Development and Application of Novel Constructs to Score C:G-to-T:A Transitions and Homologous Recombination in Arabidopsis[1][W]

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We report on the development of five missense mutants and one recombination substrate of the β-glucuronidase (GUS)-encoding gene of *Escherichia coli* and their use for detecting mutation and recombination events in transgenic Arabidopsis (*Arabidopsis thaliana*) plants by reactivation of GUS activity in clonal sectors. The missense mutants were designed to find C:G-to-T:A transitions in a symmetrical sequence context and are in that respect complementary to previously published *GUS* point mutants. Small peptide tags (hemagglutinin tag and Strep tag II) and green fluorescent protein were translationally fused to GUS, which offers possibilities to check for mutant GUS production levels. We show that spontaneous mutation and recombination events took place. Mutagenic treatment of the plants with ethyl methanesulfonate and ultraviolet-C increased the number of mutations, validating the use of these constructs to measure mutation and recombination frequencies in plants exposed to biotic or abiotic stress conditions, or in response to different genetic backgrounds. Plants were also subjected to heavy metals, methyl jasmonate, salicylic acid, and heat stress, for which no effect could be seen. Together with an ethyl methanesulfonate mutation induction level much higher than previously described, the need is illustrated for many available scoring systems in parallel. Because all *GUS* missense mutants were cloned in a bacterial expression vector, they can also be used to score mutation events in *E. coli*.

Quantitatively scorable readout systems have been developed to measure the amount of genome changes in plants. These can be exploited as bioindicators of environmental pollution (Kovalchuk et al., 1998, 2001a, 2001b) or as monitors of mutation frequencies resulting from mutant backgrounds or stress conditions in a laboratory setting (Lebel et al., 1993; Puchta et al., 1995; Kovalchuk et al., 2000, 2001b, 2003; Lucht et al., 2002; Leonard et al., 2003; Hoffman et al., 2004; Yoshihara et al., 2006). They provide either a direct or indirect way to determine the number of certain types of genome alterations. Indirect measurements use a transgenic phage or plasmid mutagenesis target that is integrated into the plant genome and that can be rescued by restriction endonuclease digestion and subsequent transformation of a suitable bacterial strain (Yoshihara et al., 2006, and refs. therein). The number of bacteria or phages carrying a mutant plasmid can be determined by positive selection of the mutant target gene encoded on this plasmid or phagemid. The advantage is that DNA sequencing of the surviving bacteria or phages allows a broad spectrum of genome alterations to be scored, including all kinds of nucleotide substitutions, insertions, and deletions, as long as they alter the functionality of the gene used for positive selection. However, such indirect measurement is cumbersome because it requires DNA isolation, plasmid rescue, high-efficiency transformation, and DNA sequencing. In addition, the number of mutations observed and the number that actually took place are not linearly correlated because one mutation happening early in plant development will result in much more mutant plant cells than does the same mutation event at a later growth stage. In contrast, direct measurement systems do not suffer from these drawbacks. In these strategies, the number of genome changes is visualized in situ in plant tissues by using a reporter gene, such as *Escherichia coli uidA* encoding GUS (Kovalchuk et al., 1998, 2001a, 2001b; Lucht et al., 2002; Leonard et al., 2003), or luciferase (Kovalchuk et al., 2003). Thereby, the number of clonal sectors in which the activity of an inactivated transgenic reporter

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gene is restored is indicative of the number of alterations that actually has taken place at a particular genome position. Application of the method is fast because only the reporter protein activity has to be visualized by providing plant tissues with a suitable substrate. However, analysis of the plants can be quite labor intensive because, in general, many plants have to be inspected visually, which is not an easy task in view of the fact that clonal sectors are often small and therefore hard to detect. The biggest disadvantage of direct measurement is the need to establish a particular transgenic plant line for each kind of mutation one wants to look for because, generally, only one particular mutation at a predefined site will restore reporter gene activity.

In the GUS-based direct measurement approach, an inactive variant of the bacterial GUS-encoding *uidA* gene is stably introduced into the plant genome. Any mutation event that restores the original active GUS-coding sequence will eventually give rise to a clonal sector in which GUS activity can be detected by staining the plant with the GUS substrate 5-bromo-4-chloro-3-indolyl-β-D-glucopyranoside (X-glu), which results in the development of a blue precipitate at the site of GUS activity (Jefferson, 1987; Jefferson et al., 1987). Hence, the number of blue clonal sectors in a given plant or plant population is a direct measurement of the mutation frequency.

Three different kinds of *GUS* modifications have been reported, all of which aim at detecting different categories of genetic alterations. First, in the so-called recombination substrates (Puchta et al., 1995; Lucht et al., 2002), a 5′ (GU) and a 3′ (US) partial GUS-coding region are placed in a tandem or inverted repeat orientation in the genome, separated by a spacer fragment. Because both these partial regions share a central overlap (U), intrachromosomal homologous recombination of *GU* and *US* restores the active GUS-coding sequence. Second, frame-shift mutations have been used to score for insertion and deletion events that restore the active GUS reading frame (Leonard et al., 2003). Finally, inactivating nucleotide substitutions designed to score for specific restoring point mutation events (Kovalchuk et al., 2000) have been introduced into the GUS-coding region.

Although none of the inactivating *gus* nucleotide substitutions from Kovalchuk et al. (2000) is designed to score for C:G-to-T:A transition events, in *E. coli*, several mutagenic treatments have been shown to result precisely in such transitions (Cupples et al., 1990). In Arabidopsis (*Arabidopsis thaliana*), C:G-to-T:A mutations initiated by deamination of methylated cytosine residues in a symmetrical CpG or CpNpG sequence context are believed to be one of the mechanisms to account for the underrepresentation of these sequences in the genome (Gentles and Karlin, 2001; Tran et al., 2005). In addition to naturally occurring phenomena, commonly used chemical mutagens, such as ethyl methanesulfonate (EMS), induce predominantly C:G-to-T:A transitions (Koornneef, 2002; Greene et al., 2003; Yoshihara et al., 2006). Currently, no indicator plants are available to assess this kind of mutation.

