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ACS Synthetic Biology

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CRAGE-Duet Facilitates Modular Assembly of Biological Systems for Studying Plant–Microbe Interactions

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Cite This: https://dx.doi.org/10.1021/acssynbio.0c00280

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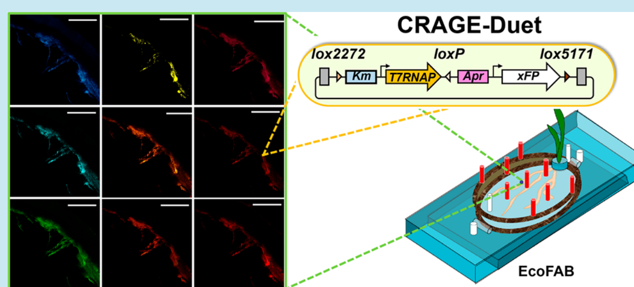
ABSTRACT: Developing sustainable agricultural practices will require increasing our understanding of plant–microbe interactions. To study these interactions, new genetic tools for manipulating nonmodel microbes will be needed. To help meet this need, we recently reported development of chassis-independent recombinase-assisted genome engineering (CRAGE). CRAGE relies on cassette exchange between two pairs of mutually exclusive *lox* sites and allows direct, single-step chromosomal integration of large, complex gene constructs into diverse bacterial species. We then extended CRAGE by introducing a third mutually exclusive *lox* site, creating CRAGE-Duet, which allows modular integration of two constructs. CRAGE-Duet offers advantages over CRAGE, especially when a cumbersome recloning step is required to build single-integration constructs. To demonstrate the utility of CRAGE-Duet, we created a set of strains from the plant-growth-promoting rhizobacterium *Pseudomonas simiae* WCS417r that expressed various fluorescence marker genes. We visualized these strains simultaneously under a confocal microscope, demonstrating the usefulness of CRAGE-Duet for creating biological systems to study plant–microbe interactions.



KEYWORDS: *bacterial strain engineering, genome engineering, genome editing, CRAGE, Cre-lox recombination, fluorescent protein*

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Understanding plant–microbe interactions is important for developing sustainable agricultural practices. To study these interactions, robust tools for genetic and genome engineering of nonmodel rhizobacteria are needed. Although the standard genetic engineering tool has been plasmid systems, their genetic instability, along with the phenotypic noise of natural population dynamics, has limited their usefulness.[1,2] Transposon-based approaches can overcome some issues associated with plasmid systems. However, random integration of transposons can negatively affect microbes' physiology and colonization capabilities,[3] so strains engineered using transposons must be carefully assessed before use.

These limitations can be avoided by integrating synthetic constructs directly into a predetermined chromosomal location. However, for many nonmodel bacteria, genome-level engineering has been cumbersome. To make it more efficient, we previously demonstrated recombinase-assisted genome engineering (RAGE), a technology for use with *Escherichia coli*.[1,2] We then modified and upgraded RAGE so it is chassis-independent, creating CRAGE.[4,5] CRAGE enables genome-level engineering of diverse bacterial species across multiple phyla, allowing researchers to avoid use of unstable plasmids and overcome phenotypic noise.

CRAGE begins with integration of a transposon containing a landing pad (LP) into recipient bacterial genomes. The LP comprises the *cre* recombinase gene flanked by two mutually exclusive lox sites. The target genes, flanked by the same set of lox sites, are introduced to the recipient strains through conjugation and inserted into the LP using cassette exchange catalyzed by Cre recombinase.[4] Using CRAGE, we previously demonstrated single-step integration of up to 60 kb of DNA fragments directly into the chromosomes of about 30 diverse bacterial species in the phyla of *Proteobacteria* and *Actinobacteria*.[4] After simple counter-selection, the integration efficiency was nearly 100%. Efficiency was not affected by the size of the DNA fragments. We expressed nine secondary metabolite biosynthetic gene clusters (BGCs) in 24 heterologous chassis using CRAGE. This approach enabled rapid activation and characterization of these BGCs.[4]

