

Fluorescence hyperspectral imaging detects an early, FLS2-independent signature of *Pseudomonas syringae* challenge in *Arabidopsis* seedlings, but not of flg22 perception

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[PENDING: date]

Abstract

Detecting plant infection before symptoms appear would help both field and controlled-environment agriculture. In 2016 we imaged *Arabidopsis* seedlings by fluorescence hyperspectral imaging (488 nm excitation, 500–800 nm emission) before and 1–48 h after treatment with a mock solution, the flagellin epitope flg22 or *Pseudomonas syringae* pv. tomato DC3000. Five genotypes were imaged: Col-0, two *fls2* receptor mutants and two *cml24* Ca²⁺-sensor mutants. We reanalysed all 1,982 hand-drawn seedling spectra of the primary experiment, and 400 raw image cubes (396 GB). Recovering the camera metadata showed that exposure time changed between timepoints: the 2016 summaries' 21% fall in intensity after treatment disappears (to within 4%) once intensities are divided by exposure. In addition, 83 exported spectra had been saved without dark subtraction; we corrected every spectrum by its own emission below the excitation wavelength. Treated and control plates also differed *before* treatment, enough for a classifier to tell them apart (plate-level AUC 1.00), so we analysed only change from each plate's own baseline. On that basis, *P. syringae* broadened the red chlorophyll emission peak from 1 h (+1.40 nm; $q = 0.018$) through 24 h, and raised emission below 640 nm from 2 h. Change-from-baseline spectra separated infected from control plates perfectly at 1, 2 and 24 h (leave-plate-out AUC 1.00; exact permutation $p = 0.029$). The pathogen signature appeared in every genotype. An independent analysis of all 1,623 seedlings segmented from the raw cubes reproduced it and added that chlorophyll emission fell from 3 h and seedlings shrank by 48 h. A smaller late rise in *cml24-4* seen in the hand-drawn spectra did not replicate at seedling level. A flg22 signal was weaker (AUC 0.89–1.00, $p \geq 0.10$). It was as strong in both *fls2* mutants, which cannot perceive flg22, as in Col-0, so in this experiment it reflects a plate-level effect rather than flg22 perception. We release the raw data, manifests, code and a reproducible pipeline as a FAIR resource, and document every data problem we found.

1 Introduction

Plants perceive bacteria through pattern-recognition receptors long before disease symptoms appear. The best-characterised receptor is FLS2, which binds the conserved flg22 epitope of bacterial flagellin [1–4]. flg22 induces defence-gene expression and resistance in wild-type plants but not in *fls2* mutants. *fls2* plants still respond to bacterial extracts through other patterns, and are more susceptible to *P. syringae* pv. tomato DC3000 sprayed on the leaf surface [5]. Pattern-triggered defence quickly lowers non-photochemical quenching [6]. *P. syringae* infection

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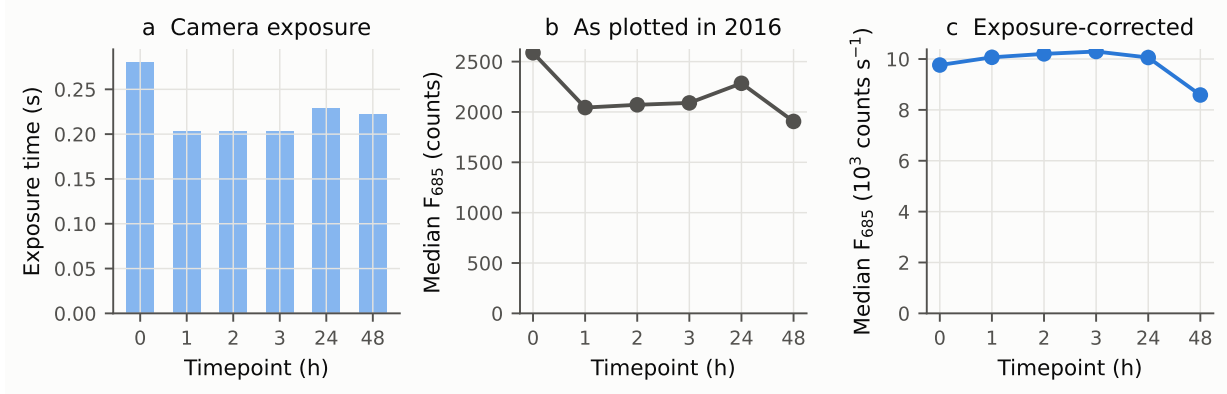