Here, we describe the development of transgenic Arabidopsis plants, each containing one of five different nucleotide substitutions in the transgenic GUS-coding region. Each of these results in a critical inactivating amino acid replacement destroying GUS activity, which can be restored by a C:G-to-T:A transition. We show that *GUS* reversion events can take place at a scorable frequency and that this frequency is indicative of the amount of C:G-to-T:A substitutions in the genome. In addition to these point mutation reporter lines, we developed an alternative *GUS* recombination substrate, which can be used to score intrachromosomal homologous recombination frequencies in Arabidopsis. The obtained indicator plants were subjected to a number of potentially mutagenic treatments to validate the system and to assess their impact on recombination and on C:G-to-T:A mutation events.

RESULTS

Transgenic Plants

For transformation of Arabidopsis plants, 14 T-DNA constructs were generated, which differ only with respect to the GUS region of the sequence encoding a translational fusion with GFP (Fig. 1). Seven different GUS-coding sequences were used, either encoding a tagged or a nontagged protein. In the tagged version, the produced GUS protein is fused to 29 amino acids of the cauliflower mosaic virus open reading frame V (CaMV ORF-V; Puchta and Hohn, 1991) at the amino terminus, followed by a hemagglutinin tag (sequence YPYDVPDYA; Kolodziej and Young, 1991), and to Strep tag II (sequence WSHPQFEK; IBA GmbH) at the carboxy terminus (Supplemental Fig. S1). The latter two tags were successfully detected in protein extracts from tagged *GUS*-expressing bacteria (Supplemental Fig. S2), and we estimated a 50% reduction in GUS units of produced GUS protein as a result of the presence of the tags (data not shown).

Of the seven *GUS* sequences, five encode a mutant protein (Fig. 1; Table I) without GUS activity, as shown in *E. coli* expression clones qualitatively (Fig. 2) and quantitatively using bacteria concentrations at least 25-fold those of the active GUS control (data not shown). The two remaining sequences represent a functional GUS-encoding construct and a tandem repeat construct that forms a functional GUS-encoding sequence only upon homologous recombination (Fig. 1). This was shown by the fact that GUS activity in transgenic plants harboring this recombination substrate was confined to clonal regions (Fig. 3), a pattern characteristic for reactivation occurring in a single cell followed by clonal expansion of that cell, exactly as observed for the *GUS* missense mutants (see below). *gus* mutant M2 (Table I) was not used in planta

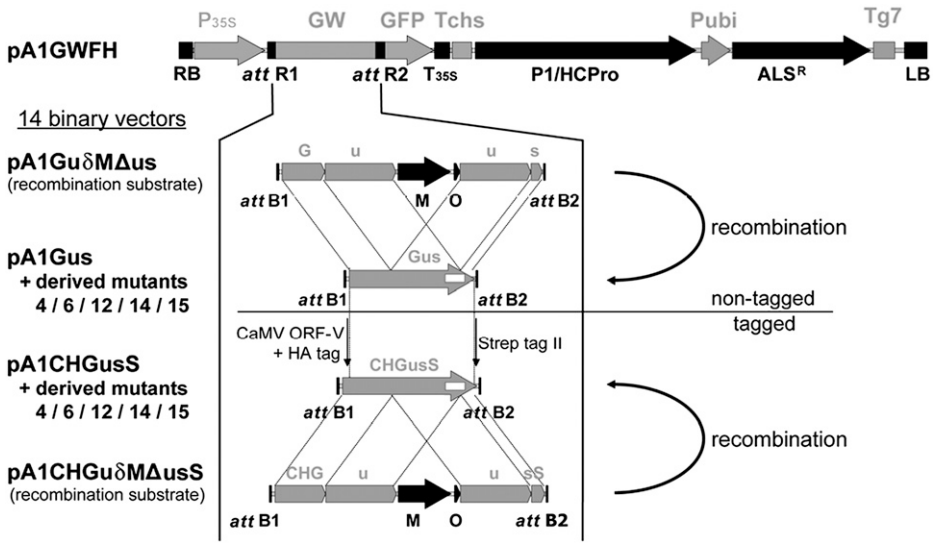

Figure 1. Overview of 14 T-DNAs used for plant transformation drawn to scale. The Gateway destination vector pA1GWFH that served as the basis for all these constructs is shown on top. During the Gateway cloning reaction, the Gateway cassette (GW) between the two recombination sites *att*R1 and *att*R2 was replaced by the different constructs shown below, whereby the recombination sites are converted into *att*B1 and *att*B2, respectively. The 14 constructs comprise the nontagged GUS-coding sequence (GenBank accession no. AM181661; see also Supplemental Fig. S1) and the five derived mutants, the nontagged *Gu-us* recombination substrate (GenBank accession no. AM181664), and the tagged versions of these seven sequences (GenBank accession nos. AM181662 and AM181663; see also Supplemental Fig. S1). The five point mutations M4, M6, M12, M14, and M15 (Table I) are situated in the white blocked region. Elements are indicated in gray or black, which are identified on top and below the cassettes, respectively (Goderis et al., 2002; Karimi et al., 2002). RB, Right border of T-DNA; LB, left border of T-DNA; P_{35S}, CaMV 35S promoter; Pubi, ubiquitin promoter; GW, Gateway recombination cassette containing the *ccdB* gene; *att*R1, R2, B1, and B2, Gateway recombination sites; GFP, green fluorescent protein (fusion protein with GUS); ALS^R, chlorosulfuron-resistant acetolactate synthase selection gene (allele *csr1-1*); P1/HCPro, inducible turnip mosaic virus P1/HCPro cassette (not relevant for this study); T_{35S}, Tchs, and Tg7, terminators; Gus, nontagged GUS-coding sequence; CHGusS, tagged GUS-coding sequence (Supplemental Fig. S1); M, mannopine synthase promoter and enhancer elements (not relevant for this study); O, start of GUS ORF (not relevant for this study). The position of the GUS-coding region and the added tags in the CHGusS relative to the Gus constructs is indicated. When in situ recombination occurs in plants containing the recombination substrates *Gu-us* (nontagged) or *CHGu-usS* (tagged) by crossover of the central tandem repeated *u* fragment, the resulting T-DNAs encode a functional GUS-GFP protein, as indicated. The *Gu* and *us* fragments correspond to the first 1,644 and last 1,187 nucleotides of the GUS-coding sequence, respectively, coinciding with the 548 amino-terminal and 395 carboxy-terminal amino acids. The overlapping *u* fragment is 1,022 nucleotides long and the fragment separating the two *u* fragments 936 nucleotides.

because some reduced GUS activity was still observed (Fig. 2).

