Reversion-Reporter Transgenes to Analyze All Six Base-Substitution Pathways in Arabidopsis[1][W]

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To expand the repertoire of Arabidopsis (*Arabidopsis thaliana*) mutation-reporter transgenes, we constructed six mutant alleles in the same codon of the β-glucuronidase-encoding *GUS* transgene. Each allele reverts to *GUS*$^+$ only via a particular one of the six transition/transversion pathways. AcV5 epitope tags, fused carboxyl terminal to the inactive GUS$^-$ proteins, enabled semiquantitative immunoassays in plant protein extracts. Spontaneous G:C→T:A transversions, previously not measured using reporter transgenes, were quite frequent. This may reflect mispairing of adenine with 8-oxoguanine in DNA attacked by endogenous oxyradicals. Spontaneous G:C→A:T was modest and other reversions were relatively low, as reported previously. Frequencies of ultraviolet C-induced T$\underline{\text{T}}$→T$\underline{\text{C}}$ and T$\underline{\text{C}}$→T$\underline{\text{T}}$ reversions were both high. With increased transgene copy number, spontaneous G:C→T:A reversions increased but ultraviolet C-induced reversions decreased. Frequencies of some reversion events were reduced among T4 versus T3 generation plants. Based on these and other analyses of sources of experimental variation, we propose guidelines for the employment of these lines to study genotoxic stress in planta.

The genetic integrity of plant DNA is threatened by replication errors and endogenous damaging agents and by environmental agents such as UV and ionizing radiation and chemical genotoxins in water, soil, and the atmosphere. Because the immobility of plants precludes physical avoidance of environmental mutagens, they must rely on protection, detoxification, and DNA-repair systems to maintain genetic integrity. Plants lack a reserved germline, so their gametes arise from meristematic cells that have divided many times while exposed to the environment. Mutations in these cells, therefore, may be passed to progeny, increasing the mutation load of future generations. It is thus important to understand how DNA lesions cause mutations and how DNA repair and other activities may suppress them.

Besides directly affecting plant growth and crop yields, impending global climate change seems likely to impact long-term plant genetic integrity, thus affecting both natural and crop ecosystems. The mutagenic effects of solar UVB irradiation will increase where and when average cloud cover decreases. In DNA, UVB efficiently induces pyrimidine dimers that block replicative polymerases. Specialized translesion polymerases synthesize DNA past these lesions, but with reduced fidelity (Kimura and Sakaguchi, 2006). Endogenous reactive oxygen species (ROS), inevitable by-products of chloroplast and mitochondrial metabolism, are increased by plant stressors such as heat and drought and are minor products of UVB radiation (Mouret et al., 2006). Besides replication errors, depurination, and cytosine deamination, mispairing of oxidized bases is a likely source of so-called spontaneous mutation (Bray and West, 2005). Transition metal ions such as Fe(II) and Cu(I) can produce highly reactive hydroxyl radicals from endogenous hydrogen peroxide via Fenton-type reactions (Cohen, 1985). Other divalent ions may displace Fe(II) from various cellular sites, leaving it free to bind to DNA. Some plant growth environments may include substantial concentrations of Zn(II), essential and not highly toxic, Cu(II), essential but toxic at high concentrations, or Cd(II), toxic but not essential (Kovalchuk et al., 2001b).

Monitoring of effects of real-world and laboratory-imposed genotoxic stress on plant genomes requires mutation assays that are convenient, reliable, sensitive, and versatile. The recessivity of most mutations in diploid organisms obviously precludes many mutation assays used in microbial studies. In plants heterozygous for a recessive mutation (albinism, say), forward mutation of the wild-type allele is readily detected, but propagation of such heterozygotes, let alone stable genetic alteration of DNA-maintenance functions in them, is cumbersome. Thus, considerable effort has

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been devoted to transgenic mutation reporters (Lebel et al., 1993; Puchta et al., 1995; Kovalchuk et al., 2000, 2001a, 2001b; Lucht et al., 2002; Leonard et al., 2003; Hoffman et al., 2004; Yoshihara et al., 2006; Van der Auwera et al., 2008). One approach is to integrate into plant genomes transgenic mutation targets that can be subsequently rescued as bacteriophages or plasmids. Introduction of the rescued DNA into suitable bacteria, under conditions where mutated targets can be positively selected, immediately yields overall forward mutation frequencies, and DNA sequencing (generally of hundreds of mutated targets) can provide informative mutation spectra. However, besides being highly laborious, this approach in effect discards a potential major advantage of in situ systems: fixed clones of mutant cells. In cultures of freely diffusing cells, the ancestry of any particular mutant cell is not known. Thus, a "jackpot" mutation early in culture growth results in a high fraction of mutant progeny and overestimation of overall mutation rates. This problem is usually addressed by fluctuation analyses, typically using 10 or more independent cultures. The same problem arises when reporter transgenes are rescued from plant DNA and mixed together, because mutations could have arisen early or late in plant development. Here, fluctuation analyses would be quite difficult. However, the progeny of any mutant plant cell typically form a single clone of contiguous mutant cells (particularly in leaves) that can be scored as a single mutation event by any in situ assay.

To overcome the recessivity problem associated with in situ detection of mutant cells (clones) rather than mutant DNA, it is necessary to score the reversion of mutant reporter genes to the wild type. Although the sensitivity of a forward mutation assay is lost, each reversion is specific for a particular mutation pathway. Thus, all six base-substitution pathways can be analyzed as a reversion of different reporter-transgene alleles. Reversion-reporter transgenes can be used to study stimulation by a particular environmental agent of a single mutation pathway, the relative frequencies of spontaneous and mutagen-stimulated reversion via different pathways, and the effects on mutation of alterations in plant genome-maintenance functions.

Hohn and coworkers (Kovalchuk et al., 2000) previously analyzed reversion mutation in Arabidopsis (*Arabidopsis thaliana*), employing various transgenes expressing bacterial GUS proteins inactivated by single amino acid changes. Reversion of the corresponding point mutations to GUS^+ resulted in clones of cells that were stained blue in situ by the GUS substrate 5-bromo-4-chloro-3-indolyl-β-D-GlcA (X-Gluc), producing readily scored spots or sectors that corresponded to defined mutation (reversion) events. They constructed transgene alleles at several different sites, one or more for each of three reversion pathways: A:T→G:C, A:T→C:G, and A:T→T:A. They identified single-transgene-copy lines and used these to measure spontaneous mutation and UV-induced T<u>T</u>→T<u>G</u> and C<u>T</u>→C<u>A</u> mutations. However, none of their transgenes could report the common UVC-induced T<u>T</u>→T<u>C</u> and T<u>C</u>→T<u>T</u> pathways, which have been observed in bacterial, yeast, and mammalian systems (Brash et al., 1987; Lawrence et al., 1993). They also observed increased A:T→C:G and G:C→A:T mutations in the presence of various metal ions (Kovalchuk et al., 2001b). However, they did not assay G:C→T:A transversion, the major mutation expected when guanine in DNA is oxidized to 8-oxoguanine, which frequently pairs with adenine (Shibutani et al., 1991). Endogenous ROS may be by-products of plant metabolism, perhaps further activated by reactions involving transition metal ions. They also used other GUS^- transgenes to monitor homologous recombination in planta (Kovalchuk et al., 2001a).

Van der Auwera et al. (2008) recently added five different mutant codons that revert by G:C→A:T transitions to the repertoire of GUS^- reversion-reporter transgenes. However, a bipyrimidine UV photoproduct was possible in only one of their five target mutant codons, and even it did not revert to GUS^+ via the common UV-induced T<u>T</u>→T<u>C</u> mutation. These workers did not measure transgene copy numbers, which can strongly affect mutation rates (Kovalchuk et al., 2000).

We have developed a set of transgenic plants that can report effects of endogenous and environmental mutagens on all possible base-substitution pathways and the roles in these pathways of plant activities that prevent or repair DNA damage or promote DNA synthesis past unrepaired damage. We constructed GUS^- transgene alleles that revert by each of the six transition and transversion pathways in the same *GUS* codon, to minimize sequence context effects in comparisons. To provide for measurement of UV-stimulated T<u>T</u>→T<u>C</u> and T<u>C</u>→T<u>T</u> mutations, pathways frequently studied in other organisms but not together accessible in any previous transgene mutation reporters, we placed the thymine and cytosine targets for A:T→G:C and G:C→A:T reversion in appropriate multipyrimidine contexts. One set of lines reports, to our knowledge for the first time, the G:C→T:A transversions characteristic of 8-oxoguanine lesions, providing a new tool to study oxidative mutagenesis. The levels of expression of reporter transgenes may affect their mutation rates (Kovalchuk et al., 2000; Van der Auwera et al., 2008). Therefore, we fused to the respective GUS^- proteins an AcV5 epitope tag (nine-residue peptide from the GP64 envelope fusion protein of *Autographa californica Multiple nucleopolyhedrosis virus*) that can be immunoassayed in plant protein extracts. This enabled us to identify different reporter lines with similarly robust expression. To avoid cosuppression between our GUS^- transcripts and cauliflower mosaic virus (CaMV) 35S-driven transcripts in other constructs that might be incorporated in the mutation-reporter lines (overexpressing DNA-repair genes, for example), we used the composite $(Aocs)_3AmasPmas$ promoter assembled by Gelvin and

colleagues (Lee et al., 2007) to express the reporter transgenes. Because high transgene copy number can also reduce expression by cosuppression while at the same time increasing the number of mutation targets, we identified lines with only one (mostly) or two transgene copies for each of the six pathways.

We measured a surprisingly high frequency of spontaneous G:C→T:A transversion, which suggests levels of endogenous oxidized guanines to be substantial in plant cells. UVC irradiation stimulated TT→TC and TC→TT mutation to similarly high levels. We observed trends toward lower spontaneous G→T and UV-stimulated TT→TC and TC→TT reversion frequencies in going from T3 to T4 generations.

