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Biochemical Analysis Leads to Improved Orthogonal Bioluminescent Tools

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Abstract

Engineered luciferase-luciferin pairs have expanded the number of cellular targets that can be visualized in tandem. While light production relies on selective processing of synthetic luciferins by mutant luciferases, little is known about the origin of selectivity. The development of new and improved pairs requires a better understanding of the structure-function relationship of bioluminescent probes. In this work, we report a biochemical approach to assessing and optimizing two popular bioluminescent pairs: Cashew/D-luc and Pecan/4′-BrLuc. Single mutants derived from Cashew and Pecan revealed key residues for selectivity and thermal stability. Stability was further improved through a rational addition of beneficial residues. In addition to providing increased stability, the known stabilizing mutations surprisingly also improved selectivity. The resultant improved pair of luciferases are >100-fold selective for their respective substrates and highly thermally stable. Collectively, this work highlights the importance of mechanistic insight for improving bioluminescent pairs and provides significantly improved Cashew and Pecan enzymes which should be immediately suitable for multi-component imaging applications.

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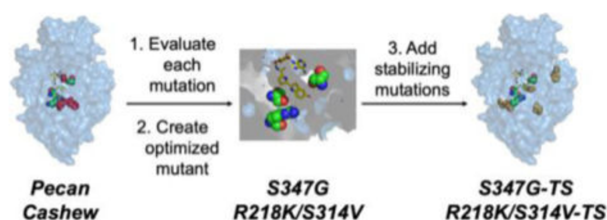
Graphical Abstract



Substrate selectivity and thermostability of mutant luciferases were analyzed using a biochemical approach. Mutants derived from two popular luciferases, Pecan and Cashew, were made and assessed to find key residues for selectivity. The mutants were optimized by layering thermal stabilizing mutations. These mutants showed increased substrate preference and activity at higher temperatures. These new enzyme-substrate pairs provide a blueprint for engineering optimized imaging probes.

Keywords

bioluminescence; biochemical characterization; luciferase; luciferin; thermostability

Introduction

Bioluminescence is a valuable imaging tool for monitoring biological processes in complex living organisms.[1] This technology relies on the oxidation of a small molecule (luciferin) that is catalyzed by an enzyme (luciferase), resulting in a photon of light. The protein firefly luciferase (Fluc) and the small molecule D-luciferin (D-luc), both derived from the North American firefly, constitute one of the most popular luciferase-luciferin pairs.[2] Fluc/D-luc has been harnessed for visualizing cell proliferation and migration in vivo, among numerous other processes.[3] While commonly used in imaging applications, Fluc/D-luc has largely been limited to monitoring one target at a time. A more detailed understanding of complex cellular events requires additional bioluminescent probes for multicomponent imaging.

Toward this end, we and others have combined chemical synthesis of D-luc analogs with directed evolution of luciferases to create orthogonal luciferase-luciferin pairs.[4] One of the most promising pairs described to date uses D-luc and 4′-BrLuc, luciferins that differ by the presence or absence of a single bromine atom (Figure 1A). The Fluc mutants Cashew (R218K), mutant 92 (R218K, M249L, S314T, G316S) and mutant 93 (R218K, M249L, S314V) preferentially emit light with D-luc, while Pecan (F243M, S347G) preferentially emits light with 4′-BrLuc (Figure 1B).[5] The mutants in these pairs out-perform most engineered bioluminescent probes due to their overall brightness, enabling tracking of smaller numbers of cells, and their ability to resolve their respective substrates to visualize two different cell populations in vitro.[5b] Additionally, it is one of a small number of multicomponent luciferase imaging systems shown to enable rapid multicomponent imaging in mice.[5a] While these enzymes represent a notable pair in the field, they still could be optimized further for key biochemical properties.[6]

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To date, the majority of biochemical studies regarding mutations and their functional impact have focused on color[7] and thermal stability.[8] These studies have been instrumental in both understanding the structure-function relationships of mutations and enabling the rational design of improved probes for applications such as in vitro dual reporter assays.[9] Recently, several thoughtful studies have comparatively analyzed combinations of luciferases and luciferins, enabling for an important comparison for scientists choosing a bioimaging tool.[6, 10] However, these studies did not examine the role of individual amino acids on the origin of unnatural substrate recognition and do not seek to improve existing tools. While there have been some noteworthy studies on how individual mutations influence modified luciferin recognition,[11] the role of active site residues in modified luciferin substrate selectivity for Fluc is poorly understood. Few studies have systematically examined how specific mutations enable recognition of one modified luciferin substrate over another.

Interestingly, the mutations found in Pecan, Cashew, mutant 92, and mutant 93 are located at six distinct amino acid positions; these positions are mutated in at least 27 published mutant luciferases with demonstrated altered modified luciferin recognition (Table S1). However, because these mutations have largely been found in the context of additional mutations, the individual contributions of these amino acids are not known despite their frequent recurrence. The roles of amino acid mutations are further complicated by potential interactions between these mutations; 13 of these 27 identified enzymes possess concurrent mutations in at least two of these six positions. A deeper understanding of the function of these amino acid positions and their interplay will facilitate the development of improved versions of Cashew and Pecan, have a broad impact on optimization of current luciferase mutants, and enable discovery of future mutant luciferases.