Figure 1: The 2016 intensity drop after treatment is an exposure artefact. (a) Exposure time per timepoint (plate mean, Control plates). (b) Median 683–687 nm emission of Control hand ROIs as plotted in 2016 and (c) divided by exposure. Data: `results/roi_spectra_A.parquet`, `metadata/cube_manifest.csv`.

changes photosynthetic chlorophyll fluorescence parameters near the infection site before visible symptoms, within 3 h for an avirulent strain [7]. Chlorophyll fluorescence imaging is therefore a candidate for disease detection and phenotyping [8–11].

Steady-state chlorophyll fluorescence emission has two bands, near 685–690 nm and 730–740 nm. Because chlorophyll reabsorbs the red band, their ratio falls as chlorophyll content rises. The ratio also depends on leaf structure, optics and photosynthetic activity [12]. Fluorescence hyperspectral imaging records the whole emission spectrum at every pixel, so subtle shape changes can be mapped, not just one or two ratios.

CML24 (*TCH2*) encodes a calmodulin-like Ca^{2+} sensor whose transcript is induced by touch, darkness and other stimuli [13]. The *cml24-4* mutant is impaired in pathogen- and pattern-induced nitric-oxide production [14]. This makes CML24 a candidate link between early Ca^{2+} signalling and downstream changes visible in fluorescence.

Here we reanalyse a 2016 experiment designed to ask whether bacterial challenge and flg22 perception leave a hyperspectral fluorescence signature, and whether that signature depends on FLS2 or CML24. Its fls2 mutants supply the key control: an flg22 signature that reflects perception should be absent in them.

2 Results

2.1 Recovering the experiment from its raw files

We inventoried 7,752 files and parsed every file name into a design record, with no exceptions. That gave 216 sample cubes for the primary July experiment (set A), 168 for an independent November replicate (set B) and 5,842 hand-drawn ROI spectra (2,486 unique by content). The headers revealed that exposure changed between timepoints (Fig. 1a). The 2016 graphs plotted raw counts, so their drop in Control intensity from 2,588 to 2,044 counts after hour 0 (Fig. 1b) is largely a camera setting. Exposure-corrected Control intensities at 1–3 h lie within 4% of hour 0 (Fig. 1c). Hour-0 scans also saturated the 12-bit detector in up to 13% of a cube’s seedling pixels (median 0.1%). A further 83 hand-ROI spectra, all from Pathogen plates (3 h plates 1–2 and 24 h plate 4), had been exported without dark subtraction. Their emission below the excitation wavelength sat at the camera’s dark level (median 109 counts, against 0.1 for all other set-A spectra). Uncorrected, this offset masquerades as broad short-wavelength “autofluorescence”. Nothing can fluoresce at wavelengths shorter than the 488 nm excitation, so we subtracted each spectrum’s own mean over 420–470 nm. We therefore based the primary analysis on exposure- and offset-independent shape features.