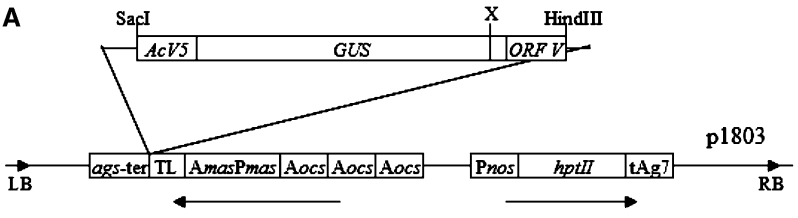
RESULTS

Establishment of a Plant System for Pathway-Specific Mutation Analysis

Generation of Plant Lines Containing Reversion-Reporter Transgenes

We used site-directed mutagenesis to generate the six *GUS* mutation constructs described in Figure 1. Each mutant codon replaces the same essential Glu codon in the GUS N terminus; each reverts to a wild-type codon only through a unique single base substitution. Figure 1 also shows examples of UV-induced or oxidative damage to DNA whose miscoding properties might engender the respective specific reversions. Protein products encoded by each mutant construct were synthesized by transcription-translation in vitro and tested for GUS activity by assaying fluorescence generated by cleavage of 4-methylumbelliferyl β-D-glucuronide (MUG; Table I). Substitution of Gln for Glu resulted in 27% activity relative to wild-type (GUS^+) proteins, and Ala and Gly substitutions resulted in about 8% activity; all other mutant proteins showed less than 2% activity. In contrast, a previous study (Kovalchuk et al., 2000) found Gln or Lys at this position to reduce GUS activity expressed by transgenes in tobacco (*Nicotiana tabacum*) protoplasts to 1% or less of that expressed by GUS^+, similar to a termination codon. We also tested the nine other amino acid substitutions that might result from base substitutions other than the six defined codon-reversion pathways. These all resulted in gene products with less than 2% activity relative to GUS^+ protein (Table I).

Our mutant GUS^- constructs were similar to those described by Kovalchuk et al. (2000). The first 87 bp of open reading frame (ORF) V from CaMV were used to replace the translation start site of the GUS-encoding *Escherichia coli uidA* gene. The encoded N-terminal leader peptide is reported to enhance *GUS* expression and activity (Swoboda et al., 1994). We fused an AcV5 epitope tag to the C terminus to allow immunological detection of even inactive gene products within transformed plants. This peptide yields a strong signal on protein immunoblots ("western" blots), and the



B

Line	Amino acid substitution	Sequence	Reversion to wild-type	Potential lesion-induced mispair
WT	(Glutamate)	CGT GAA ATC GCA CTT TAG		
MR1	Stop	CGT **T**AA ATC GCA **A**TT TAG	A:T → C:G	T[CPD]**T** A – **C**
MR2	Glycine	CGT G**G**A ATC GCA C**C**T TAG	C:G → T:A	**A** – A **C**[CPD]T
MR3	Glutamine	CGT **C**AA ATC GCA **G**TT TAG	G:C → C:G	G (8-oxoG)
MR4	Alanine	CGT G**C**A ATC GCA C**G**T TAG	G:C → T:A	A (8-oxoG)
MR5	Valine	CGT G**T**A ATC GCA C**A**T TAG	A:T → T:A	A (2-hydroxyA)
MR6	Lysine	CGT **A**AA ATC GCA **T**TT TAG	T:A → C:G	**G** – A **T**[CPD]T

Figure 1. Reversion-reporter constructs. A, Map of the p1803 binary vector T-DNA (Lee et al., 2007) used to express the *GUS* constructs. *ORF V*, Sequence from CaMV ORF V, its first 87 bases fused here to the *GUS* sequence; *GUS, uidA*-coding sequence from *E. coli*; *AcV5*, C-terminal AcV5 epitope; A*ocs*, octopine synthase upstream activation element; A*mas*P*mas*, mannopine synthase promoter and upstream activation element; TL, translational leader from tobacco etch virus 5′ untranslated region; *ags*-ter, poly(A) adenylation signal from the agropine synthase gene; P*nos*, nos promoter; *hptII*, gene conferring resistance to hygromycin; tAg7, poly(A) addition signal for T-DNA gene 7; LB, left T-DNA border; RB, right T-DNA border. Vector T-DNA sequence outside the defined promoter sequence is not known. Arrows beneath the map indicate the direction of transcription. X indicates the site of GUS base substitutions (see "Materials and Methods"). B, Reversion-reporter transgenes. For wild-type (WT; GUS^+) and six mutant GUS^- genes (*MR1–MR6*), columns show the amino acids encoded by the mutant codon, the sequence context (mutant base pair in boldface), the pathway for reversion to the wild type, and a potential premutagenic lesion base mispair resulting from UV-induced (CPD) or oxidative damage (8-oxoG, 2-hydroxyA).

Table I. *GUS activity of mutant GUS proteins synthesized in vitro*

Codon 11 Amino Acid[a]	Codon Sequence	Specific Activity[b]	Codon 11 Amino Acid[c]	Codon Sequence	Specific Activity[b]
		%			%
Glu (wild type)	GAA	(100.0)	His	CAT	0.1
Stop	TAA	0.7	Ile	ATA	1.4
Gly	GGA	8.7	Leu	CTA	1.0
Gln	CAA	27.0	Asn	AAT	1.5
Ala	GCA	7.9	Pro	CCA	0.4
Val	GTA	0.8	Arg	AGA	0.2
Lys	AAA	1.1	Ser	TCA	1.2
Asp	GAT	45.4	Thr	ACA	0.5
Frame shifted[d]	$(A)_8$	0.9	Tyr	TAT	0.04

[a]Proteins designated according to the amino acid at codon 11 of the *E. coli uidA* (*GUS*) gene, as specified by the indicated "reporter" codons. [b]Indicated (^{35}S-labeled) proteins encoded by reporter lines MR1 to MR6 (Fig. 1) were synthesized by in vitro transcription and translation. GUS activities were measured as cleavage of fluorogenic (MUG) substrate, as described in "Materials and Methods," and normalized for protein concentrations determined by SDS-PAGE and quantitative autoradiography. [c]Proteins designated according to amino acids that would be encoded by the indicated mutant codons. [d]Frame-shift mutation (TA) constructed immediately downstream of codon 11 (5′-GAAATAAAAAAAA-3′) would generate a stop codon 60 bases farther downstream.

antibody detects only a low background signal in nontransgenic plant extracts (Lawrence et al., 2003). The binary vector introduced into plants uses an *(Aocs)$_3$AmasPmas* "superpromoter" designed by Gelvin and colleagues (Lee et al., 2007) to transcribe inserted transgenes. This promoter, which includes octopine- and mannopine-synthase promoter elements, has been reported to drive expression at levels equal to or greater than the frequently used CaMV 35S promoter (Lee et al., 2007). A potential advantage is its expected lack of cosuppression with CaMV (35S)-driven transcripts of other transgenes.

We transformed Arabidopsis ecotype Columbia plants with each of the six reversion-reporter constructs and selected multiple independent hygromycin-resistant plants, designated by construct number (MR1–MR6) and line number. We semiquantitatively screened respective protein lysates for high transgene expression by blotting with anti-AcV5 antibody. We identified transformants apparently hemizygous for single transgene insertion loci (i.e. segregating out roughly 25% hygromycin-sensitive progeny). Homozygous progeny of these isolates were designated by specific subline letter and analyzed for reversion of the GUS mutation-reporter transgenes (see below). DNA blots ("Southern" blots) of restriction enzyme digests were used to estimate copy number (Supplemental Fig. S1).

Analysis of Reversion Mutation in Plants

To detect GUS activity in revertant *GUS*$^+$ transgene cells, clones of cells, or sectors in plant tissues, we treated whole plants (excluding roots) with the histochemical activity stain X-Gluc (Jefferson, 1987). Although blue (*GUS*$^+$) spots or sectors were readily identified, there were two complicating factors. First, staining of control plants expressing *GUS*$^+$ constructs suggested transcription driven by the *(Aocs)$_3$AmasPmas* superpromoter to be somewhat tissue dependent: staining (Fig. 2D) was generally stronger in more mature tissues (emergent for at least a few days) and was especially strong in vascular tissues as well as in roots (not analyzed in our mutation experiments). Similar staining patterns were observed by Gelvin and coworkers in tobacco plants expressing *(Aocs)$_3$AmasPmas*-driven *GUS*$^+$ (Ni et al., 1995). Thus, in our mutation experiments, some *GUS*$^+$-revertant cells in low-expressing tissues may not be detected, so our measurements may slightly underestimate mutation frequency. In addition, comparisons among different lines, or between

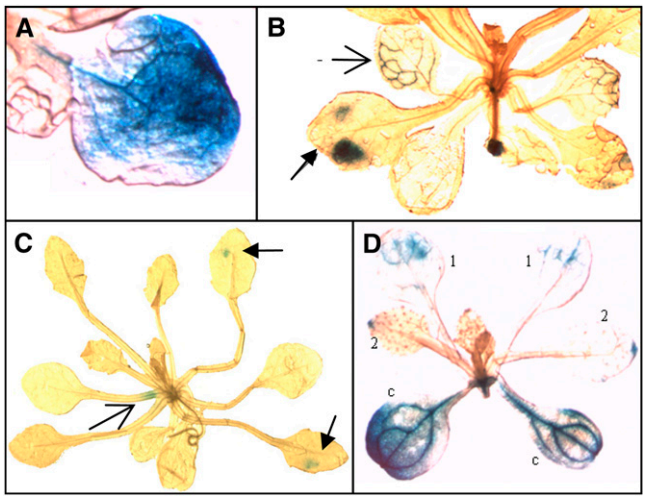


Figure 2. Representative histochemical GUS activity staining patterns. A, Large stained *GUS*$^+$ revertant sector isolated to a cotyledon. B, Complex revertant staining patterns: thin arrow, vascular-stained sector; thick arrow, paired spots. C, Simple revertant staining patterns: thin arrow, spot in leaf stem; thick arrows, single spots in leaves. D, *GUS*$^+$ control plant showing stain preferentially in mature tissue. c, Cotyledons; 1, first true leaves; 2, second true leaves.

untreated plants versus those treated with mutagens, would be most precise for the more mature tissues where the *(Aocs)$_3$AmasPmas* promoter is the most active. We only employed lines in which transgene expression levels (measured by immunostaining of the AcV5 epitope tag in protein extracts) were above a minimum threshold.