Histochemical Analyses of Transgenic *GUS* Plants Grown under Nonmutagenic Conditions

When plants originating from transformation with an active *GUS-GFP* translational fusion construct (Fig. 1) displayed a distinctly strong GFP signal (Fig. 4A), they also showed systemic intense blue GUS staining (Fig. 4B). On the contrary, in the absence of strong GFP fluorescence, plant sectors (especially leaves) often remained colorless after GUS staining (Fig. 4C). However, a low GFP signal did not appear to be indicative of poor and nonuniform X-glu staining because, in many such cases, an intense blue color was still observed over the entire plant.

For each of the 10 inactive missense *gus* constructs (M4, M6, M12, M14, and M15, both as tagged and nontagged variants), several T2 plant populations grown under normal conditions were histochemically stained with X-glu, whereby each T2 population originated from one primary transformant. The number of mutant GUS-encoding T-DNAs in these T2 plants was not determined, but because they were grown on selective medium, at least one was present. In 24 of 81 evaluated independent T2 populations from which between 58 and 873 plants were analyzed, mutation events restoring GUS activity could be detected by visualization of blue clonal spots (Fig. 3, see examples) and results are summarized in Figure 5A. In plants harboring the *GU-US* recombination substrate (Fig. 5, B and C), blue spots or clonal sectors were observed in 76% of all primary (T1) transformants in all 16 tested T1 populations and in 26 of 27 tested offspring T2 populations. In all tested inactive GUS plants, staining was limited to clonal reversion spots (Fig. 3) and very rarely to larger clonal sectors. This confirms that none of the inactivated *gus* mutants is able to hydrolyze X-glu and that mutations and recombinations are more likely to occur late in development, probably reflecting the increasing number of somatic cells. Occasionally,

Table I. *Overview of mutant E. coli GUS sequences*

Mutant[a]	Human[b]	*E. coli* Amino Acid[c]			First Codon[c,d]		Second Codon[c,d]		Reversion Nucleotide[c,e]			Accession No.[f]
		Position	Wild Type	Mutant	Wild Type	Mutant	Wild Type	Mutant	Position	Mutant	Revertant	
M2	451	413	E	G	AAC		GAA	G**G**A	1238	G	A	AM180563
M4	500	464	C	R	CTG		TGC	**C**GC	1390	C	T	AM180564
M6	504	468	Y	C	CGT	CGC	TAT	T**G**T	1403	G	A	AM180565
M12	587	549	W	R	GTA		TGG	**C**GG	1645	C	T	AM180566
M14	600	562	R	G	TTG	CTC	CGC	**G**GA	1684	G	A	AM180567
M15	605	567	K	E	AAC		AAG	**G**AG	1699	G	A	AM180568

[a]In total, 15 theoretical *gus* mutants were designed, six of which were actually obtained, tested, and listed here. Except for mutant M2, which was not used for further experiments in Arabidopsis, mutants have no GUS activity (Fig. 2). [b]Corresponding amino acid position of the mutagenized sites relative to human GUS (Oshima et al., 1987; GenBank accession no. M15182). The amino acids are identical to those shown for wild-type *E. coli* GUS (Schlaman et al., 1994; GenBank accession no. S69414). [c]Positions and mutations relative to the wild-type *E. coli* sequence from Schlaman et al. (1994; GenBank accession no. S69414). The length of the complete protein is 603 amino acids. [d]The first codon encodes the amino acid preceding the mutant amino acid, whereas the second codon corresponds to the mutant amino acid itself. The first codon was sometimes changed to achieve a symmetrical CpG or CpNpG sequence context for the mutant nucleotide in the second codon, without ever changing the amino acid at the first codon position. The first codon of the mutant sequence is listed only when it deviates from the wild-type sequence. The reversion nucleotide is underscored in the second codon. [e]The indicated base substitution at the reversion nucleotide (underscored in the second codon) position restores the original wild-type amino acid sequence and codon, except in mutant M14. In this mutant, the G-to-A transition in codon GGA results in codon AGA, which encodes the wild-type Arg without restoring the wild-type Arg codon CGC. In this case, also a GGA-to-CGA transversion restores the wild-type Arg residue. [f]Accession numbers of the *gus* missense mutants in the EBI and GenBank databases.

plants containing the recombination substrate stained completely blue, pointing to a recombination event very early in development or, in the case of T1 plants, possibly already in *Agrobacterium* prior to transformation.

Mutagenesis Experiments

The effect of EMS and UV-C mutagenesis experiments on GUS spot numbers is summarized in Table II (Supplemental Table S1 provides a more detailed version). In total, we used 13 T2 plant populations with a scorable spontaneous reversion frequency, of which, on average, 230 plants were analyzed for both the treated and control population (Supplemental Table S1). All five mutants, M4, M6, M12, M14, and M15, and the recombination substrate *GU-US* were represented, either tagged or nontagged. In addition, we included the previously published line $166_{G\rightarrow A}2$ (Kovalchuk et al., 2000), for which a high GUS spot frequency had been demonstrated as a result of the T:A-to-C:G transitions that restore GUS activity.

The EMS treatment clearly had a drastic effect ($P < 0.001$) on the number of point mutations, with induction factors ranging from seven (line $166_{G\rightarrow A}2$) up to 375 (T2 population TM15-1), as shown in Table II. Although less drastic, the percentage of plants in which reversion events occurred was also significantly increased. The effect of fungal growth in some of the EMS-treated plants (see Supplemental Protocol S3) is probably negligible. In UV-C-treated plants, the number of C:G-to-T:A mutations was significantly increased ($P < 0.001$) in all populations tested, generally by a factor of 5. When looking at the influence of UV-C irradiation or EMS treatment on plants harboring the recombination substrate *GU-US*, no statistically significant effects were observed. The remaining potentially mutagenic applications of heavy metals (Pb^{2+} and Cd^{2+}), heat and light stress (heat shock and growth at elevated temperatures), methyl jasmonate, and sodium salicylate did not significantly change the number of active GUS spots in any of the tested T2 populations carrying a missense *gus* or recombination substrate T-DNA (data not shown).