Received: May 26, 2020
Published: July 28, 2020

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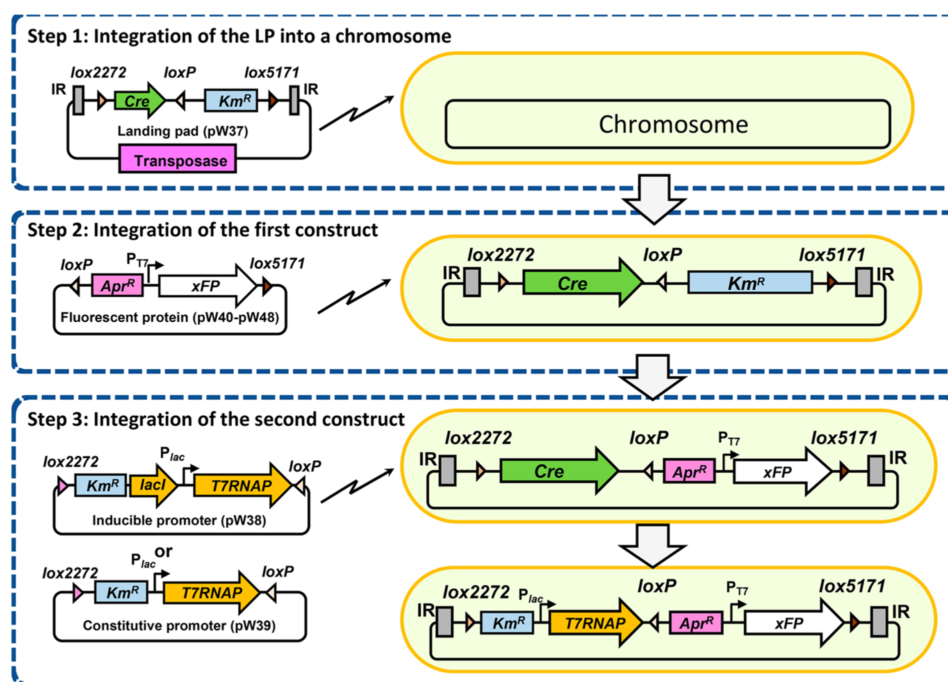

Figure 1. The CRAGE-Duet system. Step 1: The pW37 plasmid carries the LP containing three mutually exclusive lox sites, *lox2272*, *loxP*, and *lox5171*. This plasmid is conjugated from a donor strain into a chromosome of the recipient strain, mediated through transposon-based integration. Step 2: The first accessary plasmid containing the targeting genes flanked by *loxP* and *lox5171* (pW40-pW48) is conjugated into the recipient and incorporated into the first integration site, catalyzed by Cre recombinase. Step 3: The second accessary plasmid coding the inducible (pW38) or constitutive (pW39) promoter driving the expression of T7RNAP and flanked by *lox2272* and *loxP* is integrated into the second integration site, catalyzed by Cre recombinase. We used *E. coli* BW29427 as a conjugal donor strain.

We upgraded CRAGE to CRAGE-Duet by introducing a third mutually exclusive lox site (*lox2272*) to allow successive integration of two constructs[6−8] (Figure 1). CRAGE-Duet enables all the applications dual plasmid systems can enable at the genome level. To demonstrate CRAGE-Duet's ability, we used it to engineer the rhizobacteria *Pseudomonas simiae* WCS417r. This strain can robustly colonize root systems of diverse plant species, induce systemic resistance to pests, and promote plant growth.[9,10] These abilities make *P. simiae* WCS417r a good model for studying the mechanisms of microbial root colonization and beneficial plant−microbe interactions. We had previously implemented CRAGE to create a *P. simiae* WCS417r strain that expressed bacterial luciferase (*luxCDABE*),[4] which served as a tool for identifying and characterizing functions of genes responsible for plant root colonization.[10]

In this paper, we demonstrate successful implementation of CRAGE-Duet in *P. simiae* WCS417r and development of a set of strains that allow study of their spatiotemporal behavior in the rhizosphere nondestructively. Specifically, we integrated nine fluorescent proteins (FPs) with either inducible or constitutive promoters, creating a set of FP strains. Along with our companion paper,[8] these results show that CRAGE-Duet is a powerful and versatile genome engineering tool with broad potential uses in synthetic biology.

