Supporting Information

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SI Text: DNA Shuffling and Screening

Screening of ≈3,000 infection foci from the first shuffled population and 1,500 from the second population obtained from the first round of DNA shuffling identified 165 and 60 infection foci, respectively, with improved fluorescence. Infection foci were excised and individual lesions pooled into two sets. RNA was extracted, reverse transcribed and LOV2 fragments derived from *Arabidopsis PHOT2* amplified by PCR and cloned into pTMV.Asc.ΔCP to yield populations with complexities of ≈4,500 and 13,500, respectively. For the second round of shuffling, mutated LOV coding sequences were amplified separately from the two cloned populations recovered from the first round of shuffling through 15 PCR cycles with Expand HiFi (Roche). After DNase I treatment, fragments (< 75 bp) were reassembled through 30 cycles of primer-less thermocycling and the reassembled products amplified through 15 PCR cycles using primers specific to the LOV2 coding region of *Arabidopsis PHOT2*. Shuffled products were cloned into pTMV.Asc via *Asc*I and *Xho*I to allow confirmation of the observed phenotypes by passaging of infectious virions. Two libraries with complexities of ≈50,000 and 125,000 were generated and ≈5,000 infection foci were screened for further improvements in fluorescence. Fluorescent lesions were excised, passaged and their phenotypes confirmed relative to the brightest variant from the first round of shuffling. Candidates with desirable properties were used for RNA isolation, reverse transcription and PCR before cloning and DNA sequencing.

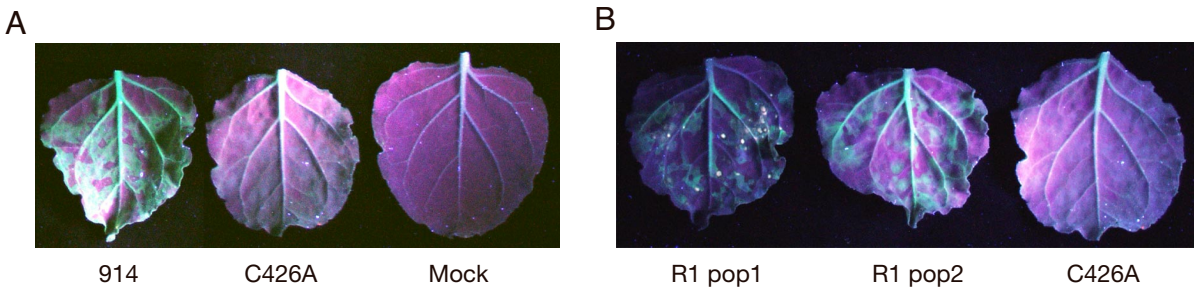


Fig. S1. Confirmation of improved fluorescence properties in virus isolates obtained from the first round of DNA shuffling. (*A*) TMV-based expression of LOV variants in leaves of *Nicotiana benthamiana*. Leaves were photographed simultaneously under UV illumination to allow direct comparison of green fluorescence intensity. Leaves were mock inoculated, inoculated with TMV vector expressing the progenitor C426A, or with the brightest variant 914 obtained from the first round of DNA shuffling. Images were taken 3 days post inoculation. (*B*) Pooled populations recovered from the first round of shuffling (R1 pop1 and R1 pop2, respectively) were assessed as in (*A*) alongside the progenitor C426A for improved fluorescence. Some fluorescent infection foci with necrotic lesions were detectable for the R1 pop1, but were less obvious for R1 pop2.