DISCUSSION

Currently, no system is available to score C:G-to-T:A transitions in plants, even though such events are assumed to occur frequently as a result of natural evolution or mutagenic conditions (Gentles and Karlin, 2001; Koornneef, 2002; Greene et al., 2003; Tran et al., 2005). Here, we describe the development and implementation of five different missense mutants of the *E. coli uidA* gene designed to detect such transitions in a symmetrical CpG or CpNpG sequence context. The lack of GUS activity of the encoded mutant proteins is in agreement with the reported active-site pocket of the human GUS (hGUSB; Oshima et al., 1987). Indeed, based on an alignment with the *E. coli uidA* gene, four (M6, M12, M14, and M15) of the five introduced inactivating amino acid substitutions (Table I) map to the catalytic cavity (Jain et al., 1996; Islam et al., 1999; Matsumura and Ellington, 2001). The position of mutation M6 (Y468C) corresponds to the catalytic Tyr-504 residue of hGUSB, whereas in mutants M12, M14, and M15, the altered amino acids have been implied to be involved in the catalytic process, albeit not by direct interaction with the substrate. As for the remaining inactive mutant M4, the modified Cys residue has not been reported to be critical, but its close proximity to the catalytic site and possible involvement in S-S bridge formation probably results in a profound distortion. The apparent drastic reduction in reaction kinetics of

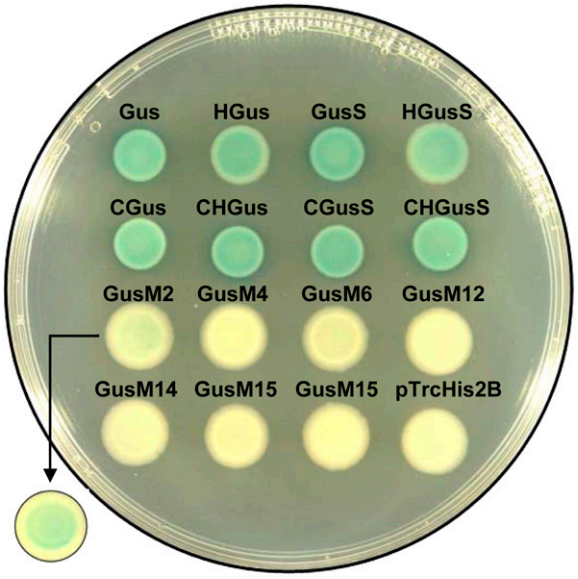

Figure 2. Visualization of GUS activity in *E. coli* GT106 cells expressing an active *GUS* sequence variant or one of the missense mutants listed in Table I. Five microliters of a glycerol stock from the bacterial strains were spotted on solid growth medium containing 100 μg/mL of the chromogenic GUS substrate X-glu, resulting in a blue precipitate when active GUS proteins are present. Proteins Gus and CHGusS (indicated above the bacterial spots) are nontagged and tagged versions of GUS (see also Fig. 1; Supplemental Fig. S1), whereby the letters C, H, and S, respectively, indicate the presence of CaMV ORF-V, the HA tag, and Strep tag II. The remaining proteins are intermediates in the cloning process, containing only one or two of these tags. Bacteria expressing mutant nontagged *gus* have been indicated by the GUS mutation as in Table I (two different clones expressing GusM15 were spotted). Bacteria harboring plasmid pTrcHis-2B (negative control) do not produce GUS proteins because this is the original expression vector used for cloning. As shown in the lower left corner, prolonged incubation of mutant M2-expressing bacteria results in light blue staining.

mutant M2 (E413G) agrees with the acid-base catalyst role of Glu-451 in hGUSB (Islam et al., 1999). Because critical amino acids are impaired, other than the targeted mutations would probably not restore the GUS activity up to a detectable level, even though we cannot exclude this possibility. Only in mutant M14, an alternative C:G-to-G:C mutation can result in the wild-type GUS amino acid as well (Table I).

In contrast to mutation-scoring strategies already reported (Puchta et al., 1995; Kovalchuk et al., 2000; Lucht et al., 2002; Leonard et al., 2003), the system presented here provides a convenient way to check the protein level of mutant GUS because the protein is produced as a GFP fusion and detectable protein tags are present at both termini of GUS in the tagged GUS plants. A good GFP signal is indicative of intense blue histochemical staining throughout functional GUS-GFP plants (Fig. 4) and hence also ensures the best conditions for visualizing GUS reversion events. A low GFP signal often correlates with poor and nonuniform X-glu staining in functional GUS-GFP plants, particularly in leaves, and can thus obscure reversion events in mutant GUS-GFP plants. The tagged constructs can be used to determine mutant GUS-GFP levels quantitatively (Supplemental Fig. S2), which is especially valuable in the CHGu-usS recombination substrate plants because *GFP* is not expressed prior to recombination (Fig. 1).

The observed GUS spot frequency in a given plant population is thus clearly not governed only by the actual mutation or recombination frequency, but also by *GUS-GFP* expression levels and the efficiency of the detection technology. The first two parameters are a function of complex interplay between the promoter sequence; the kind of point mutation or structure of the recombination substrate; the sequence context; the genomic insertion position; the copy number, locus number, and structure of the transgene; epigenetic parameters, such as the degree of methylation and chromatin condensation; the genetic background of the plants, which is also related to the ecotype; growth conditions; and experimental treatments (e.g. Meyer, 2000; Muskens et al., 2000; De Buck et al., 2001; Vaucheret et al., 2001; Hassa and Hottiger, 2005). This has important implications with respect to the analysis of mutation and recombination frequencies. First, it is of utmost importance only to look at relative frequency changes, whereby two identical plant populations are compared and only the tested parameter differs between the two. Second, despite *GUS* expression from the constitutive CaMV 35S promoter (Fig. 1), mutations or recombinations may go undetected in tissues in which this promoter is less active or when present in heavily condensed heterochromatin. Third, because reduced expression levels may obscure GUS reversion events, the GFP signal or the tags should be used to ensure comparable GUS protein levels when these are suspected to be different (e.g. for assaying the influence of symmetrical cytosine methylation).