Here, we took a biochemical approach to uncovering the origin of substrate selectivity among Cashew and Pecan as a model orthogonal pair, with an end goal of creating improved mutants. We identified critical amino acid mutations for selectivity towards D-luc and 4′-BrLuc. We then used this information to create mutants of Pecan and Cashew with improved stability and/or selectivity. Ultimately, we created versions of Cashew and Pecan that are both thermally stable and show >100-fold selectivity for their respective substrates, a substantive improvement over the original pair. The resulting mutant luciferases should find immediate use in multicomponent imaging, enable future optimization studies, and add to our growing understanding of modified luciferin recognition.

Results

Few mutations are beneficial for selective D-luc processing in Cashew and related mutants

Previous studies identified several mutant luciferases that could selectively process D-luc and 4′-BrLuc. To understand the mutational origins of selectivity, and begin optimizing these mutants, we created and evaluated a panel of mutant luciferases. We focused our attention on Cashew (R218K), mutant 92 (R218K, M249L, S314T, G316S), mutant 93 (R218K, M249L, S314V), and Pecan (F243M, S347G). To understand the role of each mutation on function, we systematically constructed Fluc mutants bearing only a single mutation derived from these previously isolated luciferases. The enzymes were then

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expressed in bacteria, the cells were lysed, and the lysate was assessed for function. We assayed these enzymes in lysate to observe the ability of luciferases to generate signal in a complex biological environment while minimizing the impact of bacterial cell penetrance by the luciferins.

First, we examined all mutations derived from the three D-luc preferring mutant enzymes (92, 93, Cashew). To understand selectivity, an equal volume of a single lysate from each mutant was incubated with either D-luc or 4′-BrLuc and light emission was measured. The selectivity is determined by the ratio of these two values, allowing for normalization of protein expression variance. Only one of the single amino acid changes, R218K, possessed a statistically significant increase in preference for D-luc over 4’Br-luc relative to Fluc (Figure 2A). Interestingly, R218K is more selective for the substrate than either 92 or 93, indicating that the additional mutations in each enzyme likely diminish selectivity, and that the enzyme may be improved by removing some mutations. All other mutations show either a nonsignificant change in substrate preference (M249L, S314V) or a decrease in preference for D-luc (S314T, G316S).

To begin to understand the origin of increased selectivity, we assessed the total light output on each individual compound. Mutants 92 and 93 both show >10-fold decrease in light emitted with D-luc, and >100-fold decrease in light emitted with 4′-BrLuc. In contrast, R218K shows a much smaller loss in brightness when using D-luc, and a >10-fold loss in brightness using 4′-BrLuc (Figure S2 and S3). These data suggest that mutations besides R218K may be decreasing the D-luc brightness, diminishing the efficacy of 92 and 93. Observing the light emitted by each individual mutant shows that G316S, S314T, and to a lesser extent S314V all appear to decrease the light emitted by D-luc while having a smaller to no effect on light emitted using 4′-BrLuc (Figure S2 and S3). Collectively, these data demonstrate that R218K improves selectivity by decreasing signal when using 4’-BrLuc, and that the brightness of 92 and 93 may be improved by removing mutations.

Considering that only R218K appears to contribute to selectivity, we wondered if the other amino acid changes may have been selected for other contributions to enzyme function. To evaluate thermal stability, supernatant from cultures expressing each mutant was heat challenged by incubating at 40 °C and subsequently measured for bioluminescence with D-luc or 4′-BrLuc at room temperature. The emission values from the samples were then compared to a control sample from the same lysate that did not undergo heat treatment, normalizing for protein expression variance. The majority of mutants produced significantly lower levels of light after treatment at 40 °C than the native enzyme (Figure 2B) demonstrating that most of the mutations destabilize the enzyme. Notably, while R218K improved substrate selectivity, it dramatically destabilized the enzyme. Both M249L and G316S significantly decreased the thermal stability as well. In contrast to the other mutations, S314T and S314V exhibited improved stability over both their respective parent mutant and Fluc. This result suggested that increased hydrophobicity at S314 may help to stabilize the luciferase enzyme at elevated temperatures. Thus, it is possible that mutations at S314 were co-selected with R218K due to their ability to offset some of the stability loss of R218K. It also suggests that stability may be improved through removal of several of the original mutations.

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To optimize these enzymes, as well as to understand how these mutations interact, we combined the beneficial mutations to create R218K/S314T and R218K/S314V mutants. Both double mutants showed selectivity for D-luc that was similar to 92 and 93 (Figure 3A). Importantly, R218K/S314V showed an additional significant increase in thermal stability (Figure 3B) relative to R218K and its parent luciferase, mutant 93. Further, R218K/S314(T/V) showed improved light output relative to their respective parent enzymes, 92 and 93, again suggesting that the additional mutations were detrimental for function (Figure S4 and Figure S5). These results suggest that these mutations can be combined to improve stability without dramatically perturbing selectivity. Considering its increased stability with only small changes to selectivity and light output, R218K/S314V is a promising improved variant of mutants 92, 93, and Cashew for use in multicomponent experiments, especially when stability is important for the experimental design.

Pecan-derived mutation confers 4′-BrLuc selectivity and stability

Next, we sought to determine how the mutations in Pecan influenced selectivity for 4′-BrLuc and overall thermostability. Single mutants were evaluated as described above. While F243M preferred D-luc with selectivity values similar to Fluc, S347G showed a preference for 4′-BrLuc similar to Pecan (Figure 4A). When examining the emission output for each compound, D-luc showed diminished light output when incubated with Pecan and S347G but did not show a significant change with F243M (Figure S6). However, the emission values of 4′-BrLuc with F243M and S347G were very similar to Fluc emission (Figure S6). Thus, it appears that only S347G contributes to selectivity for 4′-BrLuc; this is driven by diminished luminescence with D-luc.