Second, the timing of mutation events during plant development appeared to influence the pattern of *GUS*$^+$ staining and the sizes of the revertant sectors and spots. Thus, the large blue-staining leaf sectors or identical sector patterns in multiple leaves observed in more mature plants seemed likely to reflect early mutation events in meristems. Smaller blue spots most likely resulted from mutation events late in leaf development. Some leaves showed symmetrically "paired" spots, as if mutation had occurred in leaf primordia. Figure 2 shows several representative staining patterns. To provide a consistent basis for scoring mutation frequencies, we counted only once patterns of stained sectors or spots that seemed likely to have resulted from a single mutation event. This procedure seems entirely appropriate where mutation is infrequent but may slightly underestimate the numbers of mutation events in some cases. In experiments where mutation was so frequent that multiple mutation events could not be unambiguously distinguished from complex patterns due to early single events (see above), we used a second method to score mutation frequencies. The probabilities of random events that can independently "hit" any single target any number of times follow the Poisson distribution: if the mean number of events per target is x, the Poisson fraction of targets receiving zero hits is e^{-x}, the fraction receiving exactly one hit is xe^{-x}, the fraction receiving exactly two hits is $x^2e^{-x}/2!$, and so forth. By using the Poisson "zero" term to estimate the average mutation frequency (events per plant) as $x = -ln$ *(fraction of plants showing no blue sectors or spots)*, decisions about complex staining patterns were avoided. Supplemental Figure S2 shows that when mutation events were relatively frequent, the counted-mean data fitted the Poisson distribution (dotted lines) reasonably well. Counted-mean event frequencies of at least 0.05 per plant were generally in good agreement with mean frequencies calculated using the Poisson zero term (Supplemental Table S1). In a few cases, the Poisson means were less than the counted means, perhaps reflecting scoring ambiguities. In all cases, we have expressed mutation frequencies as mean events counted per plant per transgene.

Reporter-Transgene Copy Number

All reporter lines used were originally shown by segregation analysis to contain single insertion loci. All single-locus lines were selfed to yield transgene homozygotes. Copy numbers in various lines were estimated by probing DNA (Southern) blots of electrophoretically resolved restriction fragments. Genomic DNA from each line was digested with either *Bcl*I endonuclease (two sites in T-DNA, 2.4 kb apart) or *Eco*RV endonuclease (one site in T-DNA, near but not within the 2.4-kb *Bcl*I fragment). All blots were probed with a radiolabeled T-DNA-specific fragment expected to hybridize to the *Bcl*I fragment and to all *Eco*RV fragments that contain the *Bcl*I fragment. Supplemental Figure S1 shows a representative set of probed blots and summarizes the data. Visual inspection identified (homozygous) lines in which a single identical transgene copy was present in both chromosomes: a distinct but relatively faint 2.4-kb band in the *Bcl*I-digested DNA and a band of similar intensity in the *Eco*RV-digested DNA. We designated such homozygous lines to be "single copy." Lines that showed two bands in the *Eco*RV-digested DNA, each about half the intensity of the 2.4-kb band in the *Bcl*I-digested DNA, were inferred to be "two copy" (four copies in homozygotes). In one case, extension of this logic identified a likely "three-copy" line. Where blots were too complicated for this approach, copy numbers in these lines were estimated as follows. On a given gel, the mean value of the quantitatively well-defined intensities of the 2.4-kb *Bcl*I phosphorimaged bands corresponding to single-copy lines identified by inspection was assigned a relative value of 1.0. The relative intensities of 2.4-kb bands in DNA from other lines were assumed to roughly correspond to copy number, apparently as high as 28 (Supplemental Fig. S1).

Transcription of each transgene copy is likely to be affected by genome position, and the likelihood of cosuppression may increase with copy number. If position effects and/or cosuppression caused the expression of transgenes to be too low, cells containing *GUS*$^+$ revertant transgenes might not be detectable by X-Gluc staining. On the other hand, the mutation probability per plant should increase with the number of mutation targets (transgene copies). These effects on spontaneous and UV-stimulated mutation are considered below. Immunostaining for the AcV5 epitope fused to GUS revealed a trend of decreasing expression with increasing copy number (Supplemental Table S2).

Spontaneous Mutation

Supplemental Table S2 contains a complete compilation of all mutation frequency data. Lines that reported one of the six reversion pathways were grown on soil and/or defined medium for 3 weeks and spontaneous mutation frequencies were scored. Mean spontaneous mutation event frequencies among the various sublines correlated roughly with copy number (Fig. 3, white diamonds; Supplemental Table S2), even though transgene expression (AcV5 immunostaining) decreased modestly with copy number. For detection of mutation, target (copy) number thus appeared to mostly override decreases in expression at higher copy numbers and any position effects on

expression. Therefore, we normalized all spontaneous mutation frequencies (Supplemental Table S2) for copy number. Figure 4 shows a scatterplot of counted mean frequencies of mutation in several independent lines, and Table II summarizes and highlights data compiled in Supplemental Table S2. Means calculated from Poisson zero terms were similar to counted means (data not shown).

Spontaneous mutation levels were low for most lines, consistent with previous reports (Kovalchuk et al., 2000; Van der Auwera et al., 2008). However, MR4 (G→T) lines showed high levels of spontaneous mutation under most growth conditions. G:C→T:A transversions may be induced by endogenous ROS, since 8-oxoguanine (8-oxoG) bases in oxidized DNA are expected to frequently form 8-oxoG:A mispairs (Shibutani et al., 1991).

UV-Induced Mutation

We tested four different reversion pathways using UVC radiation to avoid oxidative damage of DNA by UVB and UVA (Mouret et al., 2006). The target nucleotides in lines MR1, MR2, and MR6 were embedded in runs of two, four, and four pyrimidines, respectively, and occupied 3′ positions in bipyrimidines, the more common sites of UV-induced mutations (Brash et al., 1987). The target thymine in MR5 was flanked by purines. Since UVC-stimulated frequencies of MR2 (TCC→TTC) and MR6 (TT→TC) reversion actually decreased with copy number (Fig. 3, black triangles and squares; Supplemental Table S2; see "Discussion"), only data (counted means) for single-copy sublines appear in the Figure 5 scatterplot. Similar to UVC-induced mutation reported by Kovalchuk et al. (2000), *GUS*⁻ transgene reversion in several MR2 and MR6 sublines was increased here by UVC (1 kJ m^{-2}): mean spontaneous frequencies of 0.025 ± 0.016 and less than 0.01 events per plant, UV-specific events of 0.42 ± 0.25 and 0.30 ± 0.42 per plant, respectively. The high SD values reflect both large subline-to-subline variation, also observed by Kovalchuk et al. (2000), and trial-to-trial variation (Supplemental Table S2).

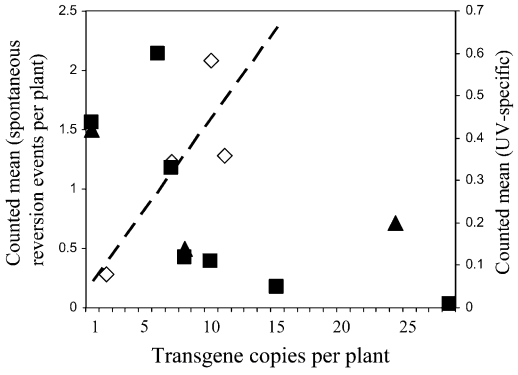


Figure 3. Dependence of apparent reversion frequencies on numbers of target transgene copies. T3 plants from MR2 (black triangles), MR4 (white diamonds), and MR6 (black squares) lines were grown on soil (all MR2 and MR6 plants, some MR4 plants; Supplemental Table S2 and Supplemental Figure S1) or MS medium (3 weeks of growth for spontaneous mutation experiments, 4 weeks of total growth for UVC-induced mutation experiments) and stained for GUS^+ activity as described in "Materials and Methods." Frequencies of revertant events per plant were combined for sublines with the same transgene copy number (Supplemental Table S2). Copy number was determined as described in "Materials and Methods" and the legend to Supplemental Figure S1. The dashed line indicates the trend for MR4 sublines (white diamonds).