As inferred from the number of GUS spots appearing in different missense mutant *gus* T2 populations, the spontaneous point mutation frequencies

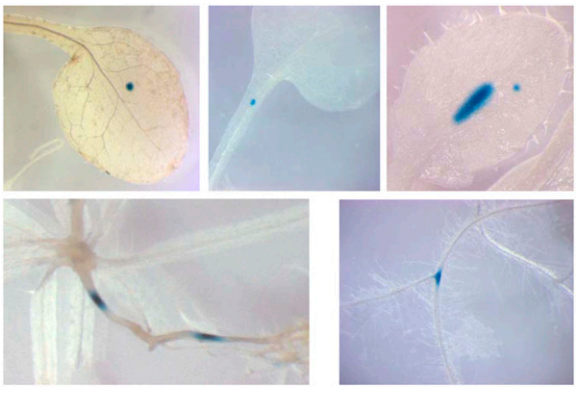

Figure 3. Visualization of GUS reversion spots after histochemical staining with X-glu in different parts of Arabidopsis plants. Top, Leaves and leaf stems; bottom, roots and hypocotyls. Images were taken under a stereomicroscope.

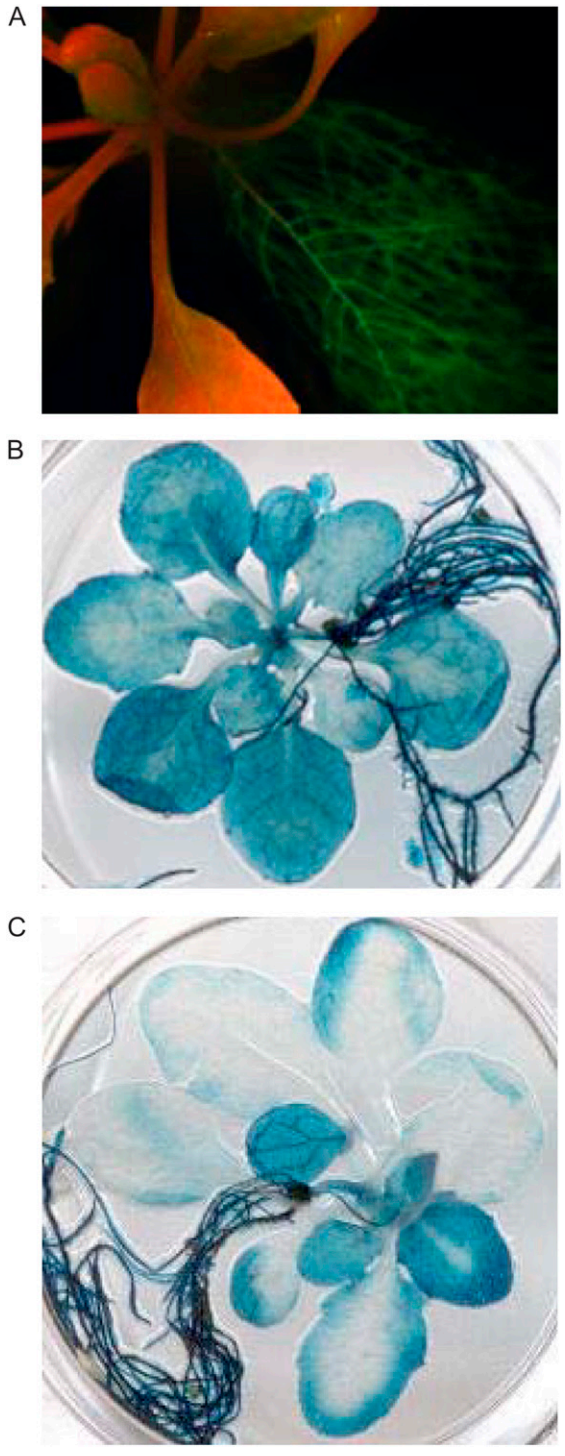

Figure 4. In situ visualization of GFP and GUS activity in Arabidopsis plants producing a translational GUS-GFP fusion protein (Fig. 1). A, GFP signals were primarily visible in roots and much less in leaves, where there is interference from the red chlorophyll background. B, Typical GUS-staining pattern in a plant with high GFP activity (as in A), expressing a functional *GUS-GFP* fusion: Blue coloring is uniform throughout the entire plant. Similar patterns are also seen in some plants without strong GFP fluorescence. C, Coloring pattern observed in many plants lacking a clear GFP signal: Leaves typically show patchy staining, whereas roots generally stain well.

are perfectly in line with those reported for different mutant GUS lines by Kovalchuk et al. (2000; Fig. 5A); also in agreement with our study, they observed that frequencies differ significantly between plant populations or plant lines even when one single-copy T-DNA was present, which clearly illustrates the above-mentioned points. The presence of a distinct GFP signal in several T2 populations, of which some with and some without spots, shows that varying expression levels alone do not account for the observed differences, meaning that the actual mutation frequencies are not identical. Also, unlinked mutations