We also assessed the thermal stability of each mutant enzyme. Both Pecan and F243M exhibited reduced light output at elevated temperatures compared to Fluc (Figure 4B). Gratifyingly, 4′-BrLuc showed increased emission after heating with S347G compared to Pecan and Fluc, demonstrating that the S347G mutant is important for selectivity without destabilizing the enzyme. Thus, Pecan can be improved by removing F243M and S347G is a promising alternative to Pecan. Once again, removing mutations led to an enzyme with improved properties.

Adding thermally stabilizing mutations to lead luciferase mutants increases stability and selectivity

To further improve these mutant enzymes, we sought to improve the thermostability of the mutants since increases in Fluc thermostability have been shown to improve cellular studies and longitudinal imaging.[12] Additionally, increased stability could ease purification and crystallization efforts.[13] Prior work from Branchini and coworkers identified a thermostable luciferase comprised of five mutations (T214A, A215L, I232A, F295L, and E354K, abbreviated as TS from this point forward, Figure S7) via random mutagenesis.[14] Notably, the TS mutations were found to increase thermostability without impacting other properties such as activity and emission wavelength[14b, 15] and were successfully incorporated into other engineered luciferases, resulting in improved stability without deleterious effects.[16]

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Taking inspiration from these studies, we sought to incorporate the stabilizing mutations into our lead minimized mutants; thus, we created R218K/S314V-TS and S347G-TS, as well as a control Fluc-TS. The substrate selectivity and thermostability of each mutant were determined as previously described. Both enzymes were significantly stabilized relative to their parent enzymes. All the TS mutants showed no activity loss upon heating, while Fluc, R218K/S314V, and S347G lose 60% - 80% of their activity upon heating (Figure 5A). In preliminary experiments, we also observed that enzymes with TS mutations added show increased stability at longer time points and at higher temperatures (Figure S8). These enzymes all appear to be substantially more stable after addition of the TS mutations.

The selectivity assay showed that R218K/S314V-TS possesses D-luc selectivity that is similar to R218K/S314V in cell lysate (Figure 5B). Thus, the gains in stability for R218K/S314V-TS did not come with a concomitant loss in selectivity. Surprisingly, addition of the TS mutations dramatically improves selectivity of S347G in cell lysate; while Pecan is ~3-fold selective for 4′-BrLuc, S347G-TS is ~200-fold selective (Figure 5B). The improvement in substrate preference can be attributed to lower reactivity in the presence of D-luc with little impact on 4′-BrLuc use (Figure S9). While not anticipated, this improvement in selectivity represents a significant improvement in Pecan; the enzyme now possesses a >100-fold preference for 4′-BrLuc. Interestingly, a similar shift does not occur for Fluc-TS or R218K/S314V-TS, indicating that one of the TS mutations likely interacts synergistically with S347G, but not with R218K or S314V.

To understand which TS mutation leads to this improvement in stability in S347G, we reverted each of the five TS mutations back to wildtype in a S347G-TS background. Reversion of L295F led to a substantial loss in overall activity on both substrates making its contribution to selectivity challenging to assess. Of the other four mutations, only one reversion mutation (L215A) showed selectivity similar to S347G (Figure S10). These data strongly indicate that A215L is largely responsible for the synergistic improvement in selectivity seen for S347G-TS; this represents a novel interaction that dramatically improves selectivity for 4′-BrLuc in the presence of S347G.

Discussion

Towards a biochemical understanding of luciferin selectivity

While the parent luciferases that were studied here had many mutations, and many of these mutations have been independently isolated in other selective luciferases, our study suggests that only a few appear to be important for selectivity. Namely, S347G and A215L increase preference for 4′-BrLuc and R218K improves preference for D-luc. Notably, these three amino acids are proximal in space, and likely interact with one another directly or through water molecules that are present in the active site. S347 lies within 5Å of the 4′ position of the luciferin; thus, it is perhaps unsurprising that mutation to the smaller glycine would increase preference for 4′-BrLuc, a larger substrate. Indeed, mutations to S347G/A have been frequently found in luciferases that prefer 4′ modified luciferins, further supporting this steric argument (Table S1). To understand this further, we used Rosetta to calculate a lowest energy structure for S347G and FLuc with both an analog of D-luc and an analog

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of 4’-Brluc. Our models appear to confirm that there is a steric clash that is relieved by mutating to the smaller glycine (Figure S11).

Less obvious though is that R218K, which is also within 5Å of the 4′ position of the luciferin, has the opposite effect. This is somewhat surprising in that R218K has been largely associated with increasing substrate recognition of larger substrates (Table S1). However, here, it appears that the opposite is the case; mutation to a smaller amino acid side chain leads to increased discrimination against the larger modified substrate. Interestingly, the larger steric modifications that appear to be facilitated by R218K mutations are on the 7′ modified luciferins and aminoluciferins, which are modified at the 6′ position; R218K is, in known crystal structures, present on the opposite face of the luciferin and in close proximity to the 4′ position of the luciferin. Based on this, an explanation beyond steric interactions must be invoked since the ability of R218K to recognize larger substrates appears to be highly substrate dependent.