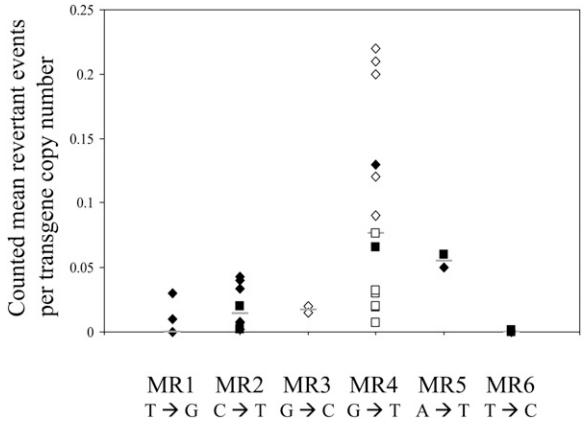


Figure 4. Spontaneous reversion frequencies. Data are from Supplemental Table S2. Plants were grown on soil (black symbols) or MS medium (white symbols) and analyzed 3 weeks after planting for reversion of *GUS*⁻ alleles to *GUS*⁺ as described in "Materials and Methods" and the text. Mean numbers of reversion events per plant, normalized for copy number (Supplemental Table S2), are shown from top to bottom for T3 (diamonds) and T4 (squares) generations. MR1 (T→G) sublines are 12D and 3F and five other sublines. Other sublines are as follows: MR2 (C→T), 8D, 10C, 12G, 15G, and 20B; MR3 (G→C), 1G and 8B; MR4 (G→T), 9F, 14A, 20C, 7D, and 5B; MR5 (A→T), 12D; MR6 (T→C), 20C, 15F, and 6B, and six other sublines. Bars indicate median values.

All MR1 (TT→TG) lines showed low levels of UVC-induced TT→TG mutation (0.09 or fewer UV-specific events per plant; Supplemental Table S2). Surprisingly, UVC radiation specifically induced 0.09 GTA→GAA events per plant in MR5 targets, where there is no site for the formation of dipyrimidine photoproducts. Van der Auwera et al. (2008) reported similar or slightly higher UV-induced reversion of purine-flanked single cytosines. Out of 61 total experiments, one MR2-12G (T4) experiment seemed anomalous and was not included in the analyses. In all other MR2 experiments, spontaneous reversion was low but detectable and UVC-stimulated reversion was relatively high.

Stimulation of G→T Mutation by Metal Ions

Transition metal ions can stimulate hydroxyl radical formation, which in turn can oxidize guanine to

Table II. *Spontaneous and UV-induced reversion frequencies*

Experiment	Line (Pathway) and Subline[a] [Generation]	Transgene Copy No.	Counted-Event Mean per Transgene Copy[b]	
			Spontaneous	UVC Specific
	MR1 (T→G)			
1–6	11A,D, 1E,A, 12D, 6A [T3]	1	<0.02	0.07
7	3F [T3]	3	0.01	0.013
	MR2 (C→T)			
8, 9, 14	10C, 8D [T3]	1	0.032	0.37
10–12	12G [T3,4]	1	0.021	0.39
15	15G [T4]	8	0.0025	0.018
16	20B [T3]	24	0.0021	0.0083
	MR3 (G→C)			
17, 18	1G, 8B [T3]	2	0.018	
	MR4 (G→T)			
19, 20	7D, 14A [T3]	2	0.14	
21, 22	5B [T3]	7	0.18	
23–30	5B [T4]	7	0.032	
31	9F [T3]	10	0.21	
32	20C [T3]	11	0.12	
	MR5 (A→T)			
33, 34	12D [T3,4]	1	0.055	0.09
	MR6 (T→C)			
35	5C [T3]	1	<0.01	0.03
36, 39	18F,G [T3]	1	<0.01	0.64
37, 38	18F [T4]	1	<0.01	0.10
40	20C [T3]	6	<0.01	0.10
41	20C [T4]	6	0.0017	0.013
42	22B [T3]	7	<0.01	0.047
43–45	15F [T3,4]	8	<0.01	0.015
46	24B [T3]	10	<0.01	0.011
47	21C [T3]	15	<0.01	0.0033
48	6B [T3]	28	0.0002	0.0004

[a]Plants from sublines (indicated generations after transformation) from the indicated mutation-reporter lines (reversion pathways indicated) were analyzed for reversion events as described in "Materials and Methods." Plants were grown in an enclosed chamber except for experiments 28 and 29, for which a walk-in growth room was used. Copy number was measured as described in "Materials and Methods." Where multiple experiments are combined, the means of the data are provided. When two generations were assayed for a particular subline, the data were combined into one mean when the difference was less than 2-fold. All experimental data are recorded in Supplemental Table S2. [b]Counted reversion events per plant (typically for 100–150 plants) per transgene copy number. Spontaneous means correspond to no mutagen (no UVC) treatment. UV-specific events (MR1, MR2, MR5, and MR6 lines) equal total reversion events in plants irradiated to 1,000 J m^{-2} UVC radiation minus spontaneous events scored in parallel experiments.

8-oxoguanine and increase G→T mutation via 8-oxoG:A mispairing. Thus, we tested Cu(II), Cd(II), and Zn(II) for effects on MR4 (G→T) reversion, using mostly the seven-copy line MR4-5B to increase sensitivity (Supplemental Table S3). Only at the highest concentration (0.055 mM) and only in one trial (Supplemental Table S3, experiment 56) did $CdCl_2$ elevate MR4 (G→T) mutation, by 0.05 events per transgene copy above the spontaneous mean (0.03 ± 0.03). In a second trial (experiment 57), 0.055 mM $CdCl_2$ had no effect. However, 0.05 mM $CuCl_2$ increased MR4 (G→T) mutation by 0.17 and 0.13 events per copy number in two trials (experiments 61 and 62, respectively). $ZnCl_2$ concentrations high enough to reduce plant viability (0.66 mM) did not elevate mutation.

Sources of Systematic Variability

When these and other plant mutation-reporter transgenes are used for various mutation studies, it is important to be aware of potential sources of systematic variation and how they might be minimized. We have examined the effects of several experimental variables on our reporter lines: transgene copy number (discussed above) and genome position, the number of prior generations of propagations of a particular line, the growth medium, and the lighting regime. We have not performed exhaustive analyses, but the preliminary experiments described below already suggest some necessary precautions.

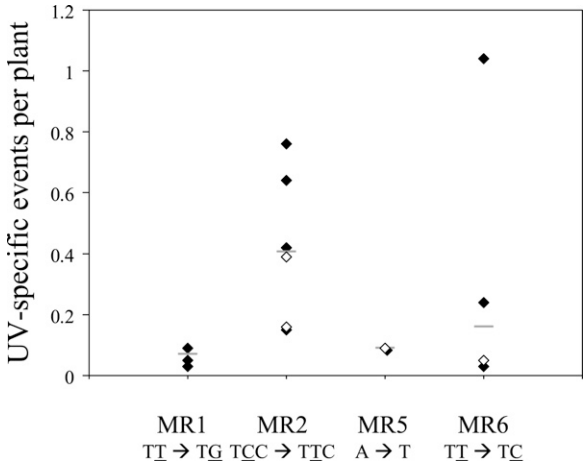


Figure 5. UV-specific reversions of single-copy transgenes. Data are from Supplemental Table S2. All plants were grown on soil. Fourteen days after planting, seedlings were UVC irradiated to 1,000 J m^{-2}, incubated 24 h in the dark, and grown under photoperiodic light (lines MR2 and MR5) or continuous light (line MR6) for a further 14 d. Plants were analyzed for reverted (GUS^+) spots as described in "Materials and Methods." Mean numbers of reversion events per plant, after subtraction of spontaneous frequencies, are shown from top to bottom for the following T3 (black diamonds) and T4 (white diamonds) sublines: MR1 (TT→TG), 1E, 11A, 11D, 1A, 12D, and 6A; MR2 (TCC→TTC), 10C, 12G, and 8D; MR5 (A→T), 12D; MR6 (TT→TC), 18F, 18G, and 5C. Bars indicate median values. Mean numbers of reversion events per plant per transgene copy for the unirradiated control plants are shown in Figure 4. Note the difference in scale.

Variability in Mutation among Different Generations and Different Lines

We described above the opposing ways in which transgene copy number affected spontaneous MR4 (G→T) mutation versus UV-induced MR2 (TCC→TTC) and MR6 (TT→TC) mutation. Kovalchuk et al. (2000) reported large differences in both spontaneous and UVC-induced mutation among a considerable number of independently transformed lines, some of which were single copy. Although possible differences in GUS^- protein levels were not addressed by those workers, transgene genome location appeared to affect mutability in at least some cases. In our experiments, spontaneous mutation frequencies were high enough to measure only for MR2 (C→T) and MR4 (G→T) lines. Using only plants of the same (T3) generation, spontaneous C→T frequencies were 0.007 to 0.04 events per plant in three single-copy lines and roughly 0.002 per copy in two multicopy lines. Among UV-treated T3 single-copy plants, the variation was proportionally similar: 0.15 to 0.76 events per plant in the three lines. For this small number of experiments, the variation among trials within a given line was as much as the variation from line to line. Thus, multiple trials seem essential in quantitative comparisons of spontaneous to mutagen-induced reversion frequencies.

We compared mutation of successive (T3 and T4) generations of several representative lines (Table II; Supplemental Table S2). In the three cases where spontaneous reversions could be scored for two generations, these were similar for MR2 (C→T) and MR5 (A→T) reversions but reduced by 1 order of magnitude from the T3 to the T4 generation of the seven-copy MR4-5B (G→T) line. UV-induced (single-copy) MR2-12G (TCC→TTC) reversions were roughly similar in T3 and T4 generations, but UV-specific (single-copy) MR6-18F (TT→TC) reversion was substantially reduced in the T4 generation. Thus, considerable caution is needed when additional markers are crossed into reporter-transgene lines (see "Discussion"). When transgene expression in T3 versus T4 generations was measured semiquantitatively by immunostaining of protein blots, no substantial differences were seen (data not shown).

Growth Conditions

Plants for mutagenesis experiments were grown on soil, except that when effects of transition metal ions were tested, plants were grown on standard Murashige and Skoog (MS) agar plates, with and without metal chlorides added to the medium (lines MR3, MR4, and MR6; Supplemental Table S3). In one case only, spontaneous mutation frequencies in plants on agar were compared with frequencies in soil (Supplemental Table S2, experiments 21–27). Plants of the seven-copy MR4-5B (G→T) line from the T3 generation showed perhaps slightly lower reversion on soil (0.13 per copy) than on plates (0.22 per copy), but T4 generation reversions were roughly similar (an average of 0.042 per copy).

