GFP Loss-of-Function Mutations in Arabidopsis thaliana

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ABSTRACT  Green fluorescent protein (GFP) and related fluorescent proteins are widely used in biological research to monitor gene expression and protein localization in living cells. The GFP chromophore is generated spontaneously in the presence of oxygen by a multi-step reaction involving cyclization of the internal tripeptide Ser65 (or Thr65)-Tyr66-Gly67, which is embedded in the center of an 11-stranded β-barrel structure. Random and site-specific mutagenesis has been used to optimize GFP fluorescence and create derivatives with novel properties. However, loss-of-function mutations that would aid in understanding GFP protein folding and chromophore formation have not been fully cataloged. Here we report a collection of ethyl methansulfonate–induced GFP loss-of-function mutations in the model plant Arabidopsis thaliana. Mutations that alter residues important for chromophore maturation, such as Arg96 and Ser205, greatly reduce or extinguish fluorescence without dramatically altering GFP protein accumulation. By contrast, other loss-of-fluorescence mutations substantially diminish the amount of GFP protein, suggesting that they compromise protein stability. Many mutations in this category generate substitutions of highly conserved glycine residues, including the following: Gly67 in the chromogenic tripeptide; Gly31, Gly33, and Gly35 in the second β-strand; and Gly20, Gly91, and Gly127 in the lids of the β-barrel scaffold. Our genetic analysis supports conclusions from structural and biochemical studies and demonstrates a critical role for multiple, highly conserved glycine residues in GFP protein stability.

GREEN FLUORESCENT PROTEIN (GFP) is a genetically encoded reporter that is intrinsically fluorescent and detectable in the absence of a substrate or cofactor (Tsien 1998; Zacharias and Tsien 2006). The chromophore of wild-type GFP, which is 238 amino acids in length, forms through autocatalytic cyclization and oxidation of the internal tripeptide Ser65-Tyr66-Gly67 of the GFP primary sequence (Zacharias and Tsien 2006; Stepanenko et al. 2013). In a commonly used variant named Thr65-GFP, Ser65 has been replaced by threonine, resulting in a six-fold increase in fluorescence through suppression of one of two absorption peaks exhibited by wild-type GFP (Heim et al. 1995; Ormö et al. 1996; Brejc et al. 1997). Determination of the crystal structures of wild-type GFP and Thr65-GFP revealed a β-barrel structure comprising 11 β-strands with lids flanking both sides and an internally positioned α-helix that harbors the chromophore (Yang et al. 1996; Ormö et al. 1996).

Various mutational strategies have been used to modify and optimize GFP. Improvements achieved in this way include increasing the fluorescence intensity by facilitating protein folding, creating different color variants, reducing the pH sensitivity, increasing thermostability, and minimizing the propensity to form dimers (Zacharias and Tsien 2006; Aliye et al. 2015). In contrast to the numerous favorable changes obtained through mutagenesis, amino acid substitutions associated with loss of fluorescence remain incompletely characterized (Yang et al. 1996; Zacharias and Tsien 2006). Although deleterious or neutral mutations are not relevant for creating novel properties or improving GFP, they nevertheless might help in understanding GFP folding and chromophore maturation (Zacharias and Tsien 2006). With this in mind, we report here a collection of GFP loss-of-function mutations retrieved in a classical genetic screen for mutants displaying diminished fluorescence of a GFP reporter gene in Arabidopsis thaliana.

KEYWORDS Arabidopsis thaliana green fluorescent protein, protein stability, protein structure, reporter gene

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MATERIALS AND METHODS

Plant line
In this study we used an Arabidopsis thaliana transgenic line in a Columbia (Col) ecotype background that is homozygous for a previously described target (T) locus encoding enhanced GFP protein (EGFP). This reporter gene is expressed primarily in shoot and root meristem regions and also in the hypocotyl (part of stem between seed leaves and root) of young seedlings (Kanno et al. 2008, 2010; Sasaki et al. 2012, 2015). EGFP was purchased from Clontech as plasmid pIRE2-EGFP. Relative to wild-type GFP, the EGFP protein contains a threonine at position 65 instead of serine and has an additional GTG (Val) codon after the initiating ATG (Met) codon to optimize translation initiation in eukaryotes. To conform to numbering of the wild-type GFP sequence (Zacharias and Tsien 2006), we consider the nucleotide and amino acids sequences of EGFP (referred to hereafter as simply GFP) without this additional Val residue throughout this article (nucleotide and amino acid sequences used are shown in Supporting Information, Figure S1). The GFP protein in the T line also has a 27 amino acid extension at the N-terminus because of the use of a cryptic promoter element upstream of the intended promoter in the transgene construct (Sasaki et al. 2015). These extra amino acids and mutations in this region are not considered in the present analysis, which focuses on loss-of-function mutations in the GFP protein coding sequence.