RESULTS AND DISCUSSION

The LP on a pW17[4] plasmid was redesigned to contain three mutually exclusive lox sites, *lox2272*, *loxP*, and *lox5171*, to yield pW37 (Supplementary Table S1).[8] The first integration site contains an antibiotic selection marker flanked by *loxP* and *lox5171*. The second integration site comprises the *cre* recombinase gene flanked by *lox2272* and *loxP* (see Figure 1). Accessory vectors including pW26 and pW5, previously built for CRAGE, are fully compatible with the first integration site of CRAGE-Duet.[4,8] pW26 has an R6Kr origin of replication and can be used to integrate constructs smaller than 10 kb, while pW5 is a pCC1-based bacterial artificial chromosome (BAC) that can be used to integrate constructs larger than 10 kbp.

We synthesized genes coding nine different FPs (Supplementary Table S1, Supplementary sequences), which can be spectroscopically resolved based on differences in excitation and emission characteristics during fluorescence imaging.[11] These genes were cloned into a pW26-based vector using Gibson assembly. Additionally, we built two accessory plasmids, pW38 and pW39, for the second integration site. These plasmids contain a T7 RNA polymerase (T7RNAP) gene under the control of an *E. coli lac* promoter inducible with isopropyl *β*-D-1-thiogalactopyranoside (IPTG) (*lacI*-P_{lac}) and a constitutive lac promoter (P_{lac}), respectively. With this system, we can modulate expression regulation without rebuilding construct variants.

Next, we systematically integrated these constructs to build a set of *P. simiae* strains (Supplementary Table S2). First, the LP was transposed into the *P. simiae* chromosome. Plant root colonization assays revealed that these transformants varied in their colonization capabilities (Supplementary Figure S1). Therefore, we selected a single LP variant strain (SB599) based on its ability to colonize *Arabidopsis* roots to a similar degree as the wild type strain. Subsequent whole genome resequencing identified the integration location NZ_CP007637 (GenBank accession): 268402 (nucleotide location) between locus tag PS417_RS26490 and 26495.