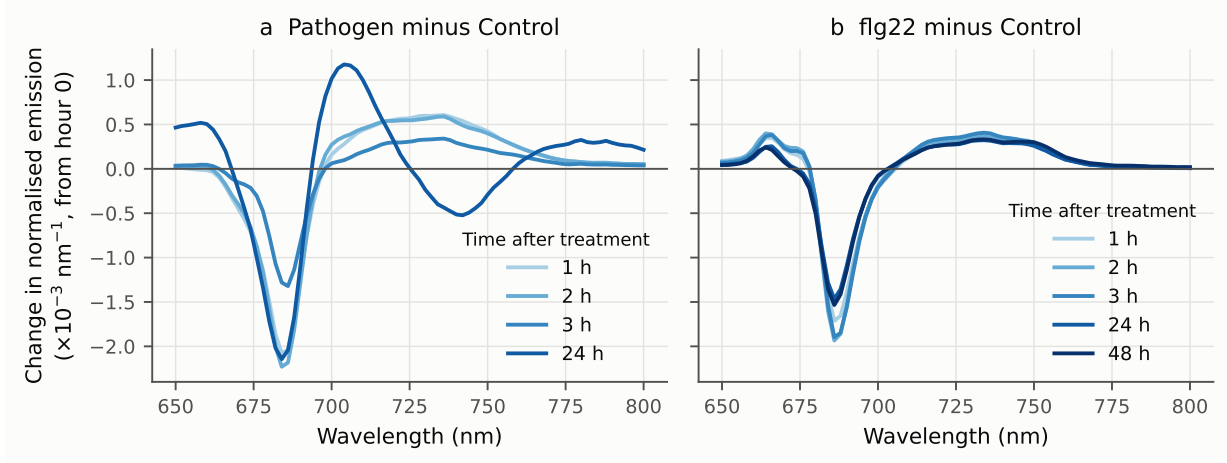


Figure 2: Treatment-induced change in emission-spectrum shape (Col-0, set A; area-normalised spectra, change from each plate’s hour 0, treated minus Control).

2.2 Plates differed before treatment

Hour 0 was imaged before treatment (FACTS R13). Even so, flg22 plates already differed from Control plates in red-peak width (-1.29 SD; $q = 5.5 \times 10^{-5}$). A classifier separated them perfectly from spectra alone (plate-level AUC 1.00, $p = 0.029$; Fig. 4a). The same held, less strongly, for Pathogen plates (AUC 0.81). Plate-to-plate differences in seedling batch, placement or imaging geometry are thus as large as the effects we want to detect. All inference below uses change from each plate’s own hour-0 baseline, with the plate as the experimental unit.

2.3 An early pathogen signature

Relative to baseline, *P. syringae* broadened the red emission peak of Col-0 seedlings by 1.40 nm at 1 h (2.07 SD, $q = 0.018$), 1.76 nm at 2 h (2.39 SD, $q = 6.8 \times 10^{-4}$), 1.02 nm at 3 h ($q = 0.005$) and 3.35 nm at 24 h ($q = 0.005$). It raised the 500–640 nm fraction of emission from 2 h (1.77 SD, $q = 0.018$) to 24 h (2.46 SD, $q = 4.3 \times 10^{-5}$; Figs 2, 3). 13 of 270 baseline-adjusted tests were significant at $q < 0.05$ (Supplementary Table). A classifier on change-from-baseline spectra separated infected from control plates perfectly at 1, 2 and 24 h (AUC 1.00, 1.00, 1.00; exact $p = 0.029$ each, close to the smallest p the 70 plate relabellings allow; Fig. 4b), and less well at 3 h (AUC 0.75, $p = 0.143$). The 2016 vendor analysis of a single infected and a single control plate reported a broad autofluorescence component in infected plants after 24 h [15]. Our short-wavelength fraction measures that component across all plates.

2.4 The flg22 signal does not depend on FLS2

flg22 also broadened the red peak at 3 h (2.18 SD, $q = 0.005$). After baseline adjustment, flg22 plates were separable from Control plates at 1–3 h (AUC 0.89–1.00; Fig. 4b). With three plates per treatment left, however, no result beat $p = 0.10$ (20 relabellings). The genetics decide what this signal is. A model trained on Col-0 flg22-versus-Control shape features gave an average flg22 score difference of 0.18 in Col-0 (out-of-fold, 1–3 h), 0.36 in *fls2-17* and 0.24 in *fls2-101* (Fig. 5a). flg22 cannot act through FLS2 in the mutants, so this signal is a property of the plates (handling, timing or seedling batch), not of flg22 perception. The pathogen signature was likewise present in all genotypes (Fig. 5b). This is expected, because bacteria are perceived through patterns other than flagellin [5].