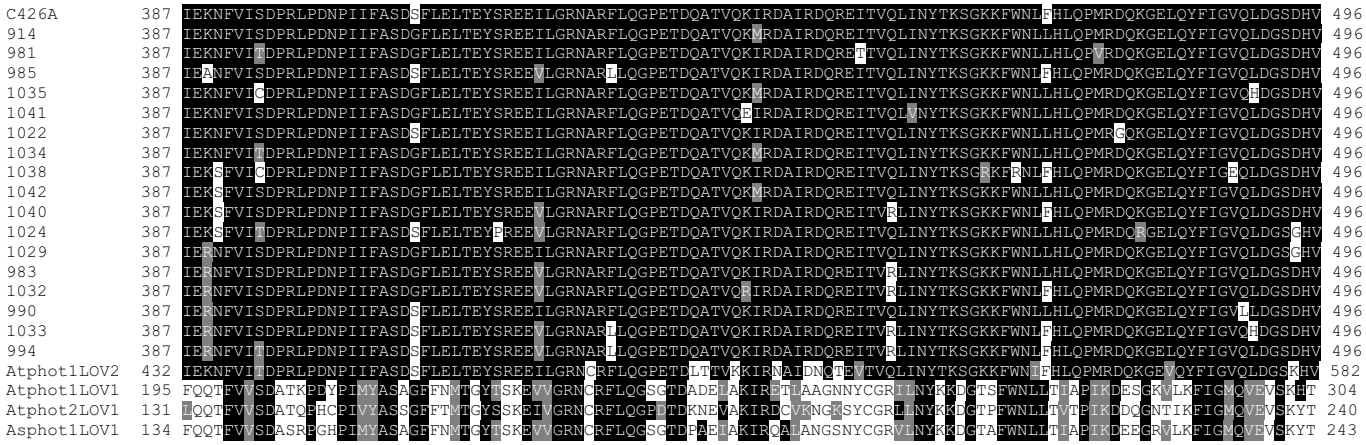


Fig. S2. Amino acid sequence alignment of individual LOV clones obtained from DNA shuffling and TMV-based expression in tobacco. A total of 16 clones were identified after the second round of DNA shuffling and were aligned with sequences of the progenitor (C426A) and the brightest variant obtained from the first round of shuffling (914). The following LOV sequences were also included in the alignment: *Arabidopsis thaliana* phot1 LOV2 (AtphotLOV2), *Arabidopsis thaliana* phot1 LOV1 (Atphot1LOV1), *Arabidopsis thaliana* phot2 LOV1 (Atphot2LOV1) and *Avena sativa* (oat) phot1 LOV1 (Asphot1LOV1). Black shading indicates 100% identity and gray 100% similarity. Amino acid residue numbers within the respective full-length phototropin protein are shown.

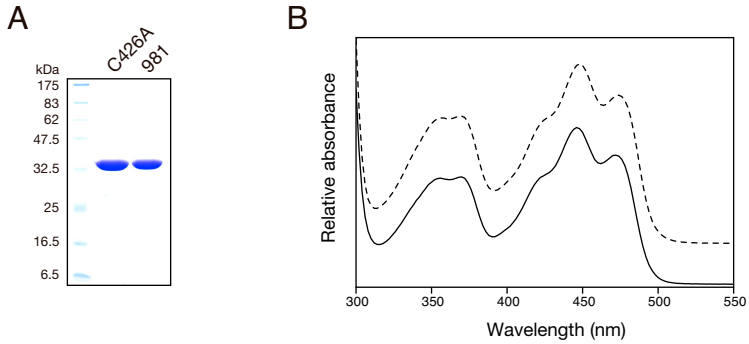


Fig. S3. Spectroscopic properties of LOV variants. (*A*) SDS/PAGE and Coomassie Blue staining of equal amounts (10 μg) of C426A and 981 expressed and purified from *E. coli* as C-terminal fusions to GST (GST). (*B*) Absorption spectra of purified C426A (solid line) and derivative 981 (dashed line). Spectra are offset for clarity.