EMS mutagenesis
Chemical mutagenesis with the alkylating agent ETHANE methylsulfonate (EMS) was performed as described previously (Kim et al. 2006). Approximately 40,000 EMS-treated seeds from the homozygous line were grown on soil (M1 generation), grown to maturity, and allowed to self-fertilize to produce M2 seeds, which comprise the first generation (Sasaki et al. 2015). The GFP protein in the T line also has a 27 amino acid extension at the N-terminus because of the use of a cryptic promoter element upstream of the intended promoter in the transgene construct (Sasaki et al. 2015). These extra amino acids and mutations in this region are not considered in the present analysis, which focuses on loss-of-function mutations in the GFP protein coding sequence.

Western blot procedure to detect GFP protein
Approximately 100 mg of 2-wk-old seedlings of the M2 or M3 generation (grown under sterile conditions on solid MS medium) were ground in liquid nitrogen in a 1.5-mL Eppendorf tube with pellet pestles (Sigma, Z359947) to a fine powder. The powder was then resuspended in 150 μL of PEB buffer (50 mM Tris, 400 mM KC1, 2.5 mM MgCl2, 1 mM EDTA, 1 mM DTT, 0.1% Triton-X, 1X protease Inhibitor, Roche 5056489001), vortexed three times for 20 sec each (between intervals place tube back on ice), and centrifuged at 15,000 rpm at 4°C for 10 min. Then, 100 μL of supernatant was combined with 100 μL PEB buffer (without KC1) in a new 1.5-mL tube. The amount of protein was quantified using the Bradford protein assay and the solution was then stored at −20°C. From each sample, 20 μg of protein were separated by sodium dodecylsulfate (SDS) polyacrylamide gel electrophoresis (PAGE) using 12% acrylamide gels. After SDS-PAGE, proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane (Bio-Rad). The membrane was then incubated in a plastic container with blocking reagent [10% w/v skim milk in 0.02% Tween 20-TBS (TBST)] for 1 hr at room temperature with gentle rocking. GFP protein was detected by incubating the membrane with the control of the mannopine synthase promoter, which confers resistance to phosphinothricin in plants. These binary vectors were each used to transform the individual gfp mutants using the floral dip procedure (Clough and Bent 1998). Transformed seedlings were selected on solid MS medium containing either gentamicin or phosphinothricin and screened for GFP expression using a stereo fluorescence microscope. Complementation was considered successful when the level of visible GFP fluorescence exceeded that observed in the original gfp mutant. In particular, we observed successful complementation of mutations that were retrieved only once (T65I, G67S, R96C, G20D, G91S, C70Y, and V112M) (Table 1), confirming that the observed loss-of-fluorescence phenotypes were due to the respective amino acid substitutions in the GFP protein.

Sequencing the GFP gene
Genomic DNA was purified using a DNeasy Plant Mini Kit (QIAGEN) according to the manufacturer’s protocol. To amplify the GFP gene, PCR was performed using Ex Taq (Takara) and the following primers: GFP-R1, TATCTGGGAACACTACACAC; and GFP-F1, GACAGAACTAATTATACCAG. The PCR conditions were as follows: 94°C for 2 min followed by 35 cycles of 94°C for 10 sec, 58°C for 20 sec, and 72°C for 90 sec, followed by a final extension for 7 min. The PCR fragments were purified using a GEL/PCR Purification Kit (Favorgen Corp., Taiwan) according to the manufacturer’s instruction followed by sequencing.