Changes in the intensity and/or spectral context of light regimes during growth might affect apparent mutation frequencies in several ways: by modulating the expression of DNA repair and DNA damage-toleration proteins, CPD-photolyase, for instance (Ahmad et al., 1997); by changing the amount of UVA/blue light available to drive photoreactivation of CPD and [6-4] photoproducts (Sancar, 1994); and by altering balances between fluxes of ROS by-products of photosynthesis and mitochondrial electron transport versus detoxification by endogenous systems, perhaps affecting levels of endogenous DNA damage responsible for spontaneous mutation. Light regime effects might be important where the numbers of plants needed to carry out several necessarily parallel experiments exceeds the number of growth chambers or where UV needs to be present in one of two growth chambers. Supplemental Table S2 shows some appreciable differences among frequencies of spontaneous G:C→T:A reversion of MR4-5B plants under different lighting regimes.

Analyses of DNA Sequences of Individual GUS Allele Cells in Tissue Samples That Showed Modest Plant-Wide GUS Staining (Light Blue) or More Intense Blue Spots

Replacement of the wild-type Glu by Gln in the MR3 (G→C) lines resulted in a protein with 27% of wild-

type GUS activity in vitro (Table I). Thus, it is not surprising that light-blue X-Gluc staining covered the entire MR3 plants in all cases (data not shown). The background staining is not likely to be due to the endogenous Arabidopsis GUS, which requires an acidic buffer for histochemical staining (Sudan et al., 2006). In the two-copy lines MR3-1G and MR3-8B, the background staining was relatively light, consistent with the lower *GUS*$^-$ protein levels measured by *GUS*$^-$-AcV5 immunostaining. In these lines, sites of reversion to *GUS*$^+$ could be scored as darker blue spots or sectors (Supplemental Table S2), but fainter revertant spots may not have been detected.

Surprisingly, MR5 (A→T) lines also showed blue plant-wide staining, even though the substitution of Val for Glu reduced in vitro GUS activity to 0.8% of the wild-type value (Table I). In all MR5 (and MR3) lines tested, PCR amplification and sequencing of plant DNA revealed only the respective mutant sequences. In one (single-copy) line, MR5-12D, background staining was low enough that darker blue, presumably *GUS*$^+$, spots could be identified (Supplemental Table S2).

Representative blue tissues from MR2 (C→T) and MR4 (G→T) plant lines were also isolated for sequence analysis. For MR2 (single-copy) samples, DNA isolated by standard techniques from tissue fragments excised from blue spots was used for PCR amplification of the transgenic *GUS* targets. Products were cloned into plasmids and transformed into *E. coli*. Sequencing of target sites in independent plasmids revealed the ratio of original (mutant) versus reverted *GUS*$^+$ codons in the isolated tissues. Eight independent transformants were analyzed from UV-irradiated subline MR2-10C (C→T) tissue, of which three were reverted. $CuCl_2$-treated MR4-5B (G→T) plant tissue was also analyzed, but in this case dilution of putative single *GUS*$^+$ targets by the 13 mutant targets (per diploid genome) also present in this seven-copy line made the identification of revertants more laborious. Therefore, we directly amplified DNA from 833 individual bacterial colonies using an allele-specific PCR technique to identify clones with revertant sequences. In all three cases where a robust band corresponding to the expected *GUS*$^+$ allele-specific product was observed, sequencing of plasmids derived from replica colonies of the bacteria identified the expected *GUS*$^+$ codon sequence. These data cannot be used to estimate the fraction of revertants among mutant codons in the blue spots. We do not know the fraction of plant cells in the blue-tissue fragments whose DNA, when originally amplified, contributed a revertant codon DNA product to the transformed bacterial colonies that were subsequently analyzed by allele-specific PCR. These data do show that reverted codons were associated with blue-staining tissues.

DISCUSSION

Here, we describe six *GUS* mutation-reporter transgenes, inserted at single Arabidopsis loci, that revert to *GUS*$^+$ by different base-substitution pathways. Our studies have expanded upon the pioneering A:T→G:C, A:T→T:A, and A:T→C:G *GUS* reversion reporters developed by Hohn and coworkers (Kovalchuk et al., 2000) and the G:C→A:T *GUS* reporter of Van der Auwera et al. (2008) in several respects. (1) We used a C-terminal epitope tag to immunoassay actual levels of *GUS*$^-$ protein in extracts from the various reporter-transgene plants. (2) As well as directly counting reverted spots and sectors, we estimated mean reversion events per plant from Poisson zero fractions; this avoids the interpretation of occasional complex staining patterns. (3) Our new MR4 (G→T) target measured high spontaneous G:C→T:A transversion. (4) Quantitative determinations of transgene copy number showed spontaneous MR4 (G→T) reversion to be directly proportional to copy number. In contrast, UV-specific MR2 (T<u>C</u>→T<u>T</u>) and MR6 (T<u>T</u>→T<u>C</u>) reversions were markedly reduced at high copy numbers. (5) We compared spontaneous MR4 (G→T) and UV-induced MR2 (C→T) and MR6 (T→C) mutation in successive T3 and T4 generations of the same lines. There was a general trend toward lower mutation frequencies in the T4 plants. (6) We made some initial tests of how growth media and lighting regimes might affect mutation frequencies, showing both to be important variables.

These mutation-reporter transgenes may be used to analyze (1) spontaneous or environmentally stimulated reversion via different mutation pathways, (2) stimulation of a particular reversion pathway by different environmental mutagens, or (3) stimulation of a particular reversion pathway by a particular mutagen in lines deficient in DNA-repair activities versus wild-type lines.

In case 1, it is essential to consider reporter-transgene copy number. The high frequencies of MR4 (G→T) reversion events in high-copy lines were roughly proportional to transgene copy number, but comparisons among different reporters (different reversion pathways) should use single-copy lines whenever possible. The strong increase in "spontaneous" G:C→T:A transversion with copy number may reflect the increased number of MR4 target guanines at risk for endogenous oxidation. Both 8-oxoguanine and formamidopyrimidine-guanine mispair frequently with adenine (Shibutani et al., 1991). The subline-to-subline variation of the relatively low spontaneous MR2 (C→T) reversion reporter did not correlate with copy number. Among the respective sets of MR4 and MR2 sublines, variation was not as strong as that observed by Kovalchuk et al. (2000), possibly because we restricted ourselves to lines for which immunoassays of the AcV5 epitope tags implied good expression of the GUS fusion proteins. Nevertheless, it is highly preferable to use at least two or three independent single-copy lines (with good GUS protein expression) for each reporter gene (each pathway) involved in comparisons. For example, chromosomal location may affect the repair of endogenous DNA damage as well as transgene expression.

To meaningfully compare UV-induced MR2 (TC→TT) and MR6 (TT→TC) reversion, it was essential to use single-copy lines. Induced mutation per copy number was depressed drastically in high-copy lines. Transcription-coupled excision repair of photoproducts in the MR2 and MR6 *GUS* template strands (Lommel et al., 1995) might be reduced if transcription were attenuated by position effects or by cosuppression caused by high copy numbers. On the other hand, the chromatin surrounding multiple copies of robustly transcribed transgenes might be relatively open, increasing accessibility to repair proteins but perhaps also increasing frequencies of photoproduct induction. Chromatin effects might be different at the different positions of the transgenes in the genomes of the respective lines. Thus, transgene copy number and position may each affect UV-specific mutation in complex and perhaps opposite ways.

Although all three of the single-copy MR2 lines showed high UV-specific TC→TT mutation (0.15–0.76 events per plant), UV-specific TT→TC mutation was high in one single-copy MR6 line (0.16–1.04 events per plant) but low in another (0.03). Thus, an unequivocal conclusion that here UV-specific TT→TC mutation is much higher relative to TC→TT than has been observed in other organisms (Brash et al., 1987) would require the study of more independent single-copy lines.

In type 2 experiments, where a given reversion target is used with different mutagens, it seems absolutely essential to use single-copy lines. Differences in expression levels do not appear to account for the strong copy-number suppression of UVC-induced mutation. Copy-number effects may be different for different mutagens. The data of Supplemental Table S2 strongly indicate the desirability of contemporaneously comparing different lines (case 1 and case 3) and/or treatments (case 2) with controls, all matched as closely as possible with respect to growth media and lighting regimes. When trials are separated by time, this careful matching of media and lighting is essential.

Type 3 experiments require the construction of new lines by crossing (necessarily single-locus) mutation-reporter lines with mutants lacking, for example, activities that repair DNA damage or synthesize DNA past DNA lesions. If T3 reporter lines are used in crosses, the transgene expression and potential for spontaneous and/or induced mutation of the T5 progeny may be different from those of their T3 progenitors. We strongly suggest that wild-type and repair-defective plants (both encoding the same reporter gene) should be T5 "siblings" from the same cross.

For all three types of experiments, it is important to recognize that cells in different plant tissues and subtissues may with different efficiencies suppress mutation via DNA repair and other genome maintenance mechanisms. Mutations in dividing cells in shoot apical meristems, floral meristems, and haploid gametophytes and gametes might be transmitted to the progeny. Here, genome maintenance functions might be particularly robust and mutation rates correspondingly low. In the differentiated leaf cells where most revertant spots are scored, some DNA-repair pathways may be less active. Indeed, reversion of a frame-shifted *GUS*$^-$ transgene by a one-nucleotide deletion ($G_7 \rightarrow G_6$) increased only 5-fold in Arabidopsis defective in mismatch repair (MMR) versus the wild type (Leonard et al., 2003), much less than the 400-fold increase in $A_7 \rightarrow A_6$ reversion seen in MMR-defective yeast. In contrast, progeny of wild-type Arabidopsis showed much less insertion/deletion mutation of short-sequence repeats (microsatellites) than MMR-defective plants, suggesting MMR to be robust in the "equivalent germline" precursors of gametes. Thus, spontaneous mutation or mutation stimulated by a set of environmental agents in a particular transgene may be maximal in somatic leaf tissues.