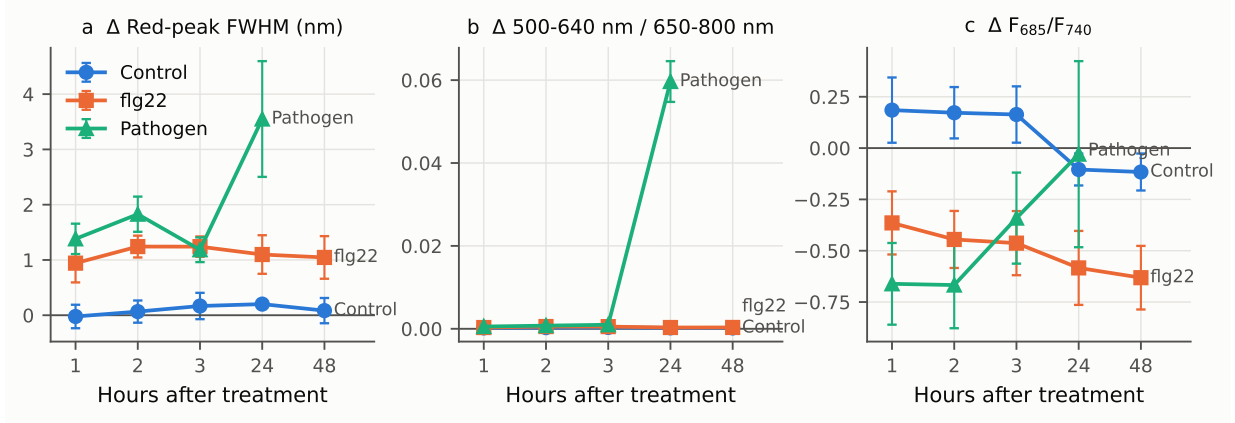


Figure 3: Time course of three emission-shape features (change from hour 0; mean $\pm$ s.e.m. over plates). Data: tables/fig3_timecourse.csv.

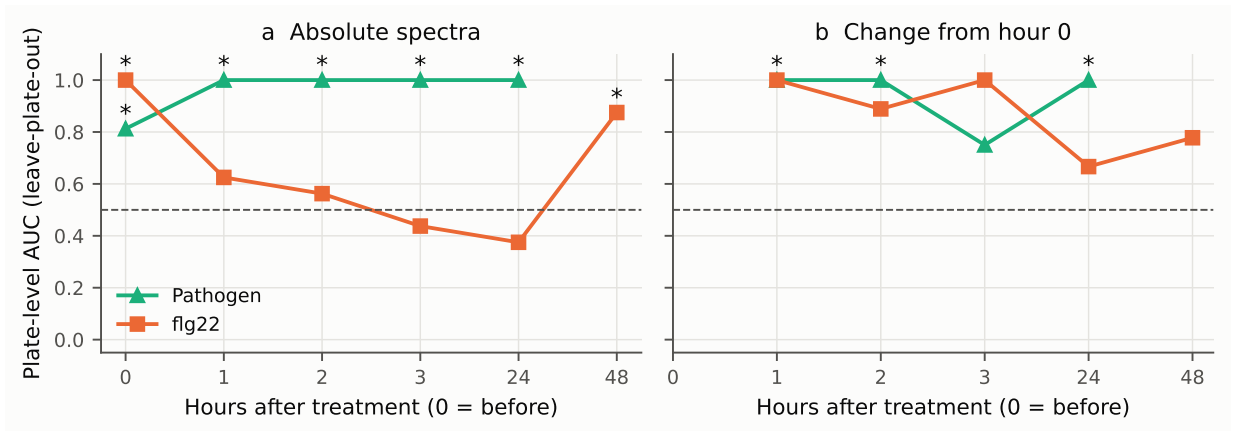


Figure 4: Plate-level classification of treatment from spectra, Col-0. (a) Absolute spectra; hour 0 is before treatment. (b) Change from hour 0. * exact permutation $p \leq 0.05$.