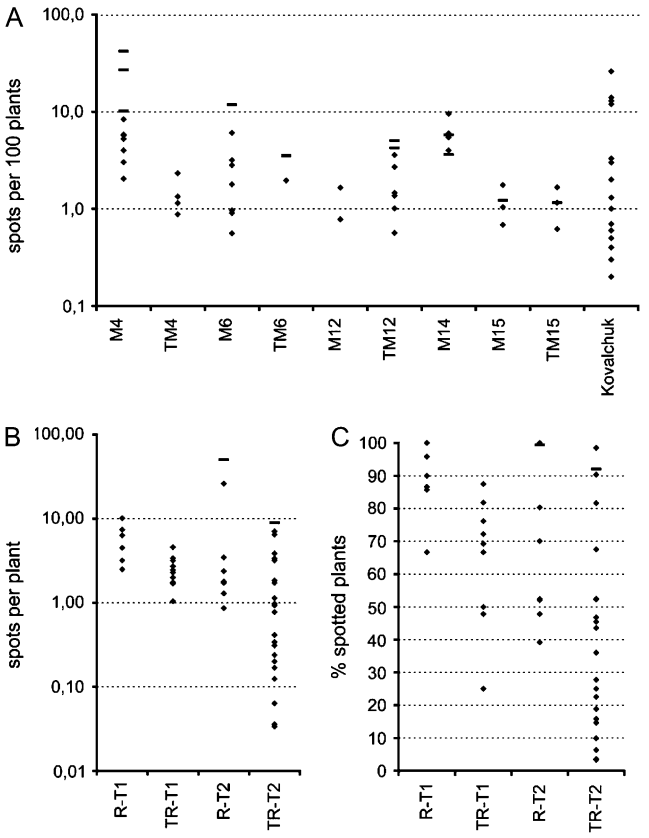

Figure 5. Number of mutation and homologous recombination events observed in several independent experiments of plants grown under nonmutagenic conditions. Each data point represents either a T1 population originating from one transformed T0 plant or a T2 population originating from a primary T1 transformant. Black diamonds and full lines refer to plants grown on solid medium and to control populations used in EMS mutagenesis experiments (partly grown in liquid medium), respectively. A, Reversion spot frequency found in T2 populations containing mutants M4, M6, M12, M14, and M15 (Table I), either as their tagged (TM) or nontagged (M) version (Fig. 1). The average number of counted mutation events per 100 plants is plotted on a logarithmic scale. As a reference, mutation frequencies above 0.1% reported for different lines by Kovalchuk et al. (2000) are shown as well. B, Average number of recombination events in T1 and T2 populations harboring the tagged (TR) or nontagged (R) recombination substrate (Fig. 1), plotted on a logarithmic scale, and expressed as a number of active GUS spots per plant. C, Percentage of plants containing recombination spots in the same experiments as B on a linear scale.

Table II. *Mutation and recombination induction by EMS and UV-C*[a]

T2[b]	Treatment	Spot Frequency Increase[c]	Spot Plant Frequency Increase[d]
M4-1°	EMS	68.44	5.10
M4-2°	EMS	25.97	4.00
M4-3°*	EMS	35.08	9.34
M6-1°	EMS	37.32	10.80
TM6-1°	EMS	134.90	27.29
TM12-1°	EMS	144.49	39.33
TM12-2°	EMS	132.65	24.01
M14-1°	EMS	318.14	29.80
M14-2	EMS	148.12	21.02
M15-1*	EMS	349.85	55.72
TM15-1°	EMS	374.64	126.81
$166_{G\rightarrow A}2$	EMS	7.42	4.81
R-1	EMS	0.65	0.99
TR-1	EMS	1.35	1.03
M4-1°	UV-C	1.86	1.84
TM6-1°	UV-C	5.37	4.98
TM12-2°	UV-C	5.95	5.17
M14-2	UV-C	4.33	4.28
M15-1*	UV-C	?	?
$166_{G\rightarrow A}2$	UV-C	3.91	3.42
R-1	UV-C	0.63	0.94
TR-1	UV-C	1.60	0.87

[a]An extended version of this table is provided as Supplemental Table S1. [b]Identification of the 13 T2 populations used and line $166_{G\rightarrow A}2$ (Kovalchuk et al., 2000). M indicates the point mutation as listed in Table I; when preceded by T it refers to a tagged construct (Fig. 1; Supplemental Fig. S1); similarly, TR and R designate the tagged and nontagged recombination substrates (Fig. 1). These indications are followed by a sequential number to differentiate between populations carrying the same T-DNA, but originating from a different primary transformant. Populations in which a *GUS* hairpin construct segregates are indicated by *. In such cases, segregation follows a single locus 3:1 ratio, as determined experimentally on phosphinothricin-containing medium. The symbol ° marks populations with a single mutant *gus* locus, as determined by segregation analysis on GLEAN. [c]Fold induction of spot frequency resulting from EMS or UV-C treatment, measured in the treated versus nontreated control population. Frequencies are measured as average number of spots per plant in the population. In T2 population M15-1, no fold induction could be determined for the UV-C treatment because spots were absent in the control population. [d]Same as C, but the frequency is measured as the fraction of plants with GUS spots in the treated versus nontreated control population.

resulting from the transformation process itself (Latham et al., 2006) may influence mutation frequencies at the *GUS* loci because these alter the plant's genetic background.

In analogy with the *GUS* constructs designed by Kovalchuk et al. (2000), we cloned the first 29 amino acids of the CaMV ORF-V upstream of the GUS-coding sequence that includes the hemagglutinin tag and Strep tag II (Fig. 1; Supplemental Fig. S1). This fragment is believed to enhance GUS protein production up to a level necessary to visualize clonal GUS spots (B. Hohn and I. Kovalchuk, personal communication). By comparing staining patterns and spontaneous GUS spot frequencies in the tagged and nontagged (in which CaMV ORF-V is not present) GUS populations of functional and mutant GUS plants, respectively, we were unable to distinguish between the presence or absence of this fragment, certainly in view of the fact that frequencies already differ largely between plant populations that do carry identical transgenes (see above and Fig. 5). However, it cannot be excluded that the protein production levels of tagged GUS would indeed be higher, which could compensate for an estimated 50% reduction of the specific activity caused by the presence of the tags.

Also in the analyzed T1 and T2 populations harboring the *GUS* recombination substrate, the spontaneous frequency at which plants with spots occur and the number of spots per plant were similar in plants containing either the tagged or the nontagged versions of *GU-US* (Fig. 5, B and C). As in the case of the *gus* missense mutants, the observed frequencies differ between populations, which can be accounted for by essentially the same phenomena. It is clear that the number of recombination events by far exceeds that of point mutations, which is in perfect agreement with earlier observations (Puchta et al., 1995; Kovalchuk et al., 2000). For example, the highest frequency of recombination spots per plant in one of the *GU-US* T2 populations is 275 times that observed for any missense mutant T2 population when considering plants grown on solid medium (Fig. 5).