To understand this phenomenon, we used Rosetta to model the lowest energy structures of R218K bound to analogs of either D-luc or 4’-Brluc. In the lowest scoring Rosetta models, when bound to D-luc, the ζ-nitrogen on R218K and the γ-oxygen of S347 face the 4’ position on D-luc and are observed to be within hydrogen bonding distance (2.8 Å) (Figure S12A). However, when binding 4’-Brluc, these side chains move substantially to accommodate the bromine atom in the 4’ position. The ζ-nitrogen on R218K and the γ-oxygen of S347 are still observed within hydrogen bonding distance (2.7 Å) in the lowest scoring model, but the rotamer of R218K is much higher in energy. Namely, the Rosetta scores of R218K bound to 4’-Brluc and D-luc are 8.43 REU and −0.222 REU, respectively (Figure S12B). While these models are speculative and will require careful experimentation to confirm, they point to a subtle change in interactions between 218 and 347 after mutation to R218K, suggesting that the interplay between these interacting amino acids, and possible active site rearrangements near the 4’ position of the luciferin, may explain the substrate selectivity of mutants possessing R218K.

It is surprising that the F243M, M249L, G316S mutations do not contribute to changes in substrate specificity of the luciferin analogs in any detectable way since these mutations have been found in many luciferases with altered substrate specificity (Table S1). With the exception of F243 mutations, which have been identified twice, the other three positions have been found in eight or more unique identified mutants (Table S1). However, at least here, removing these mutations improved the overall light output of the enzyme in lysate. It is possible that these mutations may impact a property that was not directly examined here, such as protein expression or protease stability; further *in vitro* studies with purified enzymes would be necessary to fully understand the potential contributions to these factors. However, the fact that the total light emitted increased or did not change after removing these mutations suggests that these mutations are not likely to dramatically positively impact these properties or others that would increase the effective cellular protein concentration.

Considering that removing the mutations improved Cashew and Pecan, it is worth examining whether removing these mutations from other luciferases may help improve activity as well. For example, recent studies have identified mutant G4,[17] which selectively processes a

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pi-extended luciferin, and mutant 12, which selectively processes a naphthylamino luciferin.[18] Both of these mutants have mutations at either M249 and/or G316. Additionally, Pecan (F243M, S347G) has been recently shown to efficiently process a coumarin luciferin that emits in the far red.[19] Overall, our study here strongly suggests that these and other luciferases may also be improved through careful analysis and removal of mutants as some recurring mutations in Fluc may not necessarily contribute to function.

Improved luciferases and implications for luciferase engineering

Here, we have taken a luciferase pair which represents one of the most promising in its class, and we have systematically improved the pair. By first removing detrimental mutations and then adding thermally stabilizing mutations, we have generated a pair of luciferases which are highly stable and highly selective. The selectivity that was serendipitously acquired is particularly noteworthy; there are few known engineered pairs that originate from a common enzyme that possess 100-fold selectivity for both mutants in the pair. To our knowledge, this has only been achieved with Akaluc/Akalumine and Fluc/D-luc. [20]

It is even more remarkable that the only difference in the luciferin substrates is the presence of a single bromine atom. This strongly demonstrates that very small changes in luciferin structure can, through the use of protein engineering, lead to large changes in selectivity. Many recent orthogonal engineering efforts focus on highly structurally perturbed scaffolds.[17–20] However, these engineered systems often do not emit very much light, and, if they do, it often requires many rounds of evolution to obtain suitable signal. Here, we have continued to develop a system with only a small chemical perturbation through judicious design. Perhaps because the luciferin structure is minimally perturbed, the parent enzymes generate more light than most engineered luciferase-luciferin pairs, which is why the previously unoptimized version of this pair has been frequently used in animal studies. We do not observe large losses of light with the further engineering that we have taken on here. These results further highlight the value of engineering luciferase enzymes for luciferin analogs with only small structural perturbations. Importantly, these two strategies are not mutually exclusive; these engineered enzymes can be used in tandem with luciferases engineered for larger luciferins in increasingly highly multiplexed approaches. Indeed, this has already occurred in some studies with Cashew and Pecan,[21] and it is expected that the further optimized enzymes described here will directly improve these systems.

While the layering of thermally stabilizing mutations has been used to improve brighter mutants and color altered mutants, this is, to our knowledge, the first example of adding them to substrate selective mutants. We were surprised to see that they also improved selectivity, in this case. These data have two critical implications. First, it suggests that these mutations from past studies are an untapped reservoir that may *both* improve stability and, on occasion, improve selectivity as well. This suggests that these might be fruitful positions to diversify in future library design. Second, it suggests that there might be a reservoir of synergistic mutations, which could be a good starting point for engineering efforts for future enzymes. Synergistic mutations are, by their nature, highly path dependent; the order of mutagenesis and selection pressures can and will lead to different outcomes. Using thermally stabilized enzymes as a starting enzyme for selections, rather than adding

them at the end, might unlock additional mutations and improvements in function that would otherwise go undiscovered. Indeed, this effect has been observed in other enzyme systems,[22] but has not been attempted with luciferases, to the best of our knowledge.

