes 9 and 12) also exhibit a new *Ac*-homologous band of about 8.7 kb. The individuals in lanes 10, 11, and 12, which were found to be homozygous for kanamycin resistance on progeny testing, contain the SPT::*Ac* progenitor allele (the 13-kb *Ac*-homologous Nco I fragment) on one chromosome and the excision product on the homologous chromosome. Their progenies segregated, as expected, green and variegated seedlings when plated on streptomycin. The individuals in lanes 8 and 9, which were found to be heterozygous for kanamycin resistance, lack the 13-kb band corresponding to the SPT::*Ac* progenitor allele. Their progenies segregated green and albino seedlings, instead of variegated seedlings, on streptomycin-containing media. Because the five streptomycin-resistant progeny were selected from the same fruit, the 8.7-kb bands in lanes 9 and 12 could be derived from the same premeiotic event.

Transformant 2853.2, which has two independently segregating Kan^R loci, shows two *Ac*-homologous bands of 5.3 kb and 7.6 kb (lane 2). The smaller band in 2853.2 is too small for a functional SPT::*Ac* allele to be present. This would explain why only one locus that confers variegation on streptomycin can be detected. Of six variegated 2853.2 test cross progeny examined, all contained the 7.6-kb band, but four had lost the 5.3-kb band (*9*), confirming that the larger band in the primary transformant corresponds to the active SPT::*Ac* gene. Two of the four streptomycin-resistant progeny of 2853.2 (lanes 3 and 4) show new *Ac*-homologous bands (consistent with reinsertion of *Ac* at new locations) in addition to the original 7.6-kb band. Plating progeny from both of these individuals on streptomycin verified that they were SPT/SPT::*Ac* heterozygotes. The individuals in

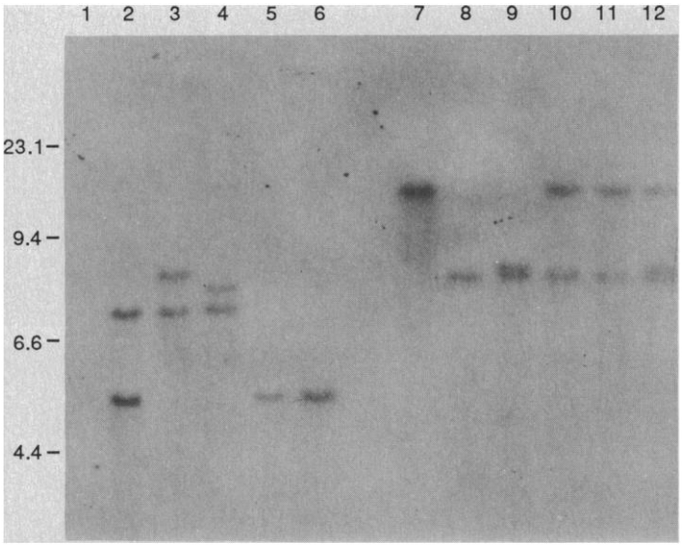

Fig. 3. DNA hybridization blot analysis of streptomycin-resistant revertants. Fully green progeny from the self matings of transformants 2853.2 and 2853.6 were selected on streptomycin. DNA was extracted from such progeny and digested with Nco I, which does not cut in *Ac*. Fragments were fractionated on a 1% agarose gel, blotted in nitrocellulose, and hybridized with an *Ac* probe (*18*). (Lane 1) Untransformed tobacco; (lane 2) 2853.2 primary transformant; (lanes 3 to 6), four different streptomycin-resistant progeny from 2853.2; (lane 7) 2853.6 primary transformant; (lanes 8 to 12), five different streptomycin-resistant progeny from 2853.6. Numbers on the left indicate fragment sizes in kilobases.

Table 1. Segregation analysis of SPT::*Ac* in progeny of transformed plants 2853.2 and 2853.6. The primary transformants were selfed and test-crossed as male (M) or female (F), and the resulting progeny were plated on streptomycin-containing media. Seedlings were classified as white, green, or variegated, and the latter were further classified on the basis of number of spots per seedling.