Detection of GFP mRNA by RT-PCR
Total RNA was extracted from approximately 2-wk-old seedlings (grown under sterile conditions on solid MS medium) using a Plant Total RNA Miniprep Purification Kit (GeneMark) followed by RQ1 DNase (Promega) treatment, according to the manufacturer’s instructions. cDNA was synthesized using Transcriptor First Strand cDNA

Complementation tests
The original transgene construct encoding GFP that was used to obtain the T line (Kanno et al. 2008) was introduced either into binary vector pPZP221, which encodes resistance to gentamicin in plants (Hajdukiewicz et al. 1994), or into pCambia binary vector 1300 (http://www.cambia.org/daisy/cambia/585/day/585_minimal_selection), in which the 35S promoter-hygromycin phosphotransferase gene was replaced by a phosphinothricin acetyltransferase gene under the control of the mannopine synthase promoter, which confers resistance to phosphinothricin in plants. These binary vectors were each used to transform the individual gfp mutants using the floral dip procedure (Clough and Bent 1998). Transformed seedlings were selected on solid MS medium containing either gentamicin or phosphinothricin and screened for GFP expression using a stereo fluorescence microscope. Complementation was considered successful when the level of visible GFP fluorescence exceeded that observed in the original gfp mutant. In particular, we observed successful complementation of mutations that were retrieved only once (T65I, G67S, R96C, G20D, G91S, C70Y, and V112M) (Table 1), confirming that the observed loss-of-fluorescence phenotypes were due to the respective amino acid substitutions in the GFP protein.
Table 1 List of gfp loss-of-function mutations identified in this screen

| Amino Acid Change | Fluorescence<sup>a</sup> | Protein Accumulation<sup>b</sup> | Highly Conserved<sup>c</sup> | Reference<sup>e</sup> |
|-------------------|---------------------------|-------------------------------|-----------------------------|----------------------|
| **PTC<sup>d</sup>** |                           |                               |                             |                      |
| W 57<sup>+</sup> (G170A) | —                          | —                             |                             | This study (batch 16); Sun et al. 2012 |
| W 57<sup>+</sup> (G171A) | —                          | —                             |                             | This study (batch 4) |
| Q 69<sup>+</sup> | —                          | —                             | —                           | Sasaki et al. 2012 |
| Q 94<sup>+</sup> | —                          | —                             | Sterile                     | This study (batch 32) |
| Q 157<sup>+</sup> | —                          | —                             |                             | Sasaki et al. 2012 |
| Q 177<sup>+</sup> | —                          | —                             | —                           | Sasaki et al. 2012 |
| Q 183<sup>+</sup> | —                          | —                             | —                           | Sasaki et al. 2012 |
| Q 184<sup>+</sup> | —                          | —                             | Sterile                     | Sasaki et al. 2012 |
| **Chromophore** |                           |                               |                             |                      |
| T 65 I            | Weak to moderate           | Weak                          | No                          | This study (batch 35) |
| G 67 S            | No                         | Weak                          | Yes                         | This study (batch 22), Sasaki et al. 2012 |
| G 67 D            | Weak                       | Weak                          | Yes                         |                      |
| **Chromophore maturation** |                     |                               |                             |                      |
| T 62 I            | Negligible                 | No                            | No                          | Sasaki et al. 2012; Sun et al. 2012 |
| R 96 C            | No                         | WT                            | Yes                         | This study (batch 31) |
| R 96 H            | No                         | WT                            | Yes                         | This study (batch 12); Sasaki et al. 2012 |
| S 205 F           | Weak                       | WT                            | No                          | Sasaki et al. 2012; Sun et al. 2012 |
| E 222 K           | Weak to moderate           | Weak                          | Yes                         | This study (batches 17, 18) |
| **Protein stability—lid residues** |                     |                               |                             |                      |
| G 20 D            | No                         | No                            | No                          | This study (batch 38) |
| G 91 S            | No                         | No                            | Yes                         | This study (batch 9) |
| G 91 D            | No                         | No                            | Yes                         | This study (batch 3); Sasaki et al. 2012 |
| G 127 D           | Negligible                 | No                            | Yes                         | This study (batches 6, 17); Sun et al. 2012 |
| **Protein stability—unknown function** |                     |                               |                             |                      |
| G 31 D            | Negligible                 | No                            | Yes                         | This study (batches 12, 31); Sasaki et al. 2012; Sun et al. 2012 |
| G 33 D            | Negligible                 | No                            | Yes                         | This study (batches 3, 28, 44) |
| G 35 S            | Weak                       | Very weak                     | Yes                         | This study (batches 23, 27, 28) |
| G 40 D            | Negligible                 | No                            | Yes                         | This study (batch 34); Sasaki et al. 2012 |
| P 56 L            | Negligible                 | No                            | No                          | This study (batch 25) |
| C 70 Y            | Weak                       | Very weak                     | No                          | This study (batch 40) |
| A 110 V           | Negligible                 | No                            | No                          | This study (batch 47); Sun et al. 2012 |
| V 112 M           | Weak                       | Weak                          | No                          | This study (batch 15) |