Conclusion

Engineered luciferase-luciferin pairs are essential tools for multicomponent imaging. As the number of engineered orthogonal pairs grow, the origin of substrate selectivity remains poorly understood, and there is still a need to continue to improve these pairs beyond their initial discovery. In this work, we sought to characterize and improve a leading luciferase pair: Cashew and Pecan. Point mutant analysis revealed that most of the mutations in Cashew and Pecan do not contribute to selectivity; only R218K is important for D-luc recognition, while only S347G contributes to selectivity towards 4′-BrLuc. While one mutation appears to improve thermal stability (S314V), removal of the remainder of the mutations improved function. We further improved the enzyme by adding previously discovered mutations that improve thermostability; the resulting enzymes show significantly improved stability and, serendipitously, improved selectivity in the case of Pecan due to the A215L mutation. This work yields a substantially improved Cashew and Pecan pair and provides key biochemical data that can be implemented to further improve thermostability and selectivity of other luciferases. Future applications of these improved probes could be expanded for longitudinal imaging studies and structure analysis.

Experimental section

Reagents

All reagents purchased from commercial suppliers were of analytical grade and used without further purification. 4’-BrLuc was prepared and used as previously described.[23]

General biological materials

Unless otherwise specified, materials (buffer salts, dNTPs, etc.) were purchased from Fisher Scientific. All PCR reactions were analyzed using gel electrophoresis with 1% agarose gels and either ethidium bromide or GelRed (Biotium) staining. Chemically competent *E. coli* cells (DH5α, Top10 or BL21, New England Biolabs) were used for transformations. Bacteria were grown in Luria-Bertani (LB, Miller) media containing 1X kanamycin A (KAN, 40 μg/mL) or ampicillin (AMP, 40 μg/mL). Luciferin aliquots (10–50 mM in 100 mM phosphate buffer, pH 7.8) were prepared and stored at −80 °C.

General bioluminescence imaging

All assays were performed in black 96-well plates (Greiner Bio One). Bacterial assays were performed using a BioTek H4 Synergy plate reader (BioTek, Vermont, USA). Autogain was performed for each image acquired. Measurements were acquired in either duplicate or triplicate, and the data were analyzed using Microsoft Excel or GraphPad Prism (version 7.0f for Macintosh, GraphPad Software).

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Stability and selectivity assays with mutant luciferases

A portion of BL21(DE3) pLysS (New England BioLabs) bacterial cells containing individual luciferase mutants were streaked on LB Agar plates embedded with 1X KAN. Starter cultures were prepared by inoculating LB media (5 mL) containing 1X KAN (40 μg/mL) with a single colony from the plate. The cultures were incubated at 37 °C with shaking overnight. A portion of each culture (150 μL) was then used to inoculate new LB media (5 mL) containing 1X KAN (40 μg/mL). Cells were grown to mid-log phase (OD_{600} = 0.8–1.0). The cultures were then induced with IPTG (0.5 mM, Gold Biotechnology) and incubated at 23 °C with shaking for 18 h. The cells were pelleted at 3500 rpm for 10 min and resuspended in 600 μL of lysis buffer (25 mM Tris•HCl, 0.5% Triton-X, 1% glycerol, 1 mg/mL lysozyme, pH 7.4). During the 20 min incubation period, ATP (3 mM) and luciferin (D-luc and 4'-BrLuc, 450 μM) working stocks were prepped and added to 96-well black plates (25 μL each). The lysed cells were spun down at 1,000 × g for 2 min. The supernatants of each luciferase were split into two PCR tubes (55 μL) and labeled as "unheated" or "heated." The heated samples were incubated in a thermal cycler for 2 min at 40 °C. The unheated samples were untreated. Both unheated and heated supernatants (25 μL) were added to the plate (150 μM luciferin and 1 mM ATP final concentration) and shaken for 5 s. The entire plate was then incubated for 5 min at 37 °C, and then chilled at 4 °C for 10 min. The plate was then shaken again for 5 s and the light output was measured. Plates were imaged and analyzed as described above.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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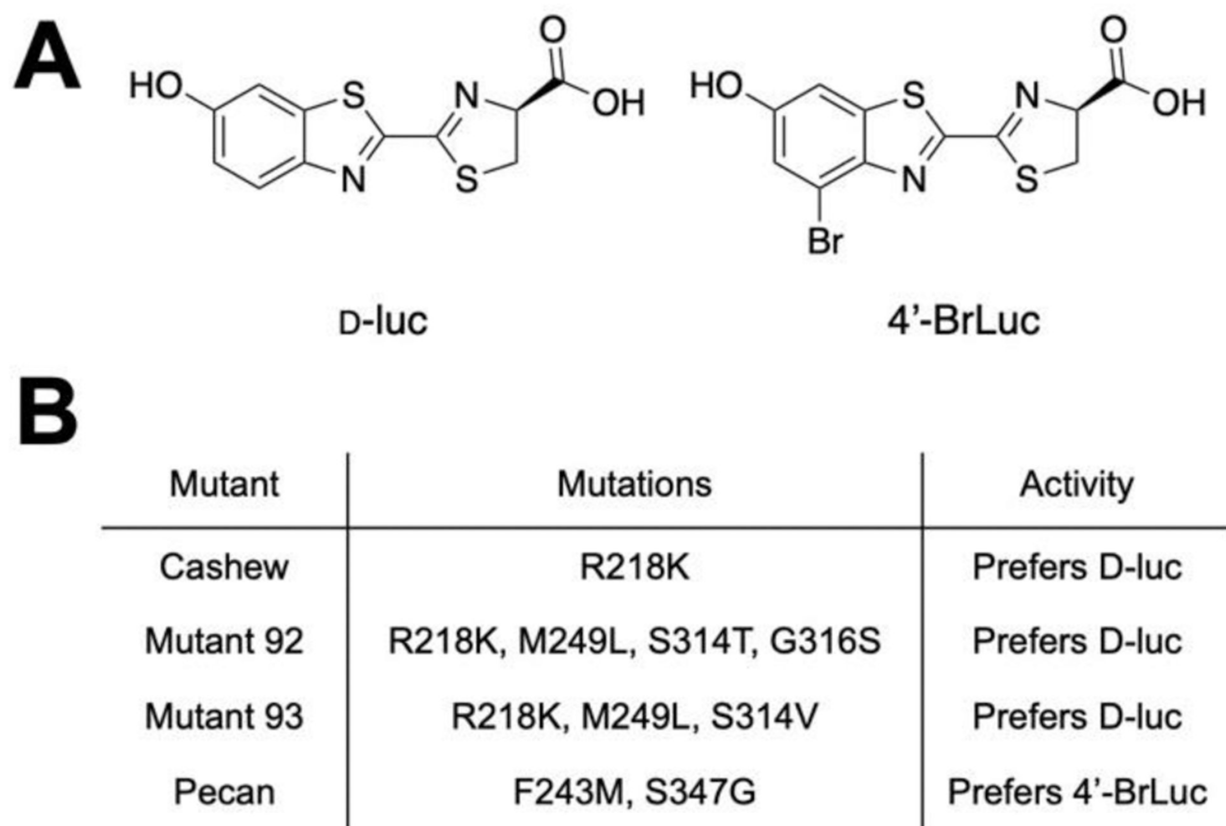