Cross	White	Variegated (number of spots per seedling)					Green	Total
		1–3	4–6	7–9	10–15	>16		
2853.2								
Self	50	68	11	8	28	27	1	193
Test (M)	41	38						79
Test (F)	82	54	5		1			142
2853.6								
Self	212	267	48	88	16		6	637
Test (M)	57	47	12					116
Test (F)	46	43	4				1	94

lanes 5 and 6 have lost the 7.6-kb SPT::*Ac* band and thus would be expected to be SPT/+ heterozygotes. This interpretation was confirmed by plating their progeny on streptomycin.

The presence of an empty site has also been shown (*9*) with a probe to the 2′ promoter of *Agrobacterium* (*11*), which lies adjacent to the *Ac* insertion site in 2853 (Fig. 1). All the data are consistent with the idea that seedlings will be green on streptomycin if there is prezygotic excision of *Ac* from an SPT::*Ac* gene. Futhermore, about half of the individuals that exhibit germinal excision of *Ac* from an SPT::*Ac* gene show evidence for reinsertion of *Ac* at new locations in the genome. Similar observations have been made with several other revertants isolated from test crosses, rather than self-matings, of transformant 2853.6 (*9*). These data show that green streptomycin-resistant individuals arise by excision of *Ac* from SPT. A reasonable inference from our data and from genetic data showing that variegation on streptomycin is linked to the SPT::*Ac* locus is that green sectors in variegated seedlings arise by somatic excisions of *Ac* during embryogenesis that restore the cell-autonomous expression of the SPT gene.

Twelve HV progeny from 2853.6 were grown to full size, selfed, and test-crossed to determine whether the highly variegated (HV) seedlings from the self-mating of the primary transformants (Table 1) were homozygous for the introduced SPT::*Ac* allele. Of the 12 HV individuals, 11 produced only kanamycin-resistant seedlings. Sample progenies from three of these Kan^R homozygous HV individuals were also plated on streptomycin. Nearly all of the self progeny showed seven or more spots per seedling, whereas more than 90% of the test cross progeny showed only one to six spots per seedling. We conclude that homozygous progeny from the 2853.6 transformant are more variegated than heterozygous progeny. This result suggests a positive dosage effect of the SPT::*Ac* allele on level of variegation.

The possibility that two copies of the SPT::*Ac* gene located in different positions of the genome also have a positive dosage effect was examined by plating on streptomycin the test cross progeny derived from the three transformants carrying two independent SPT::*Ac* loci; seedlings were classified as white (W), light variegated (LV), or HV (highly variegated). For all three T2 progenies the results are consistent with a 1:2:1 ratio of W:LV:HV. DNA hybridization blot analysis shows that the HV progeny in these platings contain two distinct *Ac*-homologous bands and that the LV progeny contain either, but not both, of these bands (*9*).

These data suggest that *Ac* in tobacco is not subject to the negative effect of dosage on transposition frequency that is observed in maize. A conspicuous difference between maize and tobacco is that in maize there are many *Ds* elements—that is, *Ac*-homologous elements that do not make an active transposase but can transpose in the presence of *Ac*. In *Drosophila*, *P* element–mediated hybrid dysgenesis is attenuated by the presence of defective *P* elements in the genome (*12*) and in maize, certain defective *En* elements encode products that reduce *En*-induced mutability (*13*). Conceivably, some of the *Ds* elements in maize encode a defective *Ac* transposase that repressses transposition through an intermolecular action potentiated or enhanced by increasing doses of effective *Ac* transposase. Alternatively, structural features of certain *Ds* elements may promote a concentration-dependent transposase interaction (dimerization) that results in a molecule that inhibits transposition. Such models could be tested in tobacco by examining the effect of a variety of modified *Ds* elements on *Ac* expression.

A practical consequence of the documented SPT::*Ac* dosage effect for transposon mutagenesis experiments is that, in addition to the green exceptions already discussed, one may be able to select as test cross progeny highly variegated seedlings in which transposition is known to have taken place.

