An Illuminated Respiratory Activity Monitoring System Identifies Priming-Active Compounds in Plant Seedlings

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Abstract

Background Growing large crop monocultures and heavily using pesticides enhances the chance of evolution of pesticide-insensitive pests and pathogens. To reduce pesticide use in crop cultivation, the application of priming-active compounds (PrimACs) is a welcome alternative. PrimACs strengthen the plant immune system and can thus help to protect plants with lower amounts of pesticides. PrimACs can be identified, for example, by their capacity to enhance the respiratory activity of parsley cells in culture as determined by the oxygen transfer rate (OTR) using the respiration activity monitoring system (RAMOS) or its miniaturized version, µRAMOS. The latter was designed for with suspensions of bacteria and yeast cells in microtiter plates (MTPs). So far, RAMOS or µRAMOS have not been applied to adult plants or seedlings which would overcome the limitation of (µ)RAMOS to plant suspension cell cultures.

Results In this work, we introduce a modified µRAMOS for analysis of plant seedlings. The novel device allows illuminating the seedlings and records the respiratory activity in each well of a 48-well MTP. To validate the suitability of the setup for identifying novel PrimAC in *Arabidopsis thaliana*, seedlings were grown in MTP for seven days and treated with the known PrimAC salicylic acid (SA; positive control) and the PrimAC candidate methyl 1-(3,4-dihydroxyphenyl)-2-oxocyclopentane-1-carboxylate (Tyr020). Twenty-eight h after treatment, the seedlings were elicited with flg22, a 22-amino acid peptide of bacterial flagellin. Upon elicitation, the respiratory activity was monitored. The evaluation of the OTR course reveals Tyr020 as a likely PrimAC. The priming-inducing activity of Tyr020 was confirmed using molecular biological analyses in *A. thaliana* seedlings.

Conclusion We disclose the suitability of µRAMOS for identifying PrimACs in plant seedlings. The difference in OTR during a night period between primed and unprimed plants was clearly distinguishable after elicitation with flg22. Thus, it has been shown, that the µRAMOS device can be used for a reliable screening for PrimACs in plant seedlings.

Background

As the world population continuously grows, there is an increased need for food, feed, fibers, and bioenergy (Oerke, 2006). These stocks are mostly derived from crops, such as corn, rice or wheat, that are usually grown in large monocultures. These are heavily sensitive to biotic and abiotic stress. Synthetic pesticides are widely used to effectively fight pests and pathogens. However, they can accumulate in the soil or plant and, thus, they raise ecological and health concerns. In addition, the heavy use of pesticides provokes the emergence of pests and pathogens with insensitivities to the chemicals (Oerke, 2006). To avoid the risks that comes with synthetic pesticides, exploiting the plant immune system emerged as a supportive, or even alternative, approach for eco-friendly plant protection (Conrath et al., 2015).

Upon attack by pathogens or after treatment with certain chemicals, plants can develop enhanced resistance to further pathogen attack (Beckers and Conrath, 2007). Upon attack by pathogens or after treatment with certain chemicals plants can develop resistance or enhance their resistance to further

pathogen attack (Beckers and Conrath, 2007). This phenomenon is referred to as induced resistance which is frequently associated with defense priming (Conrath, et al. 2015). The term refers to the enhanced capacity of plant cells to activate defense responses. Priming-associated induced resistance responses include systemic acquired resistance (SAR). SAR is a broad-spectrum immune response that is induced by salicylic acid (SA) and N-hydroxypipecolic acid (Ryals et al. 1996; Delany et al. 1994). Thus, upon treatment with N-hydroxypipecolic acid, SA or substances with a similar mode of action, plant cells are frequently primed for the enhanced activation of defense responses (Kauss et al., 1992; Thulke et al. 2015) and this usually comes with the establishment of SAR (Conrath et al., 2015. Defense priming, whether induced by SA, treatment with another chemical compound, or previous pathogen attack frequently enhances the capacity of the plant to also ward off abiotic stresses, such as salinity, drought, flooding, chilling, or heat (Janda et al. 1999; Senaratna et al. 2000; Savvides et al. 2016). Priming does not run up major fitness costs and is hardly prone to pest or pathogen adaptation (van Hulten et al., 2006; Martinez-Medina et al., 2016). Thus, triggering priming by eco-friendly chemicals represents a promising means for sustainable pest and disease control. However, identifying priming-inducing chemistry is difficult and applicable screening systems for priming activity are rare. Conrath and associates developed (Kauss et al., 1992), and recently optimized (Schillheim et al., 2018), a mid- throughput screen for compounds that prime microbial pattern-induced furanocoumarin secretion in parsley cell suspension cultures. In addition, using a respiratory activity monitoring system (RAMOS), Schilling et al. (2016) showed that priming-active compounds (PrimACs) can be identified by their capacity to enhance the respiratory activity, as measured by the oxygen transfer rate (OTR) of a parsley cell culture (Anderlei et al. 2001; Anderlei et al. 2004). For enhanced throughput, a µRAMOS device has been developed that utilizes parley suspension cells in 48-well microtiter plates (MTPs) (Flitsch et al., 2016).