A recent study by Ossowski et al. (2010) highlights possible differences between studies that measure somatic versus inherited mutations. In that work, wild-type Arabidopsis lines that had been propagated by single-seed descent for 30 generations were searched for spontaneous mutation by high-throughput sequencing of the entire genomes. A spontaneous mutation rate of 7×10^{-9} base substitutions per site per generation was observed, with a predominance of G:C→A:T transitions. The observed genomic mutation spectrum thus differs from our reporter-gene spectrum, which shows a predominance of G:C→T:A transversions. The high-throughput sequencing study focused on inherited changes, and indeed, the majority of the G:C→A:T transitions observed were silent in the third codon position (Ossowski et al., 2010). Our study focused on somatic mutation. Mutations in the "equivalent" germline (shoot and floral meristems, gametophytes, and gametes) may be subject to more stringent correction than those in differentiated somatic tissues in order to avoid deleterious changes in progeny. Thus, somatic mutations may provide a broader picture of initial mutation events as well as being much less expensive to analyze. Also, UV-induced and perhaps deamination-induced mutations might have been higher in the work by Ossowski et al. (2010) than in our study.

Because of the heterogeneity of DNA-repair and genome maintenance functions in the ancestry of any particular mutant cell, we do not consider average mutation frequencies per round of DNA replication to be meaningful. Different environmental mutagens may induce various lesions that are repaired with different efficiencies in leaf tissues, so differences in effects of these mutagens on any particular pathway must be interpreted cautiously. In leaves, even wild-type plants may already express some DNA-repair activities at low levels, so comparisons with DNA-repair-deficient plants may underestimate the effects of mutations that affect DNA-repair genes. In comparison, differences in shoot apical meristem, floral

cells, and gametophytic cells might be greater between repair-deficient versus wild-type backgrounds. One way to evaluate mutation in these cells would be to score reversion events only in floral tissues. Although discrete revertant (*GUS*$^+$) spots might be difficult to count, the Poisson zero technique could be used to estimate mean event frequencies.

Here, we have corrected the event frequencies observed in mutagen-treated plants by subtracting apparent spontaneous reversion frequencies. We did not calculate ratios ("fold increases") for several reasons. First, spontaneous frequencies were typically very low [except MR4 (G→T) events] and highly variable among independent trials, so denominators in such ratios would be very "noisy." Second, spontaneous mutations may arise at any time during growth, but induced mutations can only arise subsequent to mutagen treatment, after 2 weeks of growth in the case of UV irradiation. We suggest that mutagen-specific events always be reported as arithmetic differences, except perhaps to describe signal-to-noise ratios.

We infer that the large variety of GUS$^+$ staining patterns that we observed reflects the timing and location of the reversion events. Thus, a single acute mutagen exposure might predominantly induce isolated revertant spots, but chronic exposure might induce spots and sectors of various sizes at various locations. In these cases, mean event frequencies estimated using the Poisson zero term can nevertheless be quantitatively compared. If frequencies are so low that almost all plants show no reversion events, any staining pattern can be assumed to reflect a single event. If zero-event fractions are very small under some environmental conditions, it may be advisable to reduce the mutagen treatment. Mutagen treatment should also be reduced if the plants appear heavily stressed (e.g. increased anthocyanin production), because direct mutagen (DNA-damage) effects may be partially obscured by plant stress responses.

Kovalchuk et al. (2000) and Van der Auwera et al. (2008) placed reversion targets at different positions in 5′ and 3′ *GUS* regions, respectively. Even the same pathway showed hot and cold spots. For example, spontaneous A:T→G:C and A:T→C:G reversions were both markedly higher at codon 166 in some sublines than at other codons in any subline (Kovalchuk et al., 2000). This adds a sequence-context variable to copy-number, chromosome-location, and expression-level variables when reversions by different pathways are compared. We have minimized this variable by placing all reversion targets within the same codon. In addition, it seems valid to compare effects on different reversion pathways (type 1 experiments) only if for each pathway two or three sublines show quantitatively similar spontaneous or mutagen-induced reversion frequencies.

What generalizations can be made about the *GUS*$^-$ reversion-reporter studies by Kovalchuk et al. (2000), Van der Auwera et al. (2008), and ourselves? First, spontaneous frequencies measured in the three studies were roughly similar for plants grown on solid medium, particularly if a few outlier sublines are set aside. The slightly higher spontaneous frequencies associated with most Kovalchuk lines may reflect the 1 or 2 extra weeks of growth before analysis (GUS activity staining). Spontaneous G:C→A:T reversion was markedly elevated by growth of 2-week seedlings in liquid medium for 7 to 9 d (Van der Auwera et al., 2008). Second, UVC irradiation increased reversion via one or more pathways in all studies, but substantial differences in experimental design introduced additional variables; quantitative comparisons would seem problematic. Only in our study was the persistence of UV photoproducts promoted by (24-h) dark incubation immediately after irradiation. Van der Auwera et al. (2008) used a much lower UVC dose (80 J m^{-2}) than the 1,000 J m^{-2} employed in the other two studies. Also, most of the Van der Auwera et al. (2008) cytosine targets, designed to study deamination at CpG or CpNpG sites, were not flanked by pyrimidines and hence could not form the common bipyrimidine photoproduct. Thus, the significance of the reported UV-stimulated C→T reversion is not clear.

Third, the three groups reported different responses to transition metal ions. Kovalchuk et al. (2001b) observed higher A:T→G:C and A:T→C:G mutation during growth on six different metal ion-containing media, including Cd(II) at 0.005 to 27 μM, Zn (II) at 44 to 130 μM, Cu(II) at 0.2 to 16 μM, and Pb(II) at 1.5 to 30 μM. However, Van der Auwera et al. (2008) reported no effect of 22 μM Cd(II) or 60 μM Pb(II) on G:C→A:T mutation. We focused on MR4 (G→T) mutation, a likely result of oxidation of guanine to 8-oxoguanine by highly reactive hydroxyl radicals generated by the reaction of endogenous hydrogen peroxide with Fe(II): the Fenton reaction. In addition, other divalent cations may displace Fe(II) from intracellular sites, and Cu(II) is readily reduced to Fenton-active Cu(I) in plant tissues by ascorbate (Buettner and Jurkiewicz, 1996). At 0.05 mM, we found Cu(II) to increase G:C→T:A above the high spontaneous frequency (mean of 0.03) to 0.18 events per plant per transgene in two trials. Surprisingly, we found 0.055 mM Cd(II) to only increase G:C→T:A reversion above spontaneous levels by 0.05 events per plant per transgene in one trial and to show no effect in another trial. Even high Zn(II) concentrations affected viability but not mutation. The stimulation of mutation by both G:C and A:T base pairs by lower concentrations of Cd(II), Cu(II), and Zn (II) reported by Kovalchuk et al. (2001b) may involve DNA lesions and/or mispairing paths not previously elucidated.

CONCLUSION

The reversion-reporter transgene system described here offers several advantages. All six lines contain single transgene loci and express *GUS*$^-$ protein at moderate to high levels; single-copy or two-copy sublines are available in all six. Reversions via all six

transition and transversion pathways are analyzed in the same codon, hence in closely similar sequence contexts. The background GUS activity staining seen in MR3 and MR5 lines can be overcome by careful selection of sublines. When comparing mutation via different pathways (using lines with different targets), one should consider that modest differences may simply reflect line-to-line differences in variables such as copy number and generation number. The MR4 (G→T) line now provides a tool to study oxidative mutagenesis, and the MR2 (TC→TT) and MR6 (TT→TC) lines can now be used to compare two major UV mutagenesis pathways in planta. We provide as well some general experimental guidelines to ensure the reliability of comparison studies using mutation-reporter transgenes: multiple independent low-copy (preferably single-copy) sublines for each pathway, highly similar growth and lighting conditions, and genetic comparisons only among plants from the same generation, ideally sibling progeny from the same crosses. With careful attention to these guidelines, reversion-reporter transgenes can be powerful tools for the elucidation of mutagenesis pathways in plants.

MATERIALS AND METHODS

Construction of Reversion-Reporter Genes and Transformation into Plants

To construct the seven *GUS*$^-$ alleles shown in Figure 1, we began with plasmid pGUS23 (kindly supplied by Dr. Igor Kovalchuk, University of Lethbridge) in which the first 87 bp of CaMV ORF V replaces the translation start site of the GUS-encoding *Escherichia coli uidA* gene (Kovalchuk et al., 2000). For simplicity, we refer to these fusion genes and proteins here as *GUS*$^-$ and GUS$^-$, respectively. We used PCR amplification with primers encoding the desired mutations to generate fragments that were incorporated into complete *GUS*$^-$ genes, in the bacterial plasmid vector pCR4-TOPO (Invitrogen), flanked by restriction sites for *Hin*dIII and *Not*I restriction enzymes (abbreviated as *Hin*dIII and *Not*I henceforth). An 83-bp oligonucleotide encoding a nine-amino acid AcV5 epitope tag (Lawrence et al., 2003) and an ochre (5′TAA) stop codon were inserted at the C-terminal end of the *GUS*$^-$ coding sequences using *Avr*II and *Not*I restriction endonucleases. The tripartite genes encoding ORF V-GUS$^-$-AcV5 fusion proteins were excised from pCR4-TOPO using *Hin*dIII and *Sac*I restriction endonucleases and inserted into the corresponding sites in the binary vector p1803 (Becker et al., 1992). In the orientation indicated, *GUS*$^-$ genes were transcribed by the *(Aocs)$_3$AmasPmas* superpromoter constructed by Gelvin and colleagues (Lee et al., 2007), used in accordance with a license to Oregon State University. Kits from Qiagen were used to prepare plasmid DNA, purify PCR products, and extract DNA from agarose gels. Oligonucleotides were purchased from MWG Biotech. Restriction enzymes were purchased from New England Biolabs and Fermentas. After confirmation of the respective DNA sequences, binary vectors containing the reversion-reporter constructs were transformed into *Agrobacterium tumefaciens* strain GV3101 using kanamycin selection. The respective bacteria were used to transform Arabidopsis (*Arabidopsis thaliana*) ecotype Columbia plants by the floral dip method (Clough and Bent, 1998), with subsequent selection for growth on plates containing 20 μg mL^{-1} hygromycin.