2.5 The independent replicate

In the November replicate (set B; 405 nm excitation), hand ROIs exist for one plate per treatment, so treatment main effects cannot be separated from plate effects there. Within plates, however, flg22 changed the emission shape of Col-0 differently from *fls2-17* at 6 h (F_{685}/F_{740} interaction 1.78 SD, $q = 0.026$; 4 related features). This is the pattern expected of FLS2-dependent perception. It was not seen for *fls2-101*. The raw cubes give two or three plates per treatment. At that level no treatment effect in set B reached $q < 0.1$. One Control plate deteriorated by 48 h: its short-wavelength fraction rose by 0.19, more than on any Pathogen plate. Under 405 nm excitation, short-wavelength emission therefore tracks tissue stress in general rather than infection specifically. At 48 h, flg22 raised localised short-wavelength emission less in both *fls2* alleles than in Col-0 (*fls2-17* -0.71 SD, $q = 0.041$; *fls2-101* -0.72 SD, $q = 0.041$), which is the FLS2-dependent pattern. We regard this as tentative. The set-B lane order was confirmed against the hand-drawn spectra only on plate 1 (better in 21 of 24 comparisons); for plates 2–3 it was assumed. Bacteria-only scans showed no fluorescence of their own: at most 0.16% of pixels were above the seedling threshold, at ≤ 0.5 counts s^{-1} below 640 nm, against thousands for seedlings. The plant signal is thus not bacterial fluorescence.

2.6 Seedling-level analysis of the raw cubes

We segmented 1,623 seedlings from the set-A cubes, including the Pathogen plates at 48 h, for which no hand ROIs exist. We averaged them per plate (all genotypes) and compared changes from

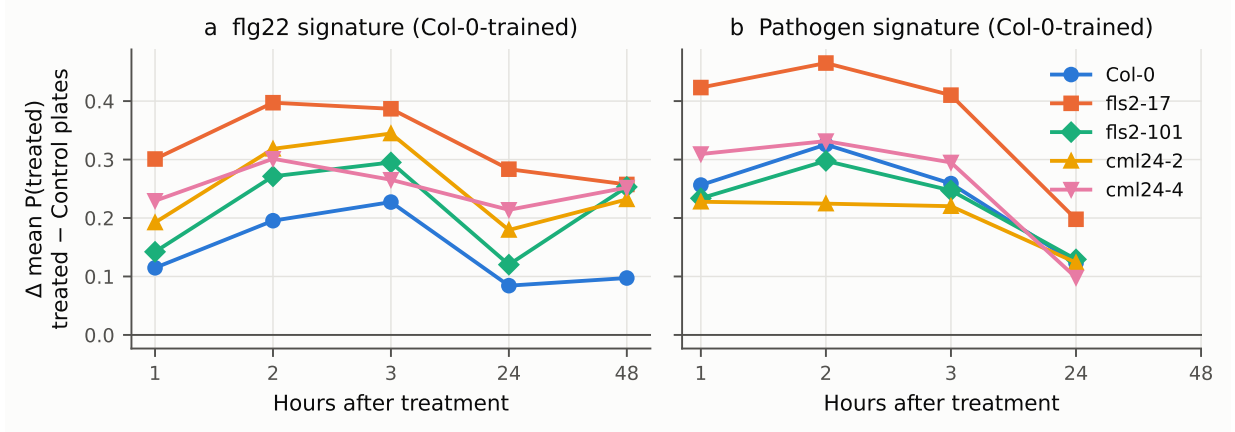


Figure 5: Genotype specificity of the signatures. Col-0-trained model applied to each genotype; difference in mean predicted probability between treated and Control plates.