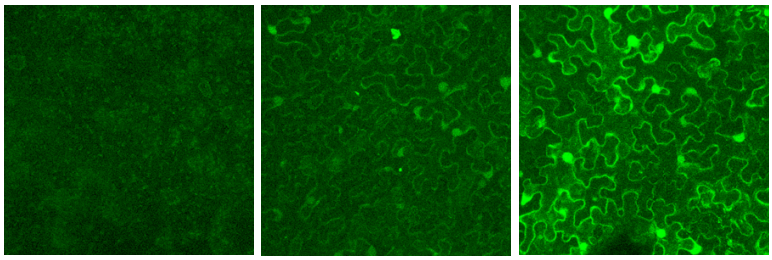


Fig. S4. *Agrobacterium*-mediated expression of LOV variants in leaves of *Nicotiana benthamiana.* Leaves were infiltrated with patches of water (control) or *Agrobacterium* containing binary expression vectors for progenitor C426A or the brightest variant obtained from the second round of DNA shuffling (981). Images of epidermal cells from different inoculation sites on the same leaf were recorded using a Leica SP2 confocal laser scanning microscope and performed using identical microscope settings to allow direct comparison of green fluorescence intensity. Images were taken 3 days post infiltration.

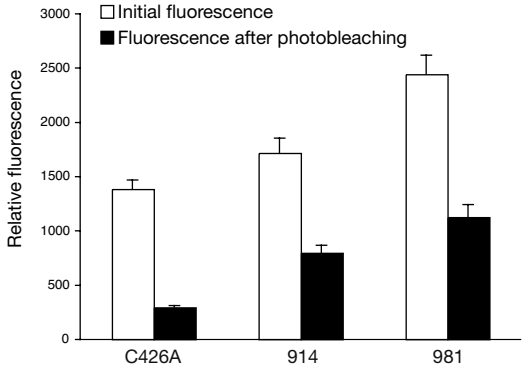


Fig. S5. Photobleaching of LOV variants expressed in tobacco epidermal cells. LOV-mediated fluorescence from nuclei was used to quantify fluorescence loss in response to prolonged scanning at 40% laser power. The first scan was used to focus and record the fluorescence level of the nuclear signal. A series of 50 images was collected every second and the final fluorescence recorded. Values represent the mean ± SE ($n = 21$).

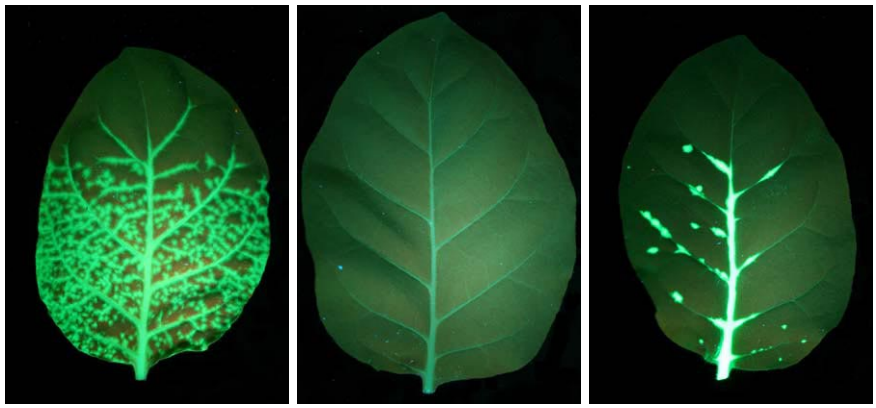


Fig. S6. Systemic movement of TMV.iLOV and TMV.GFP in tobacco. Upper leaves of *Nicotiana tabacum* at 7 days post inoculation. TMV.iLOV shows systemic spread and unloads from all major vein classes, spreading into neighboring ground tissue (left). TMV.GFP either does not cause systemic infection (center) or moves poorly, unloading only patchily from the midrib (class I) and some class II veins. Leaves were photographed simultaneously using a Hoya X1 (green filter) to allow direct comparison of green fluorescence intensity.