Some of the frequencies were measured in T2 populations that were partly grown in liquid medium (Fig. 5) because they served as controls for EMS mutagenesis (see below). The reason why the frequencies measured in these populations were often found at the high end of the spectrum remains unclear. It might merely reflect either the different growth conditions to which plants were subjected, or, alternatively, the fact that these plants were bigger than those grown on solid medium at the time of staining, leading to enlarged and more easily detectable clonal GUS spot sectors.

Treatment of plants with EMS had a tremendous effect on the mutation frequency for the five inactive missense mutants (Table II), with fold induction levels ranging from 26 (T2 population M4-2) to 375 (T2 population TM15-1). This result clearly shows that our system functions properly. As expected, the 7-fold induction in the previously published line $166_{G\rightarrow A}2$ (Kovalchuk et al., 2000) was not nearly as spectacular because EMS primarily induces C:G-to-T:A transitions (Koornneef, 2002; Greene et al., 2003; Yoshihara et al., 2006). These can restore GUS activity in our mutants, but not in the $166_{G\rightarrow A}2$ plants designed to detect T:A-to-C:G changes. Such changes were not encountered by Yoshihara et al. (2006) as a result of EMS mutagenesis. The low increase in mutation frequency in $166_{G\rightarrow A}2$ is in agreement with the poor induction of 1.1 by treatment with methyl methanesulfonate, a related mutagen (Kovalchuk et al., 2000). We did not observe a significant effect of EMS on recombination frequencies, unlike Puchta et al. (1995), who described a 2- to 3-fold induction following

methyl methanesulfonate treatment of tobacco (*Nicotiana tabacum*) plants.

By using an indirect readout system to determine mutation frequencies, Yoshihara et al. (2006) found a frequency increase from 2.5×10^{-5} to 5.7×10^{-4} as a result of EMS mutagenesis, which corresponds to a factor of 23, in contrast with the results from our five mutants, showing induction factors between 26 and 375 (Table II). Once again, this difference illustrates that one should be very careful when interpreting mutation frequencies because they can differ tremendously, depending on the characteristics of the system used to quantify them and the manner in which the plants were grown or treated. Furthermore, results from any particular system cannot be extrapolated to the whole genome. In fact, in view of these findings, it seems rather useless even to speak of the mutation frequency of an organism because this variable depends on too many factors to be of any practical use, such as the genome position or locus, the kind of mutation looked at, and the sequence context.

From all the other mutagenesis experiments, UV-C irradiation was the only one that had any scorable effect in our missense mutated GUS plants, generally resulting in a 5-fold increase in mutation frequency (Table II). This induction level is in line with that reported by Kovalchuk et al. (2000), who observed a relative increase between 1.2 and 56, with an average of 10.3. Our results are clearly on the lower end of this spectrum, because UV-C primarily causes mutations at CT or TT sequences, which can form pyrimidine dimers, and none of our mutant nucleotides are present in this context. Thus, the simultaneous availability of several different lines is very important to assess mutation frequencies because the line of choice depends on the treatment under evaluation. Testing different lines also allows identification of the kind of mutation linked to a particular stress condition, as described for bacteria (Cupples et al., 1990). In contrast to Molinier et al. (2005), who found a 7-fold increase in recombination frequency as a result of UV-C irradiation in Arabidopsis, we did not find a scorable effect of UV-C in our *GU-US*-containing plants.

Previously, an increase in mutation and recombination frequencies has been described for plants grown in the presence of heavy metals, such as lead or cadmium ions (Kovalchuk et al., 2001b). In our T2 populations, these effects were not observed, which could be explained by either a different method of application of these metals or the inherent differences between the plants used for testing. Also in another paper by Kovalchuk et al. (2005), the effect of lead on recombination frequency was no longer seen. A heat shock of 50°C or growth at elevated temperatures and increased daylength had no measurable effect on the mutation or recombination frequencies either, even though the plants were clearly affected in their growth. Addition of methyl jasmonate or sodium salicylate to the growth medium influenced the phenotype without a scorable altered base substitution or recombination frequency. This is in contrast to the reported 1.5- to 7-fold increased recombination level in two tested Arabidopsis lines as a reaction to spraying plants with the salicylic acid analogs 2,6-dichloroisonicotinic acid and benzothiadiazole (Lucht et al., 2002). Of course, these analogs might have a different effect than that of salicylic acid also because the method of application was not the same.

CONCLUSION

We have shown that our system is a valuable addition to the nucleotide substitution scoring strategy developed by Kovalchuk et al. (2000): It increases the spectrum of mutations that can be looked for, and, as shown for *E. coli* by Cupples et al. (1990), diversity of scoring systems is the key to identifying and quantifying different possible events in various circumstances. In this respect, our system is equally usable in bacteria because all the five inactive *gus* mutants are available as bacterial expression clones. However, the major disadvantage would be the current unavailability of a positive selection system to score for GUS-positive bacteria, as can be done for the β-galactosidase setup (Cupples et al., 1990). In plants, reversion frequencies remain low, but this is inherent to the fact that one looks for one particular event in a whole genome to occur. Nevertheless, by carefully selecting plant lines with a measurable reversion frequency and good mutant GUS production levels, scoring mutation frequencies in plants is a valuable system, as long as results are not overinterpreted or extrapolated to the whole genome or other settings and organisms.

MATERIALS AND METHODS

Binary Vectors and T-DNAs

In total, 14 binary T-DNA vectors were used for *Agrobacterium*-mediated floral-dip transformation of Arabidopsis (*Arabidopsis thaliana*) plants. They were obtained with the Gateway cloning technology (Invitrogen) and their respective T-DNAs are shown in Figure 1. The detailed cloning methodology is provided as Supplemental Protocol S1 and Supplemental Table S2. In short, the functional (De Loose et al., 1995) and six different mutant GUS-coding sequences (Table I) were cloned as *Escherichia coli* expression plasmids. These were all transferred to Gateway entry vectors, both as tagged and nontagged variants, which could then be used to substitute the Gateway cassette in Gateway destination vector pA1GWFH (Fig. 1). The tagged and nontagged recombination substrates were generated as Gateway entry clones and used with the same destination vector.