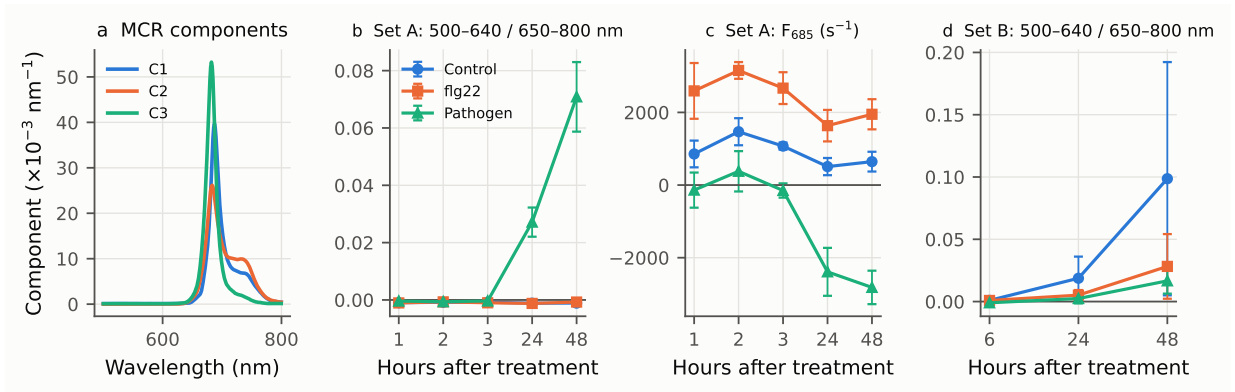


Figure 6: Seedling-level analysis of the raw cubes. (a) MCR-ALS components. (b, c) Set A, plate means of all seedlings, change from hour 0, including Pathogen at 48 h. (d) Set B, 2-3 plates per treatment. Data: `tables/seedling_plate_changes.csv`.

hour 0 (Fig. 6). This analysis is independent of the 2016 annotations, and it reproduced the early signature. At 1 h, Pathogen plates showed a broader red peak ($+0.80 \text{ nm}$, $q = 0.039$) and a lower F_{685}/F_{740} (-0.62 , $q = 0.028$). Exposure-corrected 685 nm emission fell from 3 h ($-1,222 \text{ counts s}^{-1}$, $q = 0.028$) to 48 h ($-3,462$, $q = 0.028$). By 48 h, short-wavelength emission had risen ($+0.072$, $q = 0.039$) and become patchier (per-pixel ratio s.d. $+1.05$, $q = 0.028$), and seedlings had shrunk ($-23,437 \text{ pixels}$, $q = 0.066$). In all, 37 plate-level contrasts were significant at $q < 0.05$.

Multivariate curve resolution [16] of pixels from every set-A cube resolved the emission into three components peaking at 682–687 nm. None carried more than 1.4% of its area below 640 nm, so the broad autofluorescence component reported for two plates in 2016 [15] did not emerge as an independent source of variation across all plates. The broadest, far-red-rich component rose on Pathogen plates at 1–3 h ($+0.12$ of the total at 2 h, $q = 0.028$). Genotype-resolved seedling models found no genotype $\times$ treatment interaction in set A at $q < 0.1$ (0 of all tests), so the *cml24-4* difference in the hand-drawn spectra did not replicate.

3 Discussion

Three lessons from this reanalysis apply beyond these data. First, acquisition metadata must travel with hyperspectral intensities. A camera setting that changed between timepoints produced the most striking pattern in the 2016 summaries. Second, in plate-based seedling assays the plate, not the seedling, is the treated unit. Plates can differ before treatment by as much as treatments differ afterwards, so a baseline image and leave-plate-out validation are essential. Without them,

a classifier here would have “detected” flg22 before it was applied. Third, mutants that cannot perceive the stimulus are the decisive control. Here they showed that an apparently promising flg22 signal was not perception.

What survives is a pathogen signature within 1–2 h of inoculation: a broader red emission peak and more short-wavelength emission. The timing is consistent with reports that chlorophyll fluorescence changes precede visible symptoms [7]. Because the signature appears in *fls2* mutants, it does not require flagellin perception. We found no robust effect of CML24. At 24 h the short-wavelength rise was smaller in *cml24-4* in the hand-drawn spectra (-1.74 SD, $q = 0.036$); this is the allele impaired in pattern-induced NO signalling [14]. But *cml24-2* did not differ, and the seedling-level analysis did not replicate it. Later, the signature turned into falling chlorophyll emission, patchy short-wavelength emission and tissue loss. Under 405 nm excitation, short-wavelength emission also rose on a deteriorating control plate, so on its own it marks damage rather than infection. Limitations: the growth and inoculation details come from the investigators rather than from records stored with the data (FACTS R21, R22); there were four plates per treatment; and set B used a different excitation wavelength.