PCR

PCR mixtures contained approximately 200 ng of plant DNA, *Taq* polymerase, standard PCR buffer, and appropriate primers at 5 μM (see above). Amplification reactions (30 rounds) in a Robocycler Gradient Amplification 96 thermocycler (Stratagene) followed standard procedures appropriate for the primers.

In Vitro Transcription/Translation and GUS Assays

DNA templates constructed to contain a T7 promoter and Kozak translation sequence (Kozak, 1986) were used with the TNT T7 Quick for PCR DNA system (Promega) for protein expression from PCR templates. DNA, transcription/translation enzyme Master Mix, and [^{35}S]Met were incubated at 30°C for 90 min. The resulting [^{35}S]Met-labeled-protein (73 kD) was analyzed by SDS-PAGE and autoradiography. GUS activity was measured as cleavage of the fluorogenic substrate MUG into GlcA and fluorescent 4-methylumbelliferone (Jefferson et al., 1987). Five microliters of protein synthesis reaction products was added to 495 μL of MUG assay solution containing 50 mM $NaHPO_4$, pH 7.0, 10 mM β-mercaptoethanol, 10 mM Na_2EDTA, 0.1% sodium lauryl sarcosine, 0.1% Triton X-100, and 1 mg mL^{-1} MUG. After incubation at 37°C, the reaction was stopped with excess 0.2 M sodium carbonate. Fluorescence was measured on a SpectraMAX Gemini Dual-Scanning Microplate Spectrofluorometer, with excitation at 355 nm, emission at 460 nm, and cutoff at 435 nm.

Isolation and Selection of Independent Transformants

T1 seeds harvested from *Agrobacterium*-treated T0 plants were sterilized and plated on hygromycin-selective medium at a density of 500 seeds per plate. Resistant T1 plants, roughly one to five per plate, were transplanted to soil after 1 week and allowed to self-pollinate and mature. T2 seeds from individual T1 plants were then harvested, and progeny sets of 50 T2 seeds were plated onto hygromycin plates for segregation analysis of progeny. Those T1 plant lines whose T2 progeny phenotypes indicated likely single-site insertion (segregating 3:1 for resistance) were selected for further testing. The cutoff for the probability of a line containing a single locus of transgene insertion was $P < 0.05$, as determined by the χ^2 test: [(observed resistant fraction − 0.75)^2/(0.75)] + [(observed sensitive fraction − 0.25)^2/(0.25)]. Progeny sets with χ^2 values less than 3.84 were retained for further studies. Most plants were routinely grown in a Percival PGC-105 growth chamber maintained at 22°C under cool-white fluorescent lights (Phillips F72T12/CW/VHO; 16:8-h photoperiod, output of 50 μmol m^{-2} s^{-1}; 24:0-h photoperiod, output of 40 μmol m^{-2} s^{-1}). Plants grown for harvesting and some experiments were grown in a growth room maintained at 22°C under cool-white fluorescent lights (24:0-h photoperiod, output of 75–105 μmol m^{-2} s^{-1}).

Immunoassays of *GUS*$^-$ Proteins in Plant Extracts

To prepare plant protein extracts, roughly 50 mg of tissue (three 10-d-old seedlings of four to six leaves each) were frozen in liquid nitrogen and ground using a pestle fitted to a microfuge tube. Fifty microliters of 2× protein-loading buffer (0.5 M Tris-HCl, pH 6.8, 4.4% [w/v] SDS, 20% [v/v] glycerol, 2% [v/v] 2-mercaptoethanol, and bromphenol blue) was added, and the frozen tubes were placed on ice for 5 to 15 min. Tubes were boiled for 3 min, vortexed for 30 s, sonicated for 3 min, boiled for 3 min, and vortexed for 30 s. Homogenized samples were centrifuged for 15 min at 4°C in an Eppendorf Centrifuge 5415D. Supernatants were transferred to fresh tubes and centrifuged again to minimize cellular debris. Aliquots (15 μL) were loaded onto SDS-PAGE gels without further manipulation. Protein standards were provided by lysates of *E. coli* BL21 Codon+ cells (Stratagene) that contained a pET21a vector encoding GUS$^+$-AcV5, grown overnight in tryptone/yeast extract broth with ampicillin, and induced with 0.5 mM isopropyl β-D-thiogalactopyranoside. Bacterial cultures were centrifuged, and pellets were resuspended in 2× protein-loading buffer and boiled for 3 min.

Most immunoassays of protein blots (western blots) were performed with the Bio-Rad Criterion XT system. Criterion XT polyacrylamide gels (4%–12% Bis-Tris) were used with XT MOPS running buffer for SDS-PAGE for 1 h. Protein was transferred to polyvinylidene difluoride membranes in cold transfer buffer (25 mM Trizma base, 192 mM Gly, and 20% methanol) at 100 V for 1 h. Gels were stained with Coomassie Brilliant Blue to detect residual protein not transferred. Membranes were stained with Ponceau S solution (0.1% [w/v] Ponceau S and 5.0% [v/v] acetic acid) for 5 min, then rinsed exhaustively with distilled water to minimize background stain. The Chemi Genius Bioimaging System (Syngene) was used to quantify relative amounts of protein transferred. Immunoassay signals were normalized for total protein as follows. GeneSnap and GeneTools software (Syngene) were used to quantify the predominant band (73 kD) in the extracts. This was then used as a normalization standard. Peak detection was set to "lowest slope" for Ponceau S staining (high background) and "rolling disc" for the western blots

(low background). Membranes were destained in 0.1 M NaOH for 1 min and thoroughly rinsed in distilled water and TBS-T (100 mM Trizma base, 0.68 M NaCl, pH 7.6 with HCl, with Tween 20 added to 0.1%). The membranes were blocked for 1 h with TBS-T plus 5% dried milk and incubated for 1 h in TBS-T-milk plus anti-AcV5 primary antibody (usually 0.1 μg mL^{-1} in 20 mL of TBS-T-milk). Membranes were washed five times with TBS-T, incubated for 1 h in TBS-T-milk plus anti-mouse IgG secondary antibody (typically 0.08 μg mL^{-1} in 20 mL of TBS-T-milk), and washed with TBS-T as before. SuperSignal West Pico chemiluminescent substrate (Pierce) was prepared according to the manufacturer's instructions and poured over the membranes already in the Syngene imaging apparatus. After 5 min of incubation, blots were imaged for increasing times, in most cases from 1 min to 1 h; exposures up to 5 h were used for western blots with lower signals. We used aliquots from stock solutions of monoclonal anti-AcV5 primary antibodies (prepared in mouse [Sigma-Aldrich]; 1.4 mg mL^{-1}) or ImmunoPure Goat Anti-Mouse IgG secondary antibodies (H+L, Peroxidase Conjugated [Pierce]; 0.8 mg mL^{-1}).

Isolation of Sublines Homozygous for Single Insertion Loci

Lines that showed moderate to high protein expression (typically a relative expression of 20%–100% of the most intense AcV5-immunostained band [normalized for gel loading]) and apparent single transgene insertion loci (see "Isolation and Selection of Independent Transformants" above) were selected for further analysis. Eight progeny plants for each line were transplanted to soil and allowed to mature. Each subline plant was harvested, and T3 progeny seed sets of 50 were plated on hygromycin medium for segregation analysis. T2 sublines that showed a homozygous pattern of segregation of T3 progeny (all resistant) were selected for testing. One T2 subline was tested for each selected independent T1 transformant. Four T3 progeny plants were also transplanted to soil and allowed to mature for bulk harvesting of the T4 generation. For some sublines, too few T3 seeds were recovered to use for experiments; here, bulked T4 seeds were used.

UVC-Induced Mutation

Seeds were vernalized (4°C wet incubation for 2 d), suspended in 0.1% agarose, and dispersed randomly onto pots of soil (50 seeds per pot). At the time of planting, pots were divided into "No-UV" (mock irradiated) and "UVC" groups to avoid bias. An average of 100 to 150 plants were typically assayed for each datum point. Two weeks after planting, UVC pots were exposed to a single dose of 1,000 J m^{-2} produced by a bank of six 15-W Sylvania germicidal lamps (254 nm) at a distance of 125 cm (average exposure of 6 min and 40 s at a rate of 2.5 J m^{-2} s^{-1}). Both the UVC and No-UV pots were immediately incubated in the dark for 24 h to prevent rapid removal of UV-induced dimers by endogenous enzymatic photoreactivation. UVC-irradiated plants were incubated an additional 2 weeks to fix mutations and then histochemically stained for GUS activity. No-UV plants were incubated for only 1 additional week, because they grew more rapidly than irradiated plants.