Mutant	Mutations	Activity
Cashew	R218K	Prefers D-luc
Mutant 92	R218K, M249L, S314T, G316S	Prefers D-luc
Mutant 93	R218K, M249L, S314V	Prefers D-luc
Pecan	F243M, S347G	Prefers 4’-BrLuc

Figure 1.
(A) Structures of luciferins used in this study. (B) Mutant enzymes previously identified as candidates for use in multicomponent imaging.

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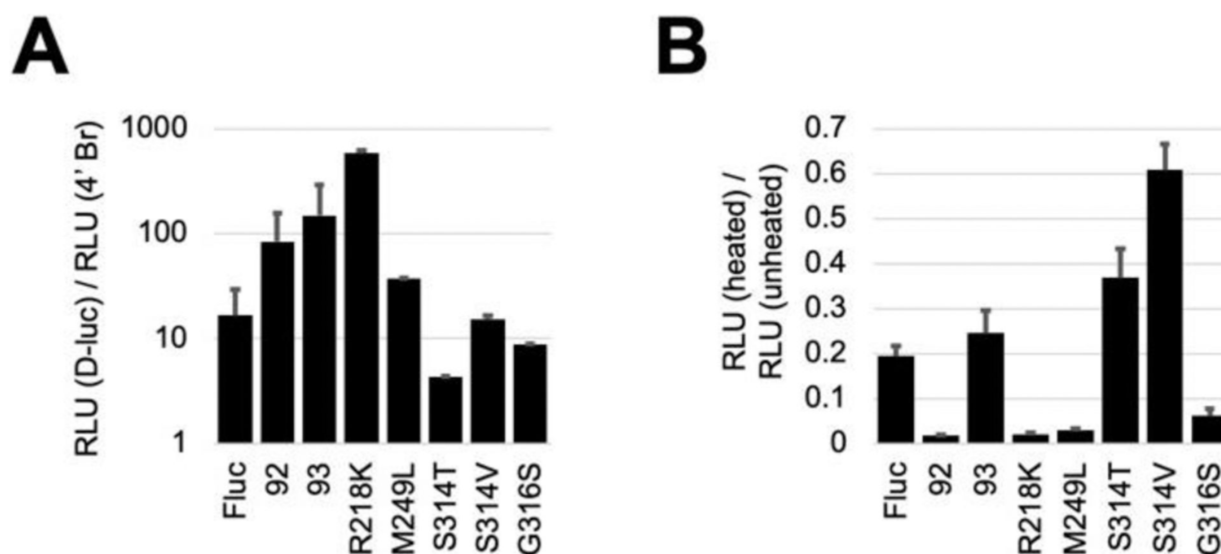

Figure 2.

Activity of luciferase mutants selective for D-luc. (A) Selectivity of luciferases. Bacterial cells expressing each mutant were lysed and the supernatant was incubated with either D-luc or 4’-BrLuc (150 μM) at 37 °C. Emission values were plotted as the ratio of D-luc activity over 4′-BrLuc light output. (B) Thermostability of luciferases. Bacterial cells expressing each mutant were lysed and a portion of the supernatant was preheated to 40 °C. The supernatant was then incubated with D-luc (150 μM) at 37 °C. Emission values were plotted as the ratio of the pre-heated sample light output over the samples at 37 °C. For all experiments, error bars represent the standard error of the mean for $n = 3$ samples.