4 Methods

4.1 Plant material and treatments

Arabidopsis thaliana seedlings of five genotypes were grown on square agar plates in five lanes per plate: Col-0 (lane A), *fls2-17* (B), *fls2-101* (C), *cml24-2* (D) and *cml24-4* (E). Lane assignments are taken from the 2016 analysis folders (FACTS R8). [PENDING: Allele origins, backgrounds and stock numbers.] *FLS2* encodes the flagellin receptor that perceives the flg22 epitope [1–4]. *CML24* encodes a calmodulin-like Ca^{2+} sensor [13] implicated in flg22-triggered nitric-oxide signalling [14]. Seedlings were grown for 7 days on $0.5\times$ Murashige–Skoog/Linsmaier–Skoog basal salts with 0.1% (w/v) sucrose and 0.5 g L^{-1} MES (pH 5.7), solidified with 0.8% Phytigel (FACTS R21) [PENDING: light, photoperiod and temperature]. Plates were treated with a mock solution, flg22 ($1\text{ }\mu\text{M}$, the laboratory’s standard working concentration; FACTS R17) or *Pseudomonas syringae* pv. tomato DC3000 (FACTS R5, R16) Bacteria were applied by flood inoculation after Ishiga et al. [17] at an OD_{600} of 0.01 in 10 mM MgCl_2 (FACTS R22). The 2016 protocol draft names Silwet L-77 as the mock [PENDING: confirm mock and flg22 application method, FACTS P2]. Four plates per treatment were imaged in the July experiment (set A).

The independent November experiment (set B; FACTS R7, R9) used Col-0, *fls2-17* and *fls2-101* only, with two or three plates per treatment, three rows per plate and three seedlings per genotype per row. A pilot experiment (February–March 2016) measured rosette leaf area from images of eight genotypes, including *glr3.3* and *glr3.4* alleles (FACTS R10–R11). It is reported in the supplement only.

4.2 Fluorescence hyperspectral imaging

Plates were imaged in the Gilroy Laboratory (UW-Madison Botany) with a MacroPhor™ fluorescence hyperspectral line-scan imager (Middleton Spectral Vision; FACTS R1). It carried an Andor Zyla 5.5 sCMOS camera in 12-bit low-noise mode (FACTS R2). Set A used 488 nm excitation and 500–800 nm emission (FACTS R3). The camera recorded 324 bands from 400.3 to 808.7 nm at about 1.23 nm spacing. Set B used 405 nm excitation and 400–800 nm emission (FACTS R4), recorded as 406 bands from 400.2 to 800.0 nm, each row-scan acquired at two excitation laser powers, 50% and 58% of maximum (Laser50, Laser58; FACTS R15). Each plate was imaged as three row-scans, one row of seedlings each. Set A was imaged at Hour0 (11:20–12:26 on 22 July 2016, about 6 h before the 1 h scans; the pre-treatment baseline, FACTS R13) and at 1, 2, 3, 24 and 48 h after infection (FACTS R6). Set B was imaged at 0, 6, 24 and 48 h. Acquisition settings were read from every ENVI header (metadata/cube_manifest.csv).

4.3 Data provenance and quality

All 7,752 files on the 2016 drive were inventoried and hashed (`scripts/00_inventory.py`). Every file name was parsed without exception: 400 hyperspectral cubes (216 set-A samples, 168 set-B samples, plus set-B bacteria-only and reference scans and one reference and one artefact scan in set A) and 5,842 hand-drawn region-of-interest (ROI) spectra (2,486 unique by content hash). All 400 raw cubes carry SHA-256 checksums in `metadata/cube_manifest.csv`. We found the following problems and corrected for each where possible.