Treatment with Metal Ions

Fifty sterile vernalized seeds were spread on standard MS plates with or without transition metal ions (0.002, 0.005, 0.027, or 0.055 mM $CdCl_2$; 0.13 or 0.66 mM $ZnCl_2$; 0.05 mM $CuCl_2$). Most metal ion plates were prepared by adding an aqueous solution of metal salt before autoclaving. Some of the 1 mg L^{-1} $CdCl_2$ plates were prepared by adding filter-sterilized aqueous cadmium chloride after autoclaving the medium (Kovalchuk et al., 2001b); no significant differences were seen. Seedlings were allowed to grow for 3 weeks before their vegetative tissues were histochemically stained for GUS activity (typically 100–150 plants per datum point).

Histochemical Staining for Transgenic GUS

Histochemical GUS activity staining was performed as described (Jefferson, 1987). Vegetative plant tissue was collected and immersed in GUS staining buffer containing 0.1 M sodium phosphate, pH 7.0 (endogenous plant GUS requires pH 4.0; Sudan et al., 2006), plus 0.1% Triton X-100, 0.05% NaN_3, and 0.63 mM X-Gluc substrate (Research Products International Corp.) from a concentrated stock solution in dimethyl formamide. Immersed tissues were vacuum infiltrated for 10 min and then incubated for 48 h at 37°C. (Vacuum infiltration is thought to provide a continuous liquid-leaf surface interface [Filkowski et al., 2004].) Samples were treated with 70% ethanol to remove chlorophyll, facilitating the visualization of X-Gluc stain. X-Gluc substrate was used as 0.64 mM solutions of the cyclohexylammonium salt or sodium salt in GUS staining buffer. No significant difference in staining efficiency was apparent.

Identification of Reverted (*GUS*$^+$) Codons

Samples of blue-staining tissue containing putative *GUS*$^+$ alleles were mechanically dissected from X-Gluc-stained leaves of UV-irradiated subline MR2-10C (C→T) plants. DNA isolated by standard techniques was used for PCR amplification of the transgenic *GUS* target. Products were inserted into the Topo vector pCR4-TOPO (Invitrogen) and transformed into *E. coli*. Sequencing of target sites in plasmids prepared from small cultures inoculated with eight individual colonies identified three independent plasmids encoding the *GUS*$^+$ codon GAA. PCR products expected to include *GUS*$^+$ target genes were similarly amplified from blue-staining tissue of $CuCl_2$-treated MR4-5B (G→T) plants and individually cloned in bacteria. In this case, dilution of putative *GUS*$^+$ targets by the 13 mutant targets (per diploid genome) made identification of revertants as described above more laborious. Therefore, we used an allele-specific PCR technique slightly modified from that described by Ye et al. (2001) to directly amplify DNA from 833 individual bacterial colonies (that had been transformed with plasmids in which the original PCR products had been cloned). In this case, we used Topo vector-specific primers (M13F and M13R) flanking the targets (expected to be randomly inserted in either orientation) and two allele-specific primers, complementary to the *GUS*$^+$ codon sequence on one strand (GAA) versus the MR4 *GUS*$^-$ codon sequence on the other strand (GCA). In all three cases where a robust band corresponding to the expected 350-bp *GUS*$^+$ allele-specific product was observed, sequencing of plasmids derived from replica colonies of the bacteria identified the expected *GUS*$^+$ codon sequence.

Statistical Analysis

Differences in apparent reversion frequency between controls and mutagen-treated plants were tested for significance ($P < 0.05$ with the Mann-Whitney [rank-sum] test). Selected data were presented in histograms and fitted to a Poisson distribution curve to confirm the random distribution of reversion events and to estimate means from the fitted distributions. Computations were performed using the VassarStats Statistical Computation Web site.

Extraction of DNA from Plants

DNA was isolated from plants for DNA blots and PCR amplification using a bead-shaker DNA apparatus (Alexander et al., 2007). Thirty to 50 mg of leaf material (equivalent to a 3-week-old [10- to 12-leaf] plant), one metal bead, and 400 μL of lysis buffer (10 mM Tris-HCl, pH 9.5, 10 mM EDTA, 100 mM KCl, 0.5 M Suc, 4 mM spermidine, 1.0 mM spermine, 0.1% [v/v] β-mercaptoethanol, and 2% [w/v] sarkosyl; final pH 9.5) were added to a microfuge tube and mixed with the aid of a reciprocating apparatus for high-velocity shaking for 30 s. Homogenized samples were centrifuged for 5 min, and the supernatant was transferred to a new tube. DNA was extracted with 300 μL of phenol: chloroform, then precipitated with ethanol (1:10 volume of 3 M sodium acetate [pH 5.2] and 2.5 volumes of 95% ethanol). Pellets were washed with 80% ethanol and dried in air, then resuspended in 100 μL Tris-EDTA buffer, pH 8.0, containing 10 μg mL^{-1} RNase.

Estimation of Transgene Copy Number

Sublines previously showing segregation consistent with single transgene insertion loci were used for copy-number analyses. Aliquots of DNA (typically 1–3 μg) extracted from each subline were digested with restriction endonuclease *Bcl*I (15 units) or *Eco*RV (20 units), both from New England Biolabs, at 50°C or 37°C, respectively, in 20-μL volumes of the recommended reaction buffer (100 mM NaCl, 50 mM Tris-HCl, 10 mM $MgCl_2$, and 1 mM ditiothreitol; pH 7.9 at 25°C). *Bcl*I cleaves two recognition sites flanking the probe-binding site within the T-DNA (Supplemental Fig. S1), yielding in transformed lines an identical 2.4-kb fragment from each copy of inserted

T-DNA. *Eco*RV cleaves at a single site 5′ to the probe-binding site and at additional sites in genomic DNA flanking the site of T-DNA insertion. Single-insertion lines yield a line-specific single *Eco*RV fragment whose size is determined by the distance from the defined T-DNA cleavage site to the nearest genomic *Eco*RV site. Typically, insertion of up to three T-DNAs can be readily identified by simple *Eco*RV band patterns. Higher copy insertions yield increasingly complex *Eco*RV band patterns, dependent on the orientation and number of T-DNA inserts, so copy number must be estimated by relative *Bcl*I signal intensities (see below). Restriction products were separated by electrophoresis on 0.8% (Tris-borate) agarose gels at 0.8 V cm^{-2}, then denatured by immersion of gels in 0.5 N NaOH + 1.5 M NaCl with gentle agitation for 30 min. After neutralization of gels by immersion for 30 min in 0.5 M Tris-HCl (pH 7.0) + 1.5 M NaCl, DNA was blotted overnight onto a Nylon Hybond N^+ membrane (Amersham/GE Healthcare) prewetted with distilled water using standard techniques (Brown, 2001) with 20× SSC (1× SSC is 0.15 M NaCl, 15 mM Na_3 citrate) as the transfer buffer. Since restaining of the final gel with ethidium bromide revealed no remaining DNA, transfer efficiencies were estimated to be greater than 90%. Membrane blots were rinsed briefly in 2× SSC and UV irradiated in a Stratalinker apparatus (Stratagene) to 1,200 J m^{-2}. Membranes were rinsed briefly in a solution of 0.1% SSC plus 0.1% SDS, blotted to remove excess liquid, transferred to a glass hybridization bottle (Fisher Scientific), and incubated in 15 mL of prehybridization solution (50% formamide, 3× SSC, 1× Denhardt's solution [0.02% Ficoll 400, 0.02% polyvinylpyrrolidone, and 0.02% bovine serum albumin], 20 μg mL^{-1} sonicated salmon sperm DNA, 5% dextran sulfate, and 2% SDS) at 50°C for 60 min with continuous rotation. The T-DNA-specific probe was generated by amplifying a 724-bp fragment spanning the joint between the GUS coding sequence and the agropine synthase terminator in plasmid p3AA-1 (reversion-reporter construction plasmid) using primers 5′-GTTCTGCGACGCTCACACC-3′ and 5′-ATCCGGTCTCAGTTACAAGC-3′ under standard PCR conditions. Roughly 1 ng of gel-purified PCR product was the template for a second PCR using the same primers and [α-^{32}P]dCTP. For hybridization, an aliquot of a mixture of unlabeled and radiolabeled probe (roughly 2×10^7 cpm) was added to 1 mL of prehybridization solution and briefly incubated in a boiling water bath, added to the hybridization bottle containing the DNA-blotted membrane and prehybridization solution, and incubated at 50°C overnight with continuous rotation. After decantation of the liquid, the membrane was rinsed briefly in 3× SSC containing 0.5% SDS and then washed with the same solution for 30 min at 55°C. Washes were repeated until background radioactivity on the blot, detected with a handheld meter, was minimal, typically two repetitions. Membranes were blotted with Whatman 3MM paper, wrapped in plastic wrap, and exposed to photographic film for 1 to 7 d at –80°C or placed in phosphorimaging cassettes for 1 to 4 d. Phosphorimaging was performed and analyzed using a Storm 820 (GE Healthcare) instrument at the Central Services Laboratory at Oregon State University, and data were reduced using ImageQuant software.

In each phosphorimage (Supplemental Fig. S1), all single-copy lines (defined by single *Eco*RV bands) were identified by inspection; the mean of their *Bcl*I band intensities (normalized for DNA loaded into each lane) was defined as relative intensity 1.0. For higher copy sublines, the ratios of their *Bcl*I band intensities to the intensity corresponding to relative intensity 1.0 were equated with T-DNA copy number. For sublines whose *Eco*RV band patterns appeared by inspection to reflect two- or three-copy insertions, estimates from relative *Bcl*I band intensities were in good agreement.

Supplemental Data

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

Supplemental Figure S1. Estimation of transgene copy number by electrophoresis and DNA (Southern) blotting.

Supplemental Figure S2. Distributions of mutation events among plant populations.

Supplemental Table S1. Comparison of methods for the estimation of frequencies of reversion events.

Supplemental Table S2. Spontaneous and UV-induced reversion frequencies.

Supplemental Table S3. Metal ion-induced mutation frequencies.

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