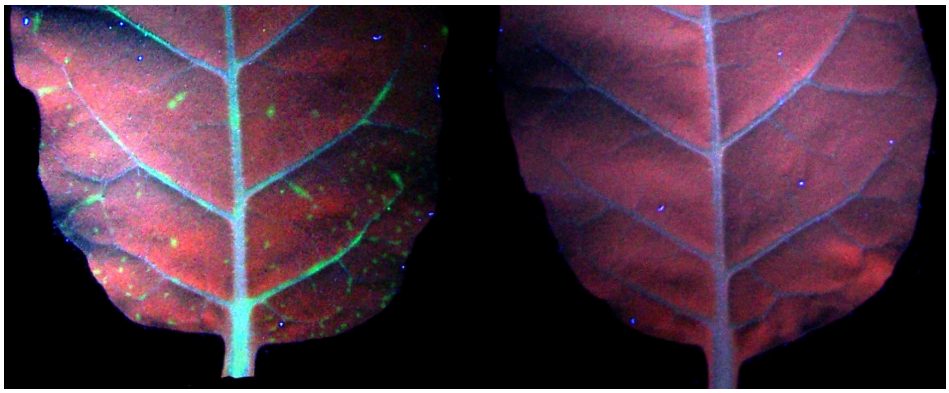


Fig. S7. Systemic movement of TMV.iLOV and TMV.H2B-iLOV in tobacco. Upper leaf halves of *Nicotiana tabacum* at 3 days post inoculation. TMV.iLOV begins to shows systemic spread, whereas TMV.H2B-iLOV does not cause systemic infection, which was confirmed by imaging at higher magnification. Leaves were photographed simultaneously to allow direct comparison of green fluorescence intensity.

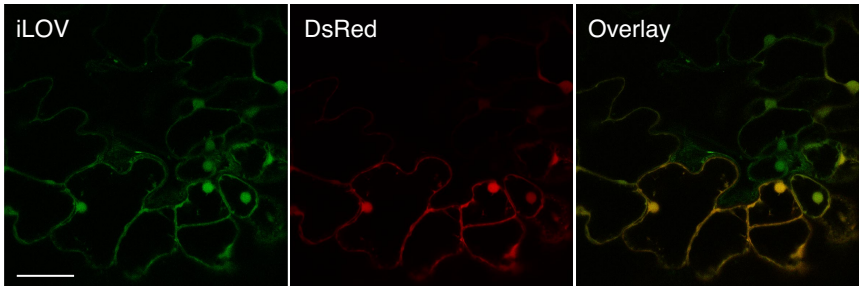
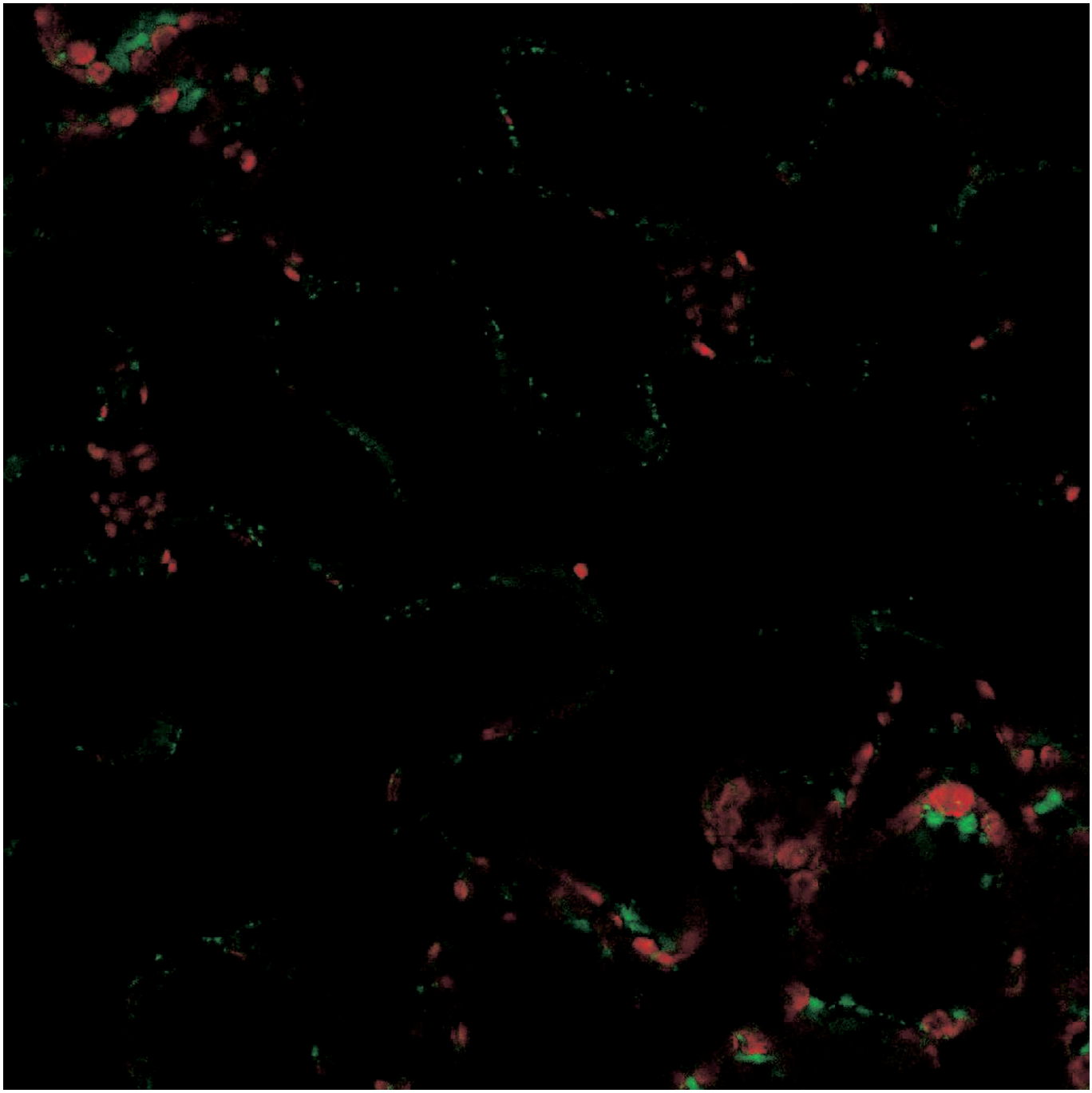