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Allelotype of Colorectal Carcinomas

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To examine the extent and variation of allelic loss in a common adult tumor, polymorphic DNA markers were studied from every nonacrocentric autosomal arm in 56 paired colorectal carcinoma and adjacent normal colonic mucosa specimens. This analysis was termed an allelotype, in analogy with a karyotype. Three major conclusions were drawn from this analysis: (i) Allelic deletions were remarkably common; one of the alleles of each polymorphic marker tested was lost in at least some tumors, and some tumors lost more than half of their parental alleles. (ii) In addition to allelic deletions, new DNA fragments not present in normal tissue were identified in five carcinomas; these new fragments contained repeated sequences of the variable number of tandem repeat type. (iii) Patients with more than the median percentage of allelic deletions had a considerably worse prognosis than did the other patients, although the size and stage of the primary tumors were very similar in the two groups. In addition to its implications concerning the genetic events underlying tumorigenesis, tumor allelotype may provide a molecular tool for improved estimation of prognosis in patients with colorectal cancer.

THE INACTIVATION OF TUMOR SUPPRESSOR genes is thought to be important in the development of many human malignancies (*1*). Inactivation of these genes, through deletion or mutation, presumably allows a cell to escape normal growth controls. Only one candidate tumor suppressor gene has been cloned (*2*), but the existence of other such genes has been inferred from experiments in which specific chromosomal regions were found to be deleted in tumors. For example, allelic deletions involving 13q, 11p, and 22q are frequently found in retinoblastomas (*3*), Wilms' tumors (*4*), and acoustic neuromas (*5*), respectively. Although initial experiments concentrated on the loss of individual chromosomal regions from specific tumors, recent reports hint at a greater complexity. In particular, allelic deletions of chromosome 11p or 13q have been reported in different studies of breast cancers (*6*); deletions of 3p, 13q, and 17p have been reported in carcinomas of the lung (*7*); and deletions of 5q, 17p, 18q, or 22q have been reported in colorectal cancers (*8, 9*). To gain a broader perspective on the prevalence of allelic deletions in a common adult tumor, we have studied markers from every nonacrocentric autosomal arm in a large number of human colorectal tumors. We have termed this analysis an allelotype, in analogy with a karyotype.

Two technical developments were necessary for the present study. First, a large number of probes useful for identifying restriction fragment length polymorphisms (RFLPs) have been generated. The variable number of tandem repeat (VNTR) probes are particularly useful for such analyses, because each probe can distinguish the two corresponding parental alleles in a high proportion of normal DNA samples (*10, 11*). Second, colorectal tumors, like many solid tumors, contain variable numbers of nonneoplastic cells that can mask allelic deletions occurring within the neoplastic tumor cell population. A cryostat sectioning method for physically fractionating such tumors to enrich for neoplastic cells has been described (*12*).

DNA was purified from cryostat sections of 56 primary colorectal carcinomas removed at surgery and compared to the DNA from normal colonic tissue of the same patients. Probes detecting RFLPs were used to determine whether one of the two parental alleles detected by each probe was specifically lost in the DNA from the tumor cells. All nonacrocentric autosomal arms were studied; the only genes known to be present on the acrocentric arms (13p, 14p, 15p, 21p, and 22p) are ribosomal. For each of these 39 chromosomal arms, enough probes were used to ensure that the two parental alleles could be distinguished in the normal tissue of at least 20 patients (that is, the informative patients).

Alleles from each chromosomal arm were lost in at least some tumors (Fig. 1A). The frequency of allelic loss varied considerably, however, with alleles from two chromosomal arms (17p and 18q) lost from more than 75% of tumors, alleles from nine arms (1q, 4p, 5q, 6p, 6q, 8p, 9q, 18p, and 22q) lost in 25 to 50% of tumors, and the remaining 28 arms lost in 7 to 24% of the tumors (Fig. 1A). There were 127 examples of allelic deletions in which the patient was informative for markers on both the p and q arms of the chromosome containing the deletion. In 65% of these cases, allelic loss occurred in only one of the two chromosomal arms. The majority of the deletions observed in this study therefore represented subchromosomal events, such as might be mediated by interstitial deletion, mitotic recombination, or gene conversion, rather than loss of a whole chromosome.

The frequency of allelic deletions was also remarkable when viewed from the perspective of individual tumors (Fig. 1B and Table

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