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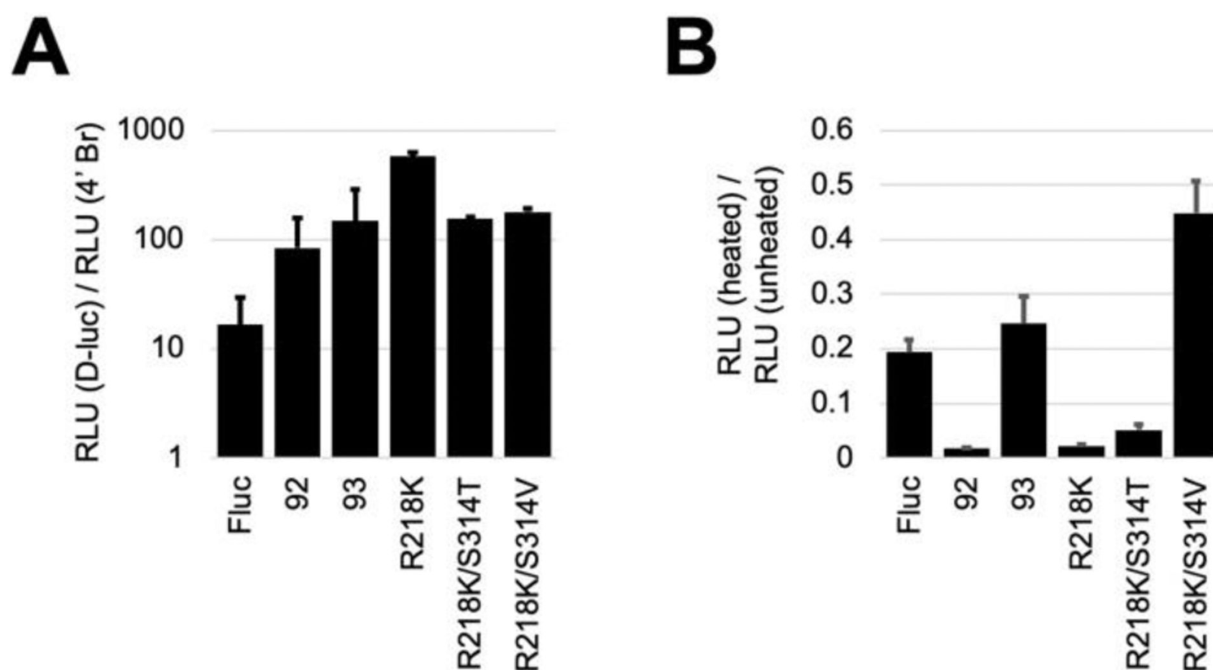

Figure 3.

Activity of improved mutants selective for D-luc. (A) Selectivity of R218K/S314T and R218K/S314V as well as their related mutants. Bacterial cells expressing each mutant were lysed and the supernatant was incubated with either D-luc or 4′-BrLuc (150 μM) at 37 °C. Emission values were plotted as the ratio of D-luc activity over 4′-BrLuc light output. (B) Thermostability of R218K/S314T and R218K/S314V as well as their related mutants with D-luc. Bacterial cells expressing each mutant were lysed and a portion of the supernatant was preheated to 40 °C. The supernatant was then incubated with D-luc (150 μM) at 37 °C. Emission values were plotted as the ratio of the pre-heated sample light output over the samples at 37 °C. For all experiments, error bars represent the standard error of the mean for n = 3 samples.

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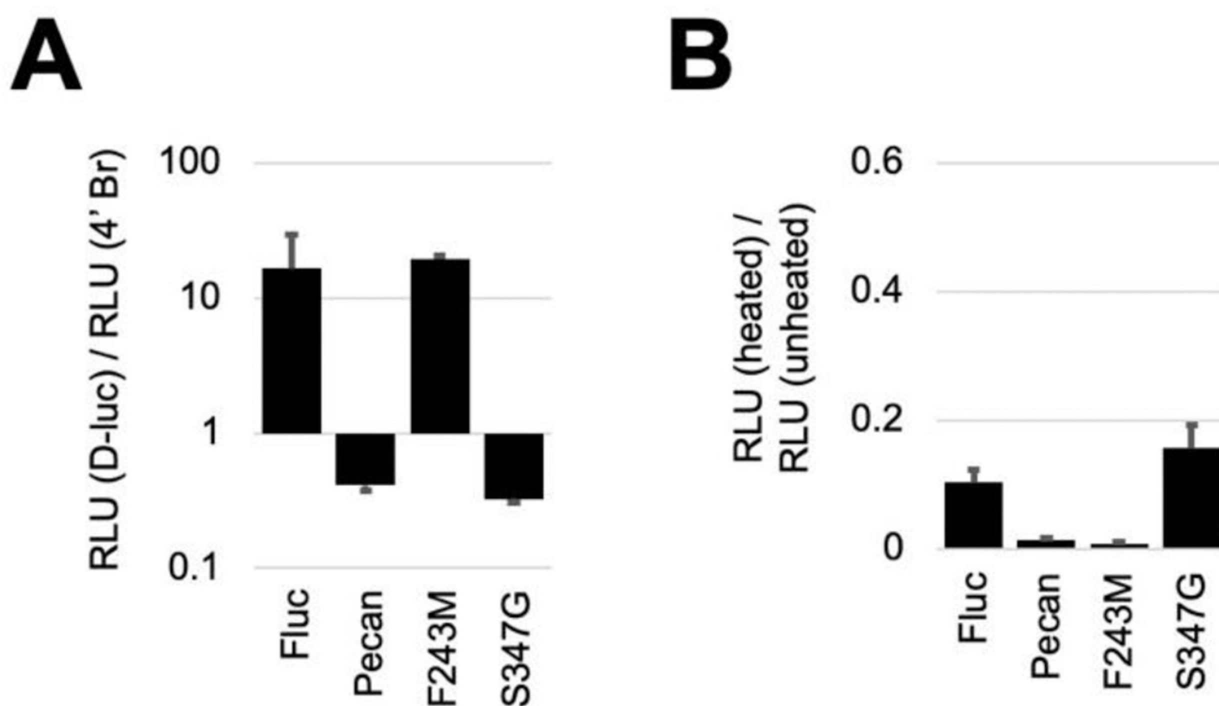

Figure 4.