Both, RAMOS and µRAMOS have been optimized for use with parsley cells in culture (Flitsch et al., 2016, Schilling et al., 2015; Schulte et al., 2018). To overcome the limitations of (µ)RAMOS to parsley cell suspensions, in this work a protocol for the use of intact plants in the µRAMOS device is developed. With the experimental setup, a light-emitting diode (LED) module is introduced, that enables each well of a 48-well MTP to be individually irradiated with white light to ensure synchronized seed germination. For a proof-of-principle a model screen for PrimACs has been performed using the newly synthesized methyl 1-(3,4-dihydroxyphenyl)-2-oxocyclopentane-1-carboxylate (Tyr020) as model test compound, which has been introduced by Krug et al. (2018). The power of the novel devise has been shown using SA as a positive control and spotting Tyr020 as a novel PrimAC in *A. thaliana*. Tyr020 was validated as a PrimAC using molecular biological analyses (Conrath, 2006).

Results

LED module - µRAMOS combination

A LED module was developed to enable the simultaneous germination of seeds, it enables adjusted light condition for germination and growth if needed (as it is the case for *A. thaliana* seedlings), an grows seedlings in each well of a 48-well MTP in controlled light condition. The module consists of 48

individually dimmable LEDs (Lumitronix), mounted on a plate of the size of a commercial 48-well MTP (**Figure 1 A)**. Optical isolators ensure that the light cannot enter neighboring wells, provided a black, clear-bottomed MTP is applied on top of the LED module. Because all wells are irradiated by an individual LED, equal conditions are maintained for each well, regardless of the well position. The µRAMOS device is applied atop the MTP (**Figure 1 B**) and enables a measurement of the respiration activity in each plate well. **Figure 1 C** displays a photograph of the setup with *A. thaliana* seedlings on top of the LED module with the attached µRAMOS device. For illustration purposes a translucent MTP has been drawn in this Figure.

Standard procedure to screen for PrimACs in plants using µRAMOS

We developed a standard procedure that enables the search for PrimACs in plants in a MTP using µRAMOS (**Figure 2**). *A. thaliana* seeds were surface sterilized with 70 % (v/v) ethanol (**Figure 2 A**). Using a seed dispenser sterilized seeds were individually transferred into the wells of an MTP (**Figure 2 B**). Sterile MS medium was added to each well (**Figure 2 C**). To ensure synchronous and effective germination of seeds, the MTP was kept for 24 h at 4 °C. Then, the MTPs were incubated for 3 weeks at 20 °C. During incubation, a 16 h day/ 8 h night cycle was applied using the LED module (**Figure 2 C**). After those 3 weeks, the seedlings were treated with SA or Tyr020 (**Figure 2 D**) and incubated for another 28 h in growth condition. Then, seedlings were sprayed with 50 pM flg22. After elicitation, the respiratory activity of the seedlings was measured for 45 h by applying the µRAMOS (**Figure 2 D**) atop of the MTP.

OTR of *A. thaliana* seedlings grown in the LED-µRAMOS combination

To validate the suitability of the novel LED-µRAMOS device for measuring the respiratory activity of *A. thaliana* seedlings, seedlings were grown in a MTP in the above described conditions in the LED-µRAMOS devise for 168 h. The OTR was monitored during the 16 h day/8 h night period. The course of the OTR over the 168 h period revealed that during the day, oxygen was produced as indicated by negative OTR values (**Figure 3**). During the night, low amounts of oxygen were consumed as obvious by positive OTR values (**Figure 3**). However, the quantities of oxygen consumed were rather low, as obvious by the OTR being about 0 mmol/L/h for most of the time in the night period. At the end of the fourth day period, 500 µL of sterile MS medium were added to each well to avoid desiccation of the seedlings. This stimulated increased oxygen consumption during the following night period, as seen in **Figure 3** at the 80 h time point. After supplementation with MS medium, the oxygen production during the following