Gateway destination vector pA1GWFH (Fig. 1) is based on construct p*7FWG2 (Karimi et al., 2002) to which the following elements were added: the *csr1-1* chlorosulfuron-resistant acetolactate synthase selection cassette ALS^R (Haughn et al., 1988; Goderis et al., 2002); an *Antirrhinum majus* chalcone synthase terminator sequence; and an inducible P1/HCPro expression cassette of turnip mosaic virus (Kasschau et al., 2003).

Functional Analyses of GUS Variants in *E. coli*

The tagged pTHCHGusS, nontagged pTHGus, and the six mutant pTHGus bacterial expression vectors (Table I; Supplemental Fig. S1) were transformed in *E. coli* strain GT106 (InvivoGen). This allows measurement of the GUS activity originating from the expression plasmid because GT106 cells do not produce endogenous functional GUS proteins. GUS activity was

measured qualitatively by plating bacteria on rich medium (Fig. 2) containing 100 μg/mL GUS substrate X-glu (Immunosource). For quantitative analysis, we used a fluorimetric kinetic assay with the substrate methylumbelliferyl-β-D-glucuronide, essentially as described by Breyne et al. (1993). To this end, bacteria were harvested at logarithmic growth phase and the GUS activity of a bacterial suspension was measured as units GUS per OD_{600} nm. Protein gel blots were used to confirm that the HA tag and Strep tag II were produced correctly from the constructed tagged GUS-coding sequence (Supplemental Fig. S2).

Plant Transformations and Characterization

Floral-dip transformations were carried out essentially as described by Clough and Bent (1998). Binary T-DNA vectors were electroporated in *Agrobacterium tumefaciens* C58C1Rif^R harboring plasmid pMP90 (Koncz and Schell, 1986). *Agrobacterium* clones were tested by PCR and several positive clones were pooled. Arabidopsis plants of line 4C-S5 (ecotype Columbia; Craft et al., 2005) were submerged in a bacterial suspension when they had approximately three 10-cm-high inflorescences, with flowers at various stages of development. Transformants were selected on medium containing 50 μg/L chlorosulfuron administered in the form of the herbicide GLEAN (DuPont). Plants from line 4C-S5 (Craft et al., 2005) contain a homozygous LhGR-N fusion protein expression transgene with a kanamycin resistance marker. In addition, some transformed plants harbored a single locus *GUS* hairpin cassette with a phosphinothricin resistance gene, without known zygosity, resulting in possible segregation over later generations derived from dipped plants (see Table II; Supplemental Table S1). Both the LhGR-N protein and *GUS* hairpin constructs are not relevant for this study. Segregation analysis on selective medium was carried out in some cases to check for the locus number and presence of the different transgenes.

In Situ GUS and GFP Analyses in Plants

To assess sites of functional GUS activity, in vitro-grown Arabidopsis plants were histochemically stained with X-glu (Jefferson, 1987; Jefferson et al., 1987) when they were 3 to 4 weeks old and analyzed for active GUS spot numbers with a stereomicroscope. Details are provided in Supplemental Protocol S2. Ferro- and ferricyanides (Jefferson, 1987) were excluded from the X-glu staining buffer, which promotes diffusion of the 5-bromo-4-chloro-indoxyl group prior to precipitation, which in turn can result in more visible larger spots. GFP activity was assayed visually under a stereomicroscope, using an excitation wavelength of 470 nm and a detection filter of 525 nm.

Evaluation of Mutation and Recombination Frequencies

Transgenic T2 seeds were isolated from primary transformants grown on GLEAN-containing medium obtained after floral-dip transformation. To assess the frequency of spontaneous reversion events in a given T2 population, on average approximately 150 T2 plants originating from one primary transformant were grown in vitro in the presence of GLEAN and analyzed histochemically for GUS spots. The frequency in T1 populations of four to 24 plants harboring the recombination substrate was tested as well, using the same methodology, with each T1 population originating from one transformed T0 plant. Mutation and recombination frequencies were expressed as the average number of spots observed per plant in a given T1 or T2 population. In addition, the number of plants containing spots versus the total number of plants tested was calculated.

The same procedure was used to check the influence of potential mutagenic or recombinogenic treatments (see below), even though in this case only T2 populations were analyzed and the number of plants in each experiment and control population averaged 230. Populations expressing missense mutant *GUS-GFP* all showed moderate to high GFP fluorescence. In several experiments, we included line $166_{G\rightarrow A}2$ (Kovalchuk et al., 2000) whose GUS activity is restored by a T:A-to-C:G transition mutation.

The effect of (potentially) mutagenic growth conditions on the number of active GUS spots was tested by subjecting about 2-week-old in vitro-grown seedlings to one of the following conditions (details in Supplemental Protocol S3): immersion in EMS (250 mM for 25 min); irradiation with UV-C (80 J/m^2 of 254 nm); growth in the presence of Cd^{2+} ions (4 mg/L), Pb^{2+} ions (20 mg/L), sodium salicylate (0.1 or 0.5 mM), or methyl jasmonate (100 μM); heat shock (50°C for 20 min); or heat and light stress (30°C and 18 h of light per day starting 7–10 d after sowing). All plants were stained for functional GUS spot counting approximately 4 weeks after germination. In each case, a control population that was not subjected to the indicated treatments was grown in parallel.

Data were analyzed using the statistical functions of *R* (see http://www.r-project.org). When a significant *F* value of $P < 0.05$ was obtained in a two-way ANOVA test, a multiple-comparison test between the treated and the control populations was conducted.

Accession Numbers

The following sequences were deposited at the EBI and GenBank sequence repositories: all six *gus* point mutants (accession nos. listed in Table I), the tagged and nontagged GUS-GFP fusion protein-coding sequences, and the tagged and nontagged *GUS-GFP* recombination substrates (see Fig. 1 for accession nos.).

Supplemental Data

Supplemental Figure S1. Cloning scheme of tagged *GUS* constructs.

Supplemental Figure S2. Detection of tags on protein gels.

Supplemental Table S1. Mutation and recombination induction by EMS and UV-C.

Supplemental Table S2. Mutagenesis oligonucleotides.

Supplemental Protocol S1. Cloning details.

Supplemental Protocol S2. In situ GUS and GFP analyses in plants (includes GUS-staining protocol).

Supplemental Protocol S3. Mutagenic treatments.

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