Amino acid residues (column 1) are numbered according to wild-type GFP. In the present study and two previous studies (Sun et al. 2012 and Sasaki et al. 2012), a modified GFP containing an additional GTG (Val) codon in position 2 after the ATG (Met) start codon was used to optimize translation initiation in eukaryotes. However, for consistency of numbering with wild-type GFP, we have treated in this article the Val in position 2 as 1a (Zacharias and Tsien 2006). Therefore, amino acid numbers in the previous studies (Sun et al. 2012, Sasaki et al. 2012) are one higher compared to wild-type GFP. Two nucleotide changes (G170A and G171A) convert W57 into a PTC (Figure S1).

<sup>a</sup> In column 2, "no" indicates no visible fluorescence under fluorescence microscope; "negligible" indicates a very faint tinge of fluorescence in the hypocotyl; "weak" indicates barely visible fluorescence in hypocotyl; "weak to moderate" indicates visible fluorescence in the hypocotyl but not shoot meristem. The wild-type T line displays fluorescence in shoot and root meristem regions and in the hypocotyl in young seedlings (Figure S2).

<sup>b</sup> In column 3, the presence of a band on a Western blot probed with an antibody to GFP is indicated as "very weak," "weak," or wild-type (WT). "No" designates no visible band on the Western blot (Figure 2). "Sterile" indicates the mutant produced few or no seeds, presumably due to second site mutations unrelated to the mutation in GFP gene, and thus progeny seedlings were not available for Western blot analysis.

<sup>c</sup> Highly conserved residues are ones among the 23 identified as the most conserved in an analysis of sequences of 250 GFP-related proteins (Ong et al. 2011).

<sup>d</sup> Mutants containing premature stop codons (PTC) did not show any GFP fluorescence or protein band on the Western blot (indicated by dashes in columns 2 and 3).

<sup>e</sup> Batch number refers to one of 54 batches of pooled M2 seeds collected from the approximately 40,000 M1 plants grown from mutagenized seed.
Synthesis Kit (Roche) using an oligonucleotide d(T) primer and 1 μg of total RNA. To detect the GFP transcripts, 1 μl of cDNA was used for RT-PCR with the following primers: forward primer, 5’-CAGCTACGGAGCTAACCATG-3’ and reverse primer, 5’-TTTACCTTGACTGCTCGTCG-3’. The PCR conditions were as follows: 94° for 2 min followed by 27 cycles of 94° for 10 sec, 58° for 20 sec, and 72° for 1 min, and finally 72° for 7 min.

Data availability
Seeds of mutants listed in Table 1 are available upon request.

RESULTS AND DISCUSSION
A transgenic Arabidopsis line (T line) that is homozygous for a stably expressed enhanced GFP (EGFP) reporter gene (referred to hereafter as simply GFP) (Kanno et al. 2008, 2010) was used in this screen. Seeds of the T line were treated with EMS and M2 seedlings, which represent the first generation when a recessive mutation can be homozygous, were visualized under a fluorescence stereo microscope to screen for GFP-negative or very weak phenotypes. DNA was isolated from these plants and the GFP gene was sequenced to identify possible loss-of-function mutations. From this screen we retrieved 20 unique gfp mutants, which were combined for further analysis with eight additional unique, uncharacterized gfp mutants recovered in a separate screen based on the same GFP reporter gene (Sasaki et al. 2012) (Table 1).