1. **Exposure time changed between timepoints.** It was 0.305 s at Hour0, 0.203 s at 1–3 h, 0.222–0.268 s at 24 h (varying within plates) and 0.222 s at 48 h. The 2016 summary graphs plotted uncorrected intensities, so their apparent drop between 0 and 1 h (about 1.5-fold) matches the exposure ratio $0.305/0.203 = 1.50$. We divide every intensity by its own exposure time, and our primary features are exposure-independent ratios (Section 4.4).
2. **Hour0 plates were scanned “inverted”,** i.e. upside down (FACTS R14), except four rescans labelled “NI” made later the same day at the 1 h settings. Hour0 is used only as a baseline and is analysed separately from the upright timepoints.
3. **Detector saturation.** At the Hour0 exposure, up to 13% of a cube’s seedling pixels (median 0.1%) reached the 12-bit ceiling (4095 counts). Saturated pixels are excluded from all spectra.
4. **Fixed scan order.** Within each timepoint plates were scanned Pathogen, then flg22, then Control, 20–40 min apart, so scan time is partly confounded with treatment. We report the drift of Control plates between consecutive hours as a bound on this effect.
5. **The plate is the experimental unit.** Treatments were applied per plate (four per treatment in set A), so treatment effects are tested at plate level. Genotype contrasts are within plates.
6. **Inconsistent dark subtraction in the 2016 exports.** 83 hand-ROI spectra (Pathogen 3 h plates 1–2 and 24 h plate 4) were exported with the camera dark level (median 109 counts) still in them; all others were dark-subtracted (median 0.1 counts). Because nothing fluoresces at wavelengths shorter than the excitation, each spectrum’s mean below the excitation line (420–470 nm in set A, 400–404 nm in set B) was subtracted from it before features were computed (`scripts/01_import_rois.py`).
7. **Missing cells.** No hand ROIs were drawn for Pathogen plates at 48 h, although the cubes exist. Our automated segmentation covers them.

4.4 Spectral processing

Each raw cube was read once, sequentially, and hashed with SHA-256 (`scripts/02_harvest_cubes.py`). The per-band dark level was taken as the median of background pixels, which sit at about 108 counts. Seedlings were segmented on the dark-subtracted 685 nm image at 10 background standard deviations, then cleaned, and cotyledon and hypocotyl components were grouped (`src/pathofluor/segment.py`). Each segmented seedling was assigned its lane and plant number from the row layout, which was validated against the 2016 hand ROIs (Supplementary Note 1). Spectral features were computed from the mean unsaturated spectrum of each seedling after Savitzky–Golay smoothing (`src/pathofluor/features.py`):

- the red/far-red ratio F_{685}/F_{740} [12];
- the position and full width at half maximum of the red emission peak;
- the spectral centroid;

- the far-red fraction of 650–800 nm emission;
- the short-wavelength fraction (500–640 nm emission relative to 650–800 nm), which captures the broad autofluorescence reported by the 2016 vendor analysis;
- exposure-normalised intensities (counts s^{-1});
- per-pixel heterogeneity of the ratio and of the short-wavelength fraction.

4.5 Statistics and classification

Hand-ROI features (primary). For each set, hour and feature we fitted $y \sim \text{treatment} \times \text{genotype} + (1 | \text{plate})$ with Control and Col-0 as references (`statsmodels` MixedLM, REML; features standardised), both on absolute values and on change from the same plate and genotype at hour 0 (plates Control-4 and flg22-2 excluded from the latter). p -values were adjusted by the Benjamini–Hochberg procedure across all features, hours and contrasts within a set [18] (`scripts/05_stats.py`). *Seedling-level features.* Seedling values were averaged per plate (and per plate and genotype), expressed as change from hour 0, and compared between treated and Control plates by Welch’s t -test, or by the same mixed model for genotype interactions, with the same FDR control (`scripts/05c_seedling_stats.py`). *Classification.* Logistic regression (L2, $C = 0.1$) and PLS-DA (three components) on the area-normalised spectrum or on the six shape features were evaluated by leave-one-plate-out cross-validation. AUC was computed on plate means of out-of-fold scores. Significance came from an exact permutation test that re-ran the whole cross-validation for every assignment of the treated label to the same number of plates (`scripts/06_models.py`). *Curve resolution.* MCR-ALS with three non-negative components was fitted to 1,500 unsaturated seedling pixels per set-A cube (500–800 nm, dark-subtracted, divided by exposure), initialised from the purest pixels (`scripts/05b_mcr.py`).

4.6 Data and code availability

Code (MIT licence), derived data, the 2016 hand-ROI spectra and all manifests are in the repository. Raw cubes are deposited on Zenodo [PENDING: DOIs]. The two 2016 Middleton Spectral Vision analysis reports [15, 19] are cited but not redistributed (FACTS R19). The metadata follow MIAPPE where applicable [20] and the FAIR principles [21].

Acknowledgements

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