Activity of Pecan and related mutants. (A) Selectivity of Pecan and its single mutants. Bacterial cells expressing each mutant were lysed and the supernatant was incubated with either D-luc or 4′-BrLuc (150 μM) at 37 °C. Emission values were plotted as the ratio of D-luc activity over 4′-BrLuc light output. (B) Thermostability of Pecan and its point mutants with 4′-BrLuc. Bacterial cells expressing each mutant were lysed and a portion of the supernatant was preheated to 40 °C. The supernatant was then incubated with either D-luc or 4′-BrLuc (150 μM) at 37 °C. Emission values were plotted as the ratio of the pre-heated sample light output over the samples at 37 °C. For all experiments, error bars represent the standard error of the mean for n = 3 samples.

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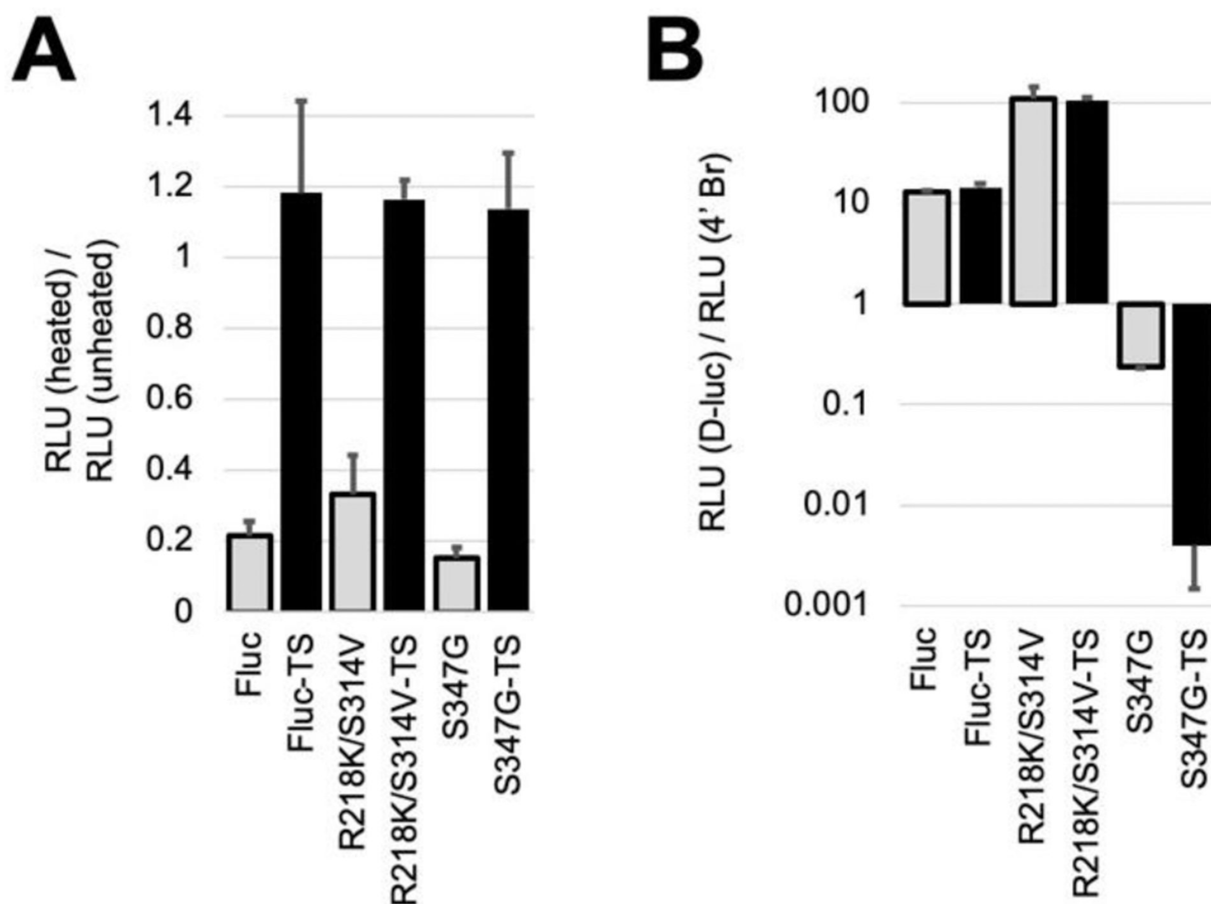

Figure 5.

TS mutants show improved stability and selectivity. (A) Thermostability of mutants selective for D-luc. Additional stabilizing mutations improved thermostability without diminishing selectivity. (A) Thermostability of mutants before (gray) and after (black) adding TS mutations. Bacterial cells expressing each mutant were lysed and a portion of the supernatant was preheated to 40 °C. The supernatant was then incubated with D-luc or 4′-BrLuc (150 μM) at 37 °C. (B) Selectivity of mutants before (gray) and after (black) adding TS mutations. Bacterial cells expressing each mutant were lysed and the supernatant was incubated with either D-luc or 4′-BrLuc (150 μM) at 37 °C. Emission values were plotted as the ratio of D-luc activity over 4′-BrLuc light output. For all experiments, error bars represent the standard error of the mean for n = 3 samples.