EMS induces almost exclusively C-to-T changes resulting in C/G to T/A transition mutations (Kim et al. 2006), thus limiting our investigation to this type of mutation. A previous analysis of the structure of more than 250 GFP-like proteins in the Protein Databank identified 23 highly conserved amino acids (Ong et al. 2011), 21 of which are present in the GFP protein used in this study (Figure S1). Many of the most highly conserved residues are situated at the lids on the top and bottom of the β-barrel and at bends between the β-sheets (Ong et al. 2011; Zimmer et al. 2014). The purpose of the lids is unclear, but the low variability displayed by these regions in GFP-like proteins suggests an important role in folding, stability, or other aspect of GFP function (Ong et al. 2011; Stepanenko et al. 2013; Zimmer et al. 2014). Our screen retrieved mutations resulting in substitutions of 11 of the 21 highly conserved amino acids present in the GFP protein used in this study (Table 1). As described below, the codons of some highly conserved amino acids in the GFP protein are not susceptible to mutagenesis by EMS. The mutations we identified can be placed into five categories and considered in the context of the 23 most highly conserved amino acids in GFP-like proteins (Ong et al. 2011).

Premature termination codons
Removing only seven amino acids from the C terminus and one from the N terminus destroys GFP function (Yang et al. 1996). In the GFP coding sequence (Figure S1), premature termination codons (PTC) can be induced by EMS only at codons for glutamine and tryptophan (Table S1). The fact that we retrieved mutations affecting the single tryptophan in the GFP protein (Trp57) and six of eight glutamine residues (only Gln80 and Gln204 were not mutated in our screens; Figure S1) suggests that the combined screens approached saturation. The truncations resulting from these PTCs far exceed those tolerated at the N and C termini of GFP, which is consistent with the lack of GFP protein accumulation (Figure 2A) and visible fluorescence in PTC mutant seedlings (Table 1).

Chromophore
From the chromogenic tripeptide (Thr65-Tyr66-Gly67), we recovered substitutions of Thr65 (T65I) and Gly67 (G67S and G67D) (Table 1, Figure 1). Because the only EMS-induced mutation of tyrosine codons produces a silent mutation (Table S1), it was not possible to obtain substitutions of highly conserved Tyr66 in our screen. Gly67 is also among the 23 highly conserved amino acids in GFP-related proteins (Ong et al. 2011). Gly67 mutant plants did not show visible GFP fluorescence (Table 1, Figure S2), although they did accumulate detectable, albeit reduced, levels of GFP protein (Figure 2A), suggesting that the G67 substitutions compromise to some extent GFP protein stability. The lack of visible fluorescence despite detectable protein accumulation is consistent with previous results showing that substituting Gly67 with any other amino acid prevents chromophore formation (Lemay et al. 2008; Stepanenko et al. 2013). Glycine is unique in having H as a side chain, in contrast to other amino acids that have a carbon, endowing glycine with exceptional conformational flexibility (Betts and Russell, 2003). Thus, glycine is the only amino acid at position 67 that permits formation of a kinked internal α-helix, which places Gly67 close to the residue at position 65 for nucleophilic attack during chromophore synthesis (Lemay et al. 2008; Stepanenko et al. 2013).

Position 65 is considered the most variable of the chromogenic tripeptide (Stepanenko et al. 2013). Accordingly, the T65I substitution we identified reduced but did not eliminate visible fluorescence in mutant seedlings (Figure S2). Similar to the G67 substitutions, the amount of GFP protein in the T65I mutant was lowered but not abolished (Figure 2A), implying reduced GFP protein stability in addition to probable inhibition of chromophore formation in this mutant.

Chromophore maturation
Chromophore synthesis requires a network of polar interactions involving amino acids of the chromogenic tripeptide as well as several closely apposed amino acids, including four that we identified in our screen: Glu222 and Arg96, which are among the 23 most highly conserved amino acids in GFP-related proteins (Ong et al. 2011), as well as Thr62 and Ser205 (Yang et al. 1996; Ormö et al. 1996). In particular, Arg96, which acts as an electrostatic catalyst, and Glu222, which behaves as a base catalyst, are considered critical features for chromophore synthesis (Stepanenko et al. 2013).

The E222K substitution we identified did not eliminate fluorescence but resulted in weak to moderate GFP fluorescence in seedlings (Table 1, Figure S2). In accordance with this finding, an E222G mutant has been reported previously to fluorescence, indicating that Glu222 is not absolutely required for chromophore formation (Lemay et al. 2008). Only low levels of GFP protein were observed in the E222K mutant (Figure 2A), suggesting an impact of the E222K substitution on GFP protein stability. In addition to a catalytic role, Glu222 has been reported to have a stabilizing function by contributing to the rigidity of the chromophore cavity (Royant and Noirclerc-Savoye 2011; Stepanenko et al. 2013).

The S205F mutant displayed weak to moderate GFP fluorescence in seedlings (Table S1) despite the accumulation of nearly wild-type levels of GFP protein (Figure 2A). These findings are consistent with the role of Ser205 in the hydrogen bonding network required for chromophore maturation (Yang et al. 1996).

The most extreme losses of fluorescence (Figure S2) accompanied by nearly wild-type levels of GFP protein (Figure 2A) were observed with the Arg96 substitutions: R96C and R96H (Table 1). Although a R96C substitution has been reported previously to fluoresce (Lemay et al. 2008), our findings with this substitution as well as R96H suggest that Arg96 is essential for chromophore formation and hence GFP fluorescence in plants. Arg96 forms a hydrogen bond with T62
Our data suggest an essential role in GFP protein stability.

Highly conserved glycine residues and GFP protein stability

There are 22 glycine residues in the GFP protein (Figure S1). Along with substitutions of Gly67 in the chromogenic tripeptide, loss-of-function substitutions were recovered for seven additional glycines, all of which are among the 23 most highly conserved amino acids in GFP-related proteins (Ong et al. 2011) (Table 1). The roles of these glycine residues have so far been unclear (Stepanenko et al. 2013), but our data suggest an essential role in GFP protein stability.

We recovered substitutions in three highly conserved "lid" residues in our screen: Gly91 is situated at the lid of the N and C termini, which are in close proximity at the same end of the barrel (Figure 1), whereas Gly20 and Gly127 are located on the opposite lid, which is referred to as the "top" of the barrel (Ong et al. 2011; Zimmer et al. 2014). Consistent with the fact that glycines can reside in parts of proteins that are unable to accommodate other amino acids, such as tight turns in structures (Betts and Russell, 2003), these three lid residues as well as Gly40, also identified in our screen, are all present in the vicinity of bends representing transitions from \(\beta\)-strands to loops in the folding pattern of GFP (Figure 1) (Zimmer et al. 2014).

Additional essential glycines identified in the screen include Gly31, Gly33, and Gly35, which are located on the second \(\beta\)-strand of the 11 \(\beta\)-strand barrel (Figure 1). These three glycines are the only conserved residues residing on \(\beta\)-strands that are not involved in chromophore formation and their function is uncertain (Ong et al. 2011; Stepanenko et al. 2013). With the exception of G35S, which showed very weak fluorescence in seedlings (Figure S2) and very low levels of GFP protein (Figure 2A), all of the glycine substitutions resulted in a lack of both visible fluorescence (Table 1) and detectable GFP protein (Figure 2A) despite normal transcription of \(gfp\) mRNA (Figure 2B). Our results implicate these highly conserved glycine residues in facilitating GFP protein folding and stability, which is consistent with a previous computational analysis of the structures of GFP and related proteins (Zimmer et al. 2014).

Nonconserved residues contributing to GFP protein folding or stability

Four loss-of-function mutations in residues that are not among the most highly conserved amino acids of GFP-related proteins were identified in our screen (Table 1). P56L, A110V, and V112M substitutions are likely to represent residues important for folding or structural stability because GFP protein did not accumulate to detectable levels in the respective mutants (Figure 2A). Pro56 is located at the beginning of the internal \(\alpha\)-helix containing the chromogenic tripeptide (Figure 1) and, together with several other proline residues, is thought to be important for maintaining kinks in the \(\alpha\)-helical backbone, which are necessary for chromophore synthesis (Stepanenko et al. 2013). Ala110 and Val112 are not at transitions in the secondary structure (Figure 1), and they are not among the 23 most highly conserved amino acid residues (Table 1) (Ong et al. 2011). The details of their contributions to GFP protein folding and stability thus remain to be clarified.

A C70Y substitution also significantly decreased accumulation of GFP protein (Figure 2A), resulting in substantially reduced visible fluorescence in seedlings (Figure S2). Cys70 is relatively close to the chromophore and to a transition between the internal \(\alpha\)-helix and a loop (Figure 1). In contrast to the C70Y loss-of-function substitution, a C70V substitution was found to improve folding properties of

Figure 1 Overall GFP fold and positions of amino acid substitutions leading to loss of fluorescence. A schematic depiction of the overall fold of Thr65-GFP protein (Yang et al. 1996) is redrawn here. The vertical arrows indicate the 11 \(\beta\) strands of the \(\beta\)-barrel structure. Amino acid residue numbers at the base and tips of the arrows indicate the beginning and ends of secondary structural elements. The chromogenic tripeptide (Thr65-Tyr66-Gly67) is positioned on an internal \(\alpha\)-helix (diagonal cylinder) extending from amino acids 56 to 72. Amino acid substitutions identified in our screen that lead to losses of fluorescence are indicated. Solid red outlines denote substitutions causing defects in chromophore formation without substantial reductions in GFP protein accumulation. Dotted red outlines indicate substitutions resulting in lowered levels of GFP protein accumulation relative to wild-type. For the remaining substitutions, no GFP protein was detected by Western blotting under the conditions used. Lid residues at the N and C termini (G91 and G127) and the opposite side (G20), which is referred to as the "top" of the barrel (Zimmer et al. 2014), are highlighted in blue.
a GFP variant (Zapata-Hommer and Griesbeck 2003). The different effects of the two mutations suggest that different substitutions of Cys70 can have either positive or negative effects on GFP folding and stability.

Highly conserved residues not identified in this genetic screen

We recovered loss-of-function substitutions in 11 of the 21 highly conserved residues found in the GFP protein used in this study (Ong et al. 2011) (Table 1, Figure S1). The remaining 10 highly conserved residues include Tyr66 of the chromogenic tripeptide, Phe27, Phe130, Leu53, and Ile136 (Figure S1). As described above, EMS-induced mutation of tyrosine codons produce only a silent change, and the same holds for the codons of phenylalanine, leucine, and isoleucine (Table S1). Therefore, EMS-induced mutagenesis cannot be used to probe the contributions of these highly conserved amino acids to GFP fluorescence and stability.

Assuming that conservation implies an essential function, it is not clear why we did not recover loss-of-function mutations in the final five highly conserved residues: Val55, Asp102, Gly104, Gly134, and Pro196 (Figure S1) (Ong et al. 2011). The codons of these amino acids are in principle targets of EMS-induced point mutations that result in amino acid substitutions (Table S1). In particular, the failure to recover substitutions of G104 and G134 is unusual, because mutations altering seven other highly conserved glycines were retrieved in the screen, in most cases more than once (Table 1). These results may indicate that our screen is not yet saturated or that substitutions in these highly conserved residues do not lead to losses of GFP fluorescence that are detectable by our visual screening procedure.

Summary

We identified a collection of GFP loss-of-function mutations that provide information about amino acids important for chromophore maturation and GFP protein stability. The mutations we identified result in substitutions of 11 of the 21 most conserved amino acids in the GFP protein used in this study as well as in seven less conserved amino acids. Mutations leading to substitutions of highly conserved Arg96 required for chromophore formation appear to substantially decrease or eliminate GFP fluorescence by impairing chromophore formation without dramatically affecting protein stability. By contrast, other mutations result in amino acid substitutions that apparently compromise protein stability and accumulation, hence leading to visible reductions in fluorescence. These substitutions affect the absolutely conserved Gly67 of the chromogenic tripeptide, seven highly conserved glycines in the lids and secondary structural transitions of the β-barrel structure (Gly20, Gly40, Gly91, Gly127) and in the second β-strand (Gly31, Gly33, Gly35), and four non-conserved residues not previously implicated in GFP protein fluorescence.
stability (Pro56, Cys70, Ala110, Val112). We also identified substitutions of amino acids involved in chromophore maturation (Thr62 and highly conserved Glu222) that are likely to influence GFP protein stability in addition to impairing fluorescence, presumably because of unsuccessful chromophore formation. These genetic findings support results from biochemical and structural analyses and contribute to a fuller understanding of amino acids, particularly numerous highly conserved glycine residues with previously unknown roles, that are essential for the function and stability of the GFP protein in a higher eukaryotic organism.

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