Fig. S8. Dual expression of iLOV and DsRed in tobacco. Epidermal cells of *Nicotiana tabacum* at the infection front of a lesion created by a TMV vector expressing both iLOV and DsRed (TMV.iLOV.DsRed). iLOV and DsRed fluorescence are indicated in green and red, respectively. The leading edge of the infection shows cells containing both fluorescent reporters (indicated by yellow fluorescence in the overlay image) behind a front of cells containing predominantly iLOV. (Scale bar, 50 μm.)



Movie S1. Fluorescent motile Golgi bodies tagged with iLOV. Ten scans were recorded consecutively with each scan lasting ≈11.5 s. The movie represents 5 frames per second.

Movie S1 (AVI)

Table S1. Frequency of nonunique amino acid substitutions occurring in the cloned progeny obtained through DNA shuffling.

Amino acid position in full-length *Arabidopsis* phot2	Substitution	% Frequency	Conservation in LOV domains of *Arabidopsis* phototropins
389	K → R	35	Q in phot1 LOV1 and phot2 LOV1
390	N → S	24	T in phot1 LOV1 and phot2 LOV1
394	S → T/C	24/12	T in *Arabidopsis* phot1 LOV2
409	S → G	65	G in phot1 LOV1 and phot2 LOV1
421	I → V	41	V in phot1 LOV1
428	F → L	18	F invariant
442	I → M	24	I invariant
455	Q → R	35	R in phot1 LOV1 and phot2 LOV1
470	F → L	65	L in phot1 LOV1 and phot2 LOV1
490	L → H	12	—
494	D → G	12	—

Amino acid positions indicated are with respect to their location within full-length *Arabidopsis* phot2. The conserved nature of the amino acid changes compared to other LOV protein sequences from *Arabidopsis* phototropins is indicated. No conservation is represented by —.