https://dx.doi.org/10.1021/acssynbio.0c00280
ACS Synth. Biol. XXXX, XXX, XXX−XXX

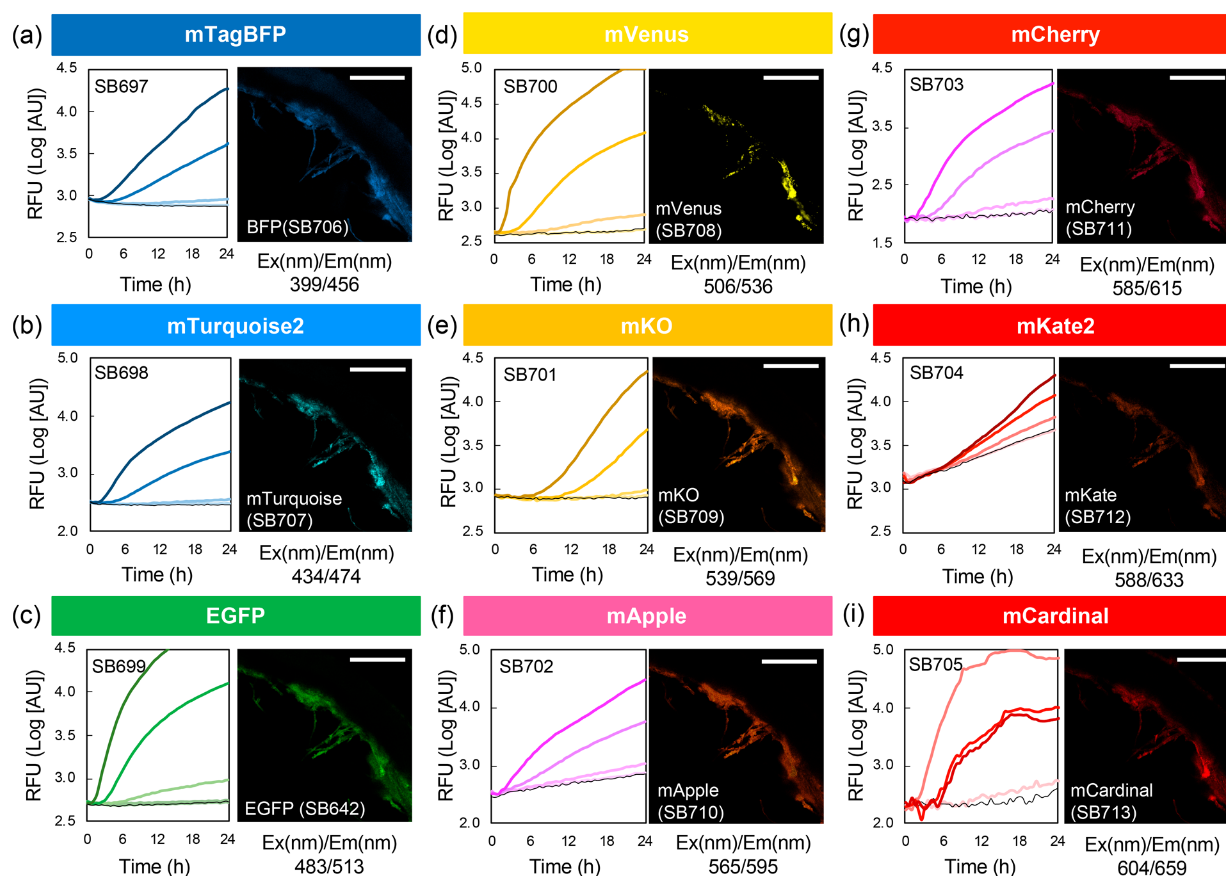

Figure 2. Expression of fluorescent proteins in *P. simiae*. Panels a−i show fluorescent activity of strains SB697−SB705 (constructs with the inducible *lac* promoter) measured by a plate reader, and colonization of the *Brachypodium* root by strains SB642 (EGFP) and SB706−SB713 (constructs with the constitutive *lac* promoter) measured under a confocal microscope. The cultures of strains SB697−SB705 were induced with four different IPTG concentrations (0, 0.01, 0.1, and 1 mM, denoted by lighter to darker colors). All plots were generated from biological triplicates. The colors used are coordinated to represent each fluorescent protein.

Second, the nine FP constructs were integrated into the first integration site to create SB730−SB738, and the *lacI*-P_{lac}-T7RNAP was subsequently integrated into the second site to create SB697−SB705. The fluorescence activity of each FP was measured (Figure 2). All FPs were successfully expressed, and FP expression was induced with a large dynamic range. In all cases, FP activity was tightly regulated without induction; the highest fluorescent activity occurred when the cultures were induced with 1 mM IPTG, except for mCardinal, for which 0.01 mM IPTG gave the strongest FP expression. The conjugal transformation efficiency was very high for all steps (>10^7 CFU/transformation). The integration efficiency was 100% after simple antibiotics selection.

Finally, we integrated P_{lac}-T7RNAP into strains SB730−SB738 in place of *lacI*-P_{lac}-T7RNAP to create SB642 and SB706−SB713, so FPs would be constitutively and highly expressed in these strains. As proof of concept, we confirmed that the fluorescence activity of strain SB642 was comparable to that of strain SB699 when its culture was induced with 1 mM IPTG (Supplementary Figure S2). Subsequently, we simultaneously inoculated all of the stains SB642 and SB706−SB713 onto *Brachypodium distachyon* root systems (BD21) planted in sterile quartz sand in an EcoFAB.[12] Using a confocal microscope, we were able to resolve all FP strains covering the root surface and confirm that their colonization was established. These results suggest that SB642 and SB706 through SB713 can be used as models to study plant−microbe interactions (Figure 2).

In conclusion, we find that the CRAGE-Duet approach for modular integration of two interdependent constructs is suitable for integration of nine different fluorescent proteins into a rhizosphere-colonizing bacterium, *P. simiae* strain WCS417r. Given our previous work showing the applicability of CRAGE to diverse species of bacteria within the phyla of *Proteobacteria* and *Actinobacteria*,[4] we anticipate that CRAGE-Duet can be a powerful approach for creating diverse collections of fluorescently labeled bacteria to study plant−microbe interactions. Promoters expressing the *cre* recombinase, antibiotic resistance, and transposase genes and their codon usages may be altered to further extend the utility of CRAGE-Duet to bacteria in other phyla. While more studies are needed, CRAGE-Duet will be useful for other synthetic biology applications such as metabolic engineering, genetic circuit engineering, and CRISPR/Cas9 genome editing,[8] especially when modular approaches are more favorable than building single-integration constructs (*e.g.*, when construct size is large or many constructs must be modified). The speed, accuracy, efficiency, versatility, and flexibility of CRAGE-Duet make it a useful tool for engineering the genomes of nonmodel bacteria and for creating important biological resources for studying plant−microbe interactions.

METHODS AND MATERIALS

Materials. The set of plasmids used for this study is listed in Supplementary Table S1; their sequences are shown as supplementary sequences in a GenBank format. The plasmid pW37 was designed and built by adding *lox2272* to the plasmid pW17.[4] We used a *Streptococcus pyogenes* Cas9 promoter for expression of the *cre* recombinase gene and a promoter commonly used for the Km^R gene. We previously determined that these promoters are active in diverse species of bacteria in the phyla of *Proteobacteria* and *Actinobacteria*. All strains built are listed in Supplementary Table S2. The One Shot Pir^+ strain and the Top10 strain (Invitrogen) were used as cloning hosts for constructs based on the R6Kr origin of replication and BAC, respectively. *E. coli* BW29427 was used as a conjugal donor strain to transfer plasmids to *P. simiae* WCS417r. Luria–Bertani (LB) medium was used for cultivation of both *E. coli* and *P. simiae*.

Implementation of CRAGE-Duet *via* Conjugation. Conjugation was performed using the method described in our previous study.[4] Donor *E. coli* strains were grown in LB medium containing 0.3 mM diaminopimelic acid (DAP) and appropriate concentrations of antibiotics (50 μg/mL for both Apr and Km) overnight. All recipient *P. simiae* strains were grown in LB medium at 28 °C overnight. Donor and recipient cells were washed three times with LB medium, then resuspended in 50 μL of LB medium containing DAP. This mixture was then transferred onto a nitrocellulose filter membrane on top of an LB agar plate containing DAP and was incubated at 28 °C for 5 to 12 h. Using a loop, the bacterial mixture grown on the membrane was streaked on an LB agar plate containing an appropriate concentration of antibiotics for overnight recovery at 28 °C. Single colonies were picked and cultured in LB medium containing appropriate concentration of antibiotics. Chromosomal integration of the LP was confirmed by colony PCR and low coverage whole genome sequencing. Chromosomal integration of the FPs and promoters was confirmed using antibiotics selection. The strains created for this study are listed in Supplementary Table S2.

Seed Sterilization/Germination of *Arabidopsis thaliana*. Seeds of *Arabidopsis thaliana* were surface-sterilized by shaking for 10 min in 70% bleach and 0.2% Tween-20, and subsequently rinsed five times with sterile distilled water. Seeds were stratified in sterile distilled water at 4 °C in the dark for 2 days. Seeds were sown and plants germinated on 10 × 10 cm plates containing 0.5× MS medium containing 0.5% sucrose solidified with 1% agar. Germination plates were left for 7 days under a 16-h-light/8-h-dark regime at 21 °C day/18 °C night.

Bacteria Growth/Colonization Assay Setup. Bacteria were grown in 2xYT medium and washed three times with 10 mM $MgCl_2$. Density of the washed cells was normalized to OD_{600} = 0.001. Plates (10 × 10 cm) containing 0.5× MS medium with 1% agar without sucrose were spread with 100 μL of OD_{600} = 0.001 washed bacteria, and 10 seedlings (7 days old) were sterilely transferred to these plates. Plates were grown under a 9-h-light/15-h-dark regime at 22 °C day/18 °C night for 12 days.

Harvest. Plant roots were cut from the shoots with a sterile razor blade and roots from two plants were transferred to a single sterile, weighed 2 mL tube containing a small amount of sand. Tubes containing roots were weighed to determine the fresh weight of the roots. Then roots were washed three times with 1 mL of 10 mM $MgCl_2$ and thoroughly vortexed to remove any loosely adhering bacteria. Washed roots were manually ground in 200 μL of 10 mM $MgCl_2$ with a tissue-grinding pestle (Corning Axygen PES-15–B-SI) against the sand in the tube. A dilution series (10^{-1} to 10^{-6}) of this solution containing ground root and colonized bacteria was prepared and 4 μL of each dilution was spotted on LB plates and grown until colonies were visible. CFUs were enumerated and the CFU/mg root fresh weight was determined.

Fluorescence Assay. Fresh LB medium containing appropriate antibiotics was inoculated in triplicate with overnight cultures of *P. simiae* strains SB697–SB705, which were then grown to an $OD_{600\ nm}$ of 0.1. Each of these cultures was transferred to four wells (100 μL each) in a 96-well black titer plate with a clear bottom (Cat# 3631, Corning Inc., NY). These cultures were induced with 0, 0.01, 0.1, or 1 mM of IPTG and were incubated in a Synergy H1 microplate reader (BioTek Instruments Inc., VT) at 28 °C in shaking mode. $OD_{600\ nm}$ and fluorescent activity were measured every 12 min for 24 h. Gain settings used for fluorescence measurements were 50 for BFP, 50 for mTurquoise, 65 for EGFP, 75 for mVenus, 100 for mKO, 100 for mApple, 100 for mCherry, 100 for mKate, and 150 for mCardinal. A gain setting of 50 for EGFP was used for experiments described in Supplementary Figure S2.

Microscope. Seeds of *Brachypodium distachyon* were surface sterilized by shaking for 30 s in 70% ethanol and for 5 min in 50% bleach, then rinsed five times with sterile distilled water. Seeds were stratified in sterile distilled water at 4 °C in the dark for 2 days. Seeds were sown and plants germinated on agar in round Petri plates for 3 days. The germinated plants were transferred to EcoFABs containing quartz sand and 10% MS under a 16-h-light/8-h-dark regime at 25 °C. The nine fluorescent strains of *P. simiae* with constitutive expression (SB642 and SB706–SB713) were inoculated at an $OD_{600\ nm}$ of 0.05 per strain on sterile, 8-day-old *Brachypodium distachyon* (BD21) seedlings that were planted in an EcoFAB[12] in sterile quartz sand and watered with 10% MS media.[13] The plants and bacteria were incubated under growth lights for 7 days at 25 °C prior to imaging. The roots were excised from the EcoFAB and mounted on a glass slide in preparation for confocal imaging. A Zeiss LSM 710 confocal microscope was used in lambda mode with excitation and emission wavelengths over the nine fluorescent proteins used, as illustrated in Figure 2.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acssynbio.0c00280.

> Supplementary Tables S1 and S2 listing plasmids and strains used in this study; Supplementary Figure S1 containing colonization efficiency of *P. simiae* strain WCS417r on the root of *Arabidopsis thaliana*; Supplementary Figure S2 containing expression of GFP from SB642 and SB699; sequences in the GenBank format for all plasmids used in this study (pW37–pW48); all plasmids will be distributed upon request (PDF)

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Author Contributions

[¶]B.W. and Z.Z. contributed equally to this work.

Notes

The authors declare the following competing financial interest(s): Z.Z., G.W., D.R., J.F.C., and Y.Y. filed a patent application (20190048354) related with the CRAGE-Duet technology.

■ ACKNOWLEDGMENTS

The work was done by the U.S. Department of Energy Joint Genome Institute (a DOE Office of Science User Facility), the Laboratory Directed Research and Development Program of Lawrence Berkeley National Laboratory, and m-CAFEs Microbial Community Analysis & Functional Evaluation in Soils (m-CAFEs@lbl.gov), SFA led by Lawrence Berkeley National Laboratory (a DOE Office of Science, Office of Biological & Environmental Research), all under U.S. Department of Energy Contract No. DE-AC02-05CH11231. This work was supported by NSF grant IOS-1917270 to J.L.D., who is an investigator of the Howard Hughes Medical Institute and is supported by the HHMI. We thank Holly Aaron, Director of the Molecular Imaging Center at UC Berkeley, for her guidance on operating in lambda mode for the confocal imaging. We thank Anita Wahler for reviewing and editing the manuscript.

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