Daily humidity oscillation regulates the circadian clock to influence plant physiology

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Early circadian studies in plants by de Mairan and de Candolle alluded to a regulation of circadian clocks by humidity. However, this regulation has not been described in detail, nor has its influence on physiology been demonstrated. Here we report that, under constant light, circadian humidity oscillation can entrain the plant circadian clock to a period of 24 h probably through the induction of clock genes such as CIRCADIAN CLOCK ASSOCIATED 1. Under simulated natural light and humidity cycles, humidity oscillation increases the amplitude of the circadian clock and further improves plant fitness-related traits. In addition, humidity oscillation enhances effector-triggered immunity at night possibly to counter increased pathogen virulence under high humidity. These results indicate that the humidity oscillation regulates specific circadian outputs besides those co-regulated with the light-dark cycle.

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Life on earth is fundamentally dictated by the daily and yearly rhythms produced as a result of Earth’s rotation and revolution around the Sun. Living organisms perceive these rhythms and use biological circadian clocks to help tell time and schedule physiological processes in anticipation of regular changes in the environment\textsuperscript{1–4}. In plants, the circadian clock is a complex network of transcriptional and translational feedback loops\textsuperscript{5,6}. At the core of the plant circadian clock are two partially redundant morning-phased transcription factors (TFs), CIRCADIAN CLOCK ASSOCIATED 1 (CCA1) and LATE ELONGATED HYPOCOTYL (LHY), which repress their own expression as well as the expression of their repressors, the PSEUDO-RESPONSE REGULATORS (PRR1, PRR5, PRR7 and PRR9)\textsuperscript{8}. PRR1, better known as TIMING OF CAB EXPRESSION 1 (TOC1), is an evening-phased TF, which, together with CCA1 and LHY, forms the core feedback loop\textsuperscript{5,9}. This CCA1/LHY-TOC1 core loop is further connected with the morning loop (i.e., PRR3, PRR7 and PRR9) and the evening loop (i.e., PRR3, EARLY FLOWERING 3, EARLY FLOWERING 4, LUX ARRHYTHMO and GIGANTEA) to create the complex architecture of the plant circadian clock\textsuperscript{8}.

Early investigations had alluded to effects of light, temperature and air humidity on circadian clocks\textsuperscript{10,11}. Yet, most studies of plant and animal circadian clocks have been conducted using only light or temperature as a clock entraining signal even though correlations between humidity changes and potential clock outputs, including metabolism\textsuperscript{12–15}, animal behaviours (e.g., the biting activity of mosquitoes\textsuperscript{16–19}) and incidence of plant diseases\textsuperscript{20,21} have been reported. The extent to which daily humidity oscillation directly regulates the circadian clock has not been investigated. Our study reveals that in the absence of light and temperature cues, air humidity entrains the plant circadian clock probably through high humidity-mediated induction of CCA1; whereas, in the presence of light-dark cycles, oscillating air humidity increases the amplitude of clock genes, further improves plant fitness-related traits and enhances resistance against evening-inoculated pathogens. Therefore, this study not only demonstrates humidity oscillation as a regulator of the circadian clock but also shows its specific impacts on plant physiology.

**Results**

**Humidity oscillation regulates physiological clock outputs.** To test whether humidity is a regulator of the plant circadian clock, we first examined the influence of daily humidity oscillation on plant physiology. We kept the temperature constant at 22 °C so that relative humidity (RH) could be used as a proxy for vapour pressure deficit (VPD), which is known to regulate plant transpiration\textsuperscript{22–24}. Additionally, we performed our experiments in constant light (LL) to exclude the influence of the light-dark cycle. We first compared plants grown under 22 °C in either constant RH of 50% or 90% to plants grown under 22 °C in 12 h 50%/12 h 90% oscillating RH. The oscillating RH was designed to simulate weather conditions observed in Harvard Forest (latitude: +42.53311, longitude: −72.18968) during the equinoxes, which had a 24-h period and 40% RH range (Supplementary Fig. 1a-c). To minimize confounding results due to temperature changes, we grew plants in humidity- and temperature-controlled Percival chambers (Supplementary Fig. 2a, b; Supplementary Table 1). We found the biggest increases in leaf expansion and biomass in plants grown under 12 h 50%/12 h 90% oscillating RH (Fig. 1a, b), suggesting beyond the known effects of high humidity on plants, such as the opening of stomata\textsuperscript{12,25} and increasing CO\textsubscript{2} assimilation\textsuperscript{26,27}, humidity oscillation has additional influences on plant physiology. To test whether these influences could be circadian clock-regulated, we compared wild-type (WT) plants to the clock core loop triple mutant cca1 lhy toc1. Plants were grown in LL at 22 °C under either 12 h 50%/12 h 90% oscillating RH or 70% constant RH, so that they were exposed to the same total amount of humidity. We found that WT plants accumulated more biomass and flowered earlier under oscillating humidity than under 70% RH and that these effects were dependent on core clock genes (Fig. 1c, d). These results suggest that the daily oscillation of air humidity could, in principle, be a regulator of the plant circadian clock.

**Humidity oscillation entrains the circadian clock in LL.** Next, we investigated whether humidity could entrain the circadian clock, i.e. synchronize the clock in the absence of other environmental signals. We examined synchronicity of clock gene expression in transgenic Arabidopsis plants carrying luciferase

Fig. 1 Humidity oscillation influences plant physiology in a core clock gene-dependent manner under constant light conditions. a, b Photograph (a) and weight (b) of leaf-rosette of 3-week-old plants grown under constant light (LL) with constant 50% relative humidity (RH), 90% RH, or 12 h 50%/12 h 90% oscillating RH. Data are shown as mean ± s.d. of n ≥ 11 plants; one-way ANOVA followed by Bonferroni’s multiple comparisons correction, **p < 0.01, ***p < 0.001; ns not significant. This experiment was repeated two times with similar results. c Dry weight of leaf-rosette of 3-week-old WT and the core clock gene mutant cca1 lhy toc1 plants grown under LL with constant 70% RH or 12 h 50%/12 h 90% RH. Data are shown as mean ± s.d. of three experimental replicates representing at least 45 plants from a single parent line. Data were combined using linear mixed effect model (lme4) with experiment as random effects; two-way ANOVA, *p < 0.05. d Flowering time of plants grown under LL with constant 70% RH or 12 h 50%/12 h 90% RH. Data are shown as mean ± s.d. of three experimental replicates representing at least 78 plants from a single parent line. Data were combined using linear mixed effect model (lme4) with experiment as random effects; two-way ANOVA, ***p < 0.001
reporters driven by promoters of the clock genes CCA1 (CCA1p:LUC), LHY (LHYp:LUC) and TOC1 (TOC1p:LUC). In order to distinguish the influence of humidity oscillations from those of temperature and light cycles on the circadian clock, we first grew plants in the humidity- and temperature-controlled Percival chambers (Supplementary Fig. 2a,b) and then imaged them in a custom-built chamber containing two compartments in which we could independently oscillate humidity while keeping temperature within a range of 1 °C (Supplementary Fig. 2c), which is well below the lowest temperature range (4 °C) reported to entrain the Arabidopsis circadian clock38.

From seed germination, we grew plants in two Percival growth chambers set to LL 22 °C conditions with humidity kept at constant 50% RH in one chamber and oscillating (12 h 50%/12 h 90% RH) in the other chamber. After 3 weeks, plants were moved to the imaging chamber and clock gene expression was measured by luciferase reporter activities in the area around the shoot apical meristem, which displays robust and synchronous waveforms39. We found persisting CCA1p:LUC, LHYp:LUC and TOC1p:LUC rhythms in plants entrained with oscillating humidity even after they were released into free-running conditions (LL 22 °C, 50% RH) (Fig. 2a). A larger fraction of plants pre-grown under oscillating humidity were rhythmic and their phases were more synchronized when compared to those of plants pre-grown under constant humidity (Fig. 2b; Supplementary Table 2), which indicates entrainment. This entrainment was verified by quantitative reverse transcription-PCR to rule out possible influence of humidity on the luciferase reporter activity (Supplementary Fig. 3a–d). In addition to the core circadian clock genes, the humidity oscillation also entrained clock output genes, such as the morning-phased CATALASE 2 (CAT2) and the evening-phased COLD, CIRCADIAN RHYTHM and RNA BINDING 2 (CCR2) and CATALASE 3 (CAT3) (Fig. 2c, d; Supplementary Table 2; Supplementary Fig. 3e, f), suggesting that entrainment by humidity oscillation influences clock functions.

In the entrainment experiments shown in Fig. 2a–d and Supplementary Fig. 3, we were intrigued by the phases of the clock and clock output genes, which were at their respective antiphases, i.e., morning-phased genes CCA1, LHY and CAT2 peaked at the low-to-high humidity transition, which corresponded to dusk in nature, whereas the opposite occurred to the evening-phased genes TOC1, CAT3 and CCR2. To confirm that humidity oscillation could set the phase of the clock, we grew two populations of plants from germination at 22 °C and 50% RH but under antiphasic light regimens (LD or DL). After 2 weeks, plants were temporarily moved to free-running conditions (LL and 50% RH) at their respective dawn for imaging and then moved to LL 12 h 50%/12 h 90% RH humidity oscillation conditions, which would have corresponded to LD in nature (Fig. 2e). We found that the humidity oscillation shifted the phase of plants initially pre-grown under UD to their antiphase whereas plants initially pre-grown under DL did not experience a phase-shift (Fig. 2f), supporting the hypothesis that humidity oscillation sets the phase of the circadian clock under LL.

To gain insight into the mechanism by which humidity sets the phase of the clock, we investigated whether humidity could perturb the endogenous rhythm of the core clock genes. Arabidopsis plants carrying luciferase reporters were grown for 3 weeks under 12 h light/12 h dark (LD) and 50% RH, and then released to DD and 50% RH. After 54 h under DD, i.e., 6 h after the subjective dawn when humidity is unlikely to be high in nature, we exposed these plants to a treatment of 2-h 90% RH and detected an immediate induction of the morning-phased CCA1p:LUC reporter (Fig. 2g, h) as well as the CCA1 mRNA (Fig. 2i). Interestingly, the other morning core clock gene LHY was insensitive to the 2-h pulse of high humidity treatment (Fig. 2g–i) even though it could be entrained by repeated 12 h 50%/12 h 90% RH cycles (Fig. 2a, b, f; Supplementary Fig. 3). These results suggest that high humidity could set the phase of the clock through the induction of CCA1.

Influence of oscillating humidity on the prr7 prr9 mutant. To investigate whether oscillating humidity potentially entrains the clock via changes in temperature, a known entraining signal, we tested the prr7 prr9 double mutant, which is unresponsive to 12 °C/22 °C temperature entrainment30. We subjected the double mutant to humidity oscillation (12 h 50%/12 h 90% RH) and, as a control, to air temperature oscillation (12 h 21 °C/12 h 22.5 °C), which covers the range of ambient temperature fluctuations observed during humidity oscillation in our chambers (Supplementary Fig. 2). We found that while both humidity and the 1.5 °C air temperature cycles could entrain LHYp:LUC in WT plants, only humidity oscillation could entrain the clock reporter in prr7 prr9 (Supplementary Fig. 4a,b; Supplementary Table 2).

Light, air temperature and humidity are also known to influence leaf temperature through radiation, heat exchange and transpiration, respectively. Therefore, it is possible for their perception to partially converge on leaf temperature. To test this hypothesis, we subjected the prr7 prr9 mutant to humidity oscillation and air temperature oscillation, which produced similar oscillations in leaf temperature. Using a FLIR A320sc infrared (IR) camera, we determined the relationship between air temperature/humidity and leaf temperature in our growth chamber (Supplementary Fig. 4c). We found that the range of leaf temperature oscillation under 21 °C and 50%/90% RH corresponded to the one under 21 °C/22.5 °C and 50% RH (Supplementary Fig. 4d). Yet, we observed entrainment of prr7 prr9 under 21 °C with 12 h 50% 12 h 90% RH, but not under 50% RH with 12 h 21 °C/12 h 22.5 °C (Supplementary Fig. 4b,e).

Humidity increases the amplitude of core clock genes in LD. Since light and humidity cycles coexist in nature, we hypothesized that similar to the synergistic effect by simulated light and temperature cycles on the Drosophila’s clock31, humidity oscillation may increase the amplitudes of CCA1, LHY and TOC1, and improve fitness-related traits in LD. To test this hypothesis, we compared CCA1p:LUC, LHYp:LUC and TOC1p:LUC expression in plants grown under LD with either constant 50% RH or 12 h 50% RH/12 h 90% RH cycle with the 2-h overlap observed in nature (i.e., 2-h 90% RH with light at dawn and 2-h 50% RH with darkness at dusk; Supplementary Fig. 1d). For the remainder of the manuscript, we term this combined humidity and light cycles as “simulated natural conditions”. We found that the simulated natural conditions increased the expression amplitudes of core clock genes as determined by the clock-driven reporter activities as well as the endogenous clock gene mRNA levels (Fig. 3a–c; Supplementary Fig. 5). Interestingly, the 2-h overlap between light and humidity cycles appears to be required for the interplay between light and humidity on the circadian clock, because when the humidity cycles were set in complete antiphase with the light cycles, little amplitude increase was observed (Fig. 3d–f). Although the humidity-sensing mechanism has yet to be elucidated, these data suggest that humidity may increase the amplitude of the light-driven clock by targeting some morning clock genes such as CCA1.

Simulated natural conditions improve fitness-related traits. Next, we investigated the influence of simulated natural conditions on several physiological traits previously shown to be affected by the clock32–35. We compared biomass accumulation, flowering time and seed production in plants grown under light-dark cycles with either simulated natural conditions or...
constant 70% RH. We found that simulated natural conditions increased plant biomass accumulation and shortened flowering time in WT (Fig. 4a, b). More importantly, despite having no detectable influence on seed quantity, humidity oscillation improved seed quality as determined by seed mass (Fig. 4c, d). These influences were not observed in the cca1 lhy toc1 triple mutant (Fig. 4a, b, d), which is known to have near WT growth phenotype in LD. These results indicate that simulated natural conditions influence plant physiology in a core clock gene-dependent manner.

**Simulated natural conditions influence plant immunity.** High humidity is known to correlate with disease outbreaks in...
Fig. 3 Simulated natural conditions increase the amplitude of the circadian clock under the light-dark cycle. **a-c** CCA1p:LUC (**a**), LHYp:LUC (**b**) and TOC1p:LUC (**c**) activity rhythms (top) and calculated periods, circadian phases and amplitudes (bottom) in WT grown under LD with constant 50% RH (grey) or simulated natural conditions (black). Simulated natural conditions represent 12 h 50% RH/12 h 90% RH cycles with the 2-h overlap of high humidity and light in the morning as observed in nature. Luciferase activity was normalized to the peak value between 40 and 60 h. Periods, circadian phases and amplitudes were calculated by FFT-NLLS analysis of the data between 60 and 132 h. White and black bars represent light and dark, respectively; white and blue bars represent 50% RH and 90% RH, respectively; black arrow, start of humidity entrainment conditions. Data are shown as mean ± s.e.m. and mean ± s.d. for activity rhythms and their calculated parameters, respectively; \( n = 24 \) plants; \( t \)-test, ***\( p < 0.001 \). Relative amplitude error values of \( \leq 0.7 \) were considered rhythmic. This experiment was repeated three times with similar results.

**d-f** CCA1p:LUC (**d**), LHYp:LUC (**e**) and TOC1p:LUC (**f**) activity rhythms (top) and calculated periods, circadian phases and amplitudes (bottom) in WT grown under LD with constant 50% RH (grey) or 12 h 50%/12 h 90% RH (blue) with no overlap of light and high humidity in the morning. Data are shown as mean ± s.e.m. and mean ± s.d. for activity rhythms and their calculated parameters, respectively; \( n = 24 \) plants. Relative amplitude error values of \( \leq 0.7 \) were considered rhythmic. This experiment was repeated three times with similar results.
The daily oscillation of air humidity regulates the plant circadian clock and influences plant physiology in a core clock gene-dependent manner. In the absence of the light cycle, humidity oscillation entrains the circadian clock to its antiphase (Fig. 2, Supplementary Figs. 3 and 4), whereas under simulated natural conditions, it increases the amplitude of the clock and further improves fitness-related traits (Figs. 3 and 4). Our entrainment results support the existence of a humidity-sensitive oscillator within the circadian clock, but with CCA1 and LHY, the two parallel morning genes, responding to humidity differently; CCA1 responded rapidly to humidity, whereas LHY did not (Fig. 2g). Rhythmic expression of LHY and TOC1 seen in the oscillating humidity could conceivably be driven by the altered expression of CCA1. This suggests that the humidity regulation of the circadian clock might be distinct from the regulation by light and temperature, which both involve phytochromes as sensors and have similar effects on clock regulation by light and temperature, which both involve phytochromes as sensors and have similar effects on

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due to changes in VPD, we investigated whether humidity entrainment was dependent on leaf temperature, a consequence of leaf transpiration. Temperature is a known entraining signal. If a correlated oscillation in leaf temperature caused by the known effect of humidity on transpiration was sufficient to entrain the clock, it would be expected to cause rhythms in the same phase as observed in our experiments and the prr7 prr9 double mutant, which is unresponsive to changes in air temperature, may also be unresponsive to changes in humidity. However, our data showed that humidity is still able to entrain rhythms in the temperature-insensitive prr7 prr9 mutant (Supplementary Fig. 4). We, therefore, suggest that humidity may act via a temperature-independent mechanism. However, a pleiotropic effect of the mutant background on the clock cannot be completely ruled out. Even if humidity does not regulate the clock through leaf temperature, it is still possible that the humidity perception involves other effects of leaf transpiration or sensors shared with the temperature signalling similar to what was recently reported in *Drosophila*.

It also remains unknown how, under simulated natural conditions, oscillating humidity increases the amplitude of the light-driven clock without perturbing its phase. Elucidating this mechanism may require identification of all the humidity targets in the clock, besides CCA1, and mathematical modelling of the clock signalling network. Such information may also shed light on the question whether humidity is a novel environmental zeitgeber distinct from light and temperature.

Our study supports previous findings that the circadian clock not only allows plants to anticipate infection at the time when it is most likely to occur but also gates the immune response when infection does occur to minimize conflict with growth and development. Previously, we found that untimely induction of defence by the immune signal salicylic acid (SA) at night could lead to a severe loss of plant fresh weight. This was likely due to SA-mediated repression of genes in water transport, such as 9 of the 26 *Arabidopsis* aquaporin genes. Water availability is not only essential for plant growth but also critical for pathogen virulence as recently demonstrated for *Pst* whose proliferation in the apoplast requires both establishment of aqueous conditions and...
by its effectors and high humidity in the environment. Therefore, humidity may function as a cue to allow scheduling of defence and growth activities with minimal fitness cost. The daily oscillation of humidity is a universal natural phenomenon experienced by all terrestrial organisms. A thorough investigation of humidity as a regulator of circadian clock in other species may further advance the field of chronobiology by uncovering new clock components, or even organisms to which humidity might be a primary zeitgeber. In addition to our finding that high humidity can activate T3SS in bacteria (Supplementary Fig. 6a), air humidity is known to greatly influence organisms such as insects with high surface to body volume ratios, which make them vulnerable to rapid desiccation. This is in line with the early observations that insect activity cycles are influenced by the diurnal humidity fluctuations reported by Colin Pittendrigh, a founder of chronobiology, in his work on malaria-transmitting mosquitoes. We also anticipate that our study in plants may lead to more in-depth investigation of humidity influences on mammalian physiology, as multiple studies have reported a correlation between humidity and disease susceptibility in humans.

Methods

Materials and growth conditions. LHY::LUC and TOC1::LUC lines in WT and in prr7-3 prr9-1 were generated by Salomé and McClung whereas the CCA1::LUC line was described in Pruneda-Paz et al. The cal1 hy14 toc1 triple mutant was provided by Mizio, T. and generated in Ito et al. Plants were grown in 2 l air circulation chambers (AR models) where light, temperature and humidity could be controlled independently. Light provided by Philips AltoII tubes was set at 100 µmol m$^{-2}$s$^{-1}$ during the day or LL and 0 µmol m$^{-2}$s$^{-1}$ at nights; temperature and air humidity were set according to experimental designs. Plants were grown in MetroMix 360 soil in 72-well flats (Hummer 11-0705) and watered every 2-3 days to keep the soil volumetric water content (soil moisture) between 20% and 40% so that they did not experience drought stress. When applicable, plant genotypes were randomized to minimize position-specific effects and conditions in Percival chambers were switched between experiments (e.g., 50% constant RH versus 50%/90% RH oscillating humidity) to minimize chamber-specific effects. When three humidity conditions were needed, e.g., when comparing leaf-rosette biomass accumulation (Fig. 1a, b), plants were exposed to oscillating humidity by manually moving them between the 50% RH chamber and the 90% RH chamber.

LUCIFERASE IMAGING CHAMBER AND ASSAY CONDITIONS. To simultaneously image luciferase reporter activities in two sets of soil-grown mature plants (up to 192) under LL and oscillating humidity, respectively, while controlling light and humidity conditions and images, we custom-built an imaging chamber with two compartments. Humidity was fed to the chamber by ultrasonic humidifiers (one Holmes humidifier for each compartment) and humidity in the room was kept at 50% RH using a dehumidifier (General Electric). Light was provided by red and blue light-emitting diode lights in the ratio of 2 to 1, respectively, and the fluence rate set at 100 µmol m$^{-2}$s$^{-1}$. Temperature fluctuations in the chamber were kept within 1°C by providing a constant flow of air at 22°C into the chamber with and without the mist. Three-week-old transgenic plants carrying luciferase reporter were sprayed with 2.5 mM luciferin (Gold Biotechnology) in 0.02% Triton X-100 (Sigma) at both mist. Three-week-old transgenic plants carrying luciferase reporters were sprayed with 2.5 mM luciferin and 12 h before being moved into the imaging chamber. In the imaging chamber, plants were kept under their growth conditions for at least 1 day to stabilize gene expression before imaging at 2 h intervals. Gene expression was determined by bioluminescence emission of the area around the shoot apical meristem. For each plant, bioluminescence data were monitored to its mean expression value or its peak expression value before humidity treatment and periodicity analysis was performed using the Biodare’s FFT-NLLS and LS tools with an expected period between 18 and 36 h. Rhythms with relative amplitude error ≤ 0.7 were considered rhythmic and their calculated amplitudes and circadian phases were averaged peaks relative to time 0 were reported. Supplementary Table 2 contains periodicity analysis using the Biodare’s LS tool and the synchronization index, which was calculated using the formula:

\[
\text{Sync} = \frac{1}{N} \sum_{k=1}^{N} \left[ \cos(2\pi k/N) + i \sin(2\pi k/N) \right]
\]

with \(k\) the calculated circadian phase and \(N\) the number of oscillators.

RNA extraction and quantitative PCR. Plant RNA extraction was performed according to protocols provided by the manufacturers of the TRIZOL reagent (Ambion) and the Direct-zol (Zymo). cDNA construction was performed using superscript III reverse transcriptase enzyme (Invitrogen); and quantitative PCR (SYBR Green PCR kit, Roche) was carried out according to the manufacturers’ protocols. Total bacterial RNA was extracted using the RNaseasy kit (Qagen) and the Bacteria Protect Reagent (Qiagen) following the manufacturer’s instructions. Brieﬂy, bacteria were re-suspended, washed twice in cold phosphate-buffered saline containing the Bacterio Protect Reagent and mechanically disrupted by 10 min of sonication (Bioraptor300). RNA was puriﬁed from the extract using the RNaseasy kit and DNA was removed by treating total RNA with two rounds of the Ambion DNAse I kit. cDNA was synthesized (Roche) and supercript III reverse transcriptase enzyme (Invitrogen) according to the enzyme protocol. Primer pairs (Supplementary Table 3) were selected to amplify regions of approximately 120 bp. Plant housekeeping gene UBIQUITIN 5 (UBQ5) and bacterial genes gyrA and gap1 were used as internal controls. When indicated, experimental replicates were combined using a linear mixed effect model (lme4 package in CRAN) before significant differences were determined by Student’s t-test or analysis of variance (ANOVA). Results are reported as mean ± s.d. of three independent experiments.

Fitness-related trait measurements. Plants derived from a single parent were grown in soil under the indicated conditions and randomized in 72-well flats with respect to their genotypes to minimize position-specific effects. Conditions in Percival chambers were switched between experiments to minimize chamber-specific effects. Leaf rosettes from three plants were harvested together 3 weeks after germination. Fresh weight was measured immediately after harvest and dry weight was measured after drying at 65°C for 7 days. Flowering time was reported as days to bolt (1 cm inflorescence). For seed mass and seed count measurements, plants were watered until leaf senescence was visible to at least 50% of leaves and then left to dry. Seeds from at least 12 plants were pooled and stored in a paper bag for at least 2 weeks before seed mass was measured using an analytical balance. Per experiment and condition, three samples of dry seed pools were weighed and seeds in each pool were counted to determine the average seed mass. Experimental replicates of seed mass and seed count were combined using a linear mixed effect model (lme4 package in CRAN) before significant differences were determined by Student’s t-test or ANOVA. Results are reported as mean ± s.d. of three independent experiments.

Pathogen infection. Whole leaves of 3-week-old soil-grown plants were infiltrated with Pst (OD600 = 0.001), Pst AvrRpt2 (OD600 = 0.0001) or Pst hrp1 (-) (OD600 = 0.001) in 10 mM MgSO4 and tissue collected 3 days after inoculation. Colony-forming units were counted according to Durrant et al. Ion leakage measurement. Ion leakage was determined by measuring electrolytes leaked from leaves inoculated with Pst AvrRpt2 (OD600 = 0.0001) or Pst hrp1 (-) (OD600 = 0.001). Ion leakage measurements were performed at 36 h post inoculation when ETI-associated PCD started to occur. Similar to the method described in Woo et al., two leaves per plant were immersed in 5 mL of 400 mM mannitol with gentle shaking for 2 h, after which the sample conductivity was measured using Orion Star series meter (Thermo scientific) and normalized to total conductivity determined after boiling samples for 10 min. Six samples per growth condition were reported collectively as mean ± s.d. The experiment was repeated three times with similar results.

Statistical analysis. Statistical analyses were performed using GraphPad Prism 6 or the R programming language (R version 3.3.1). All centre values shown in figures are means of biological or experimental replicates. When mixed linear model was used, statistical analysis was performed on the mean estimates and their standard deviations.

Data availability. The data that support the findings of this study are available in Supplementary Data 1 and in the Biodare repository.
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Author contributions

M.M. and X.D. designed the experiments. M.M., L.L., S.K, J.M. and E.M.M. performed the experiments and M.M. performed statistical analyses. N.E.B. and X.D. supervised the project. M.M., S.K., N.E.B. and X.D. wrote the paper with input from all co-authors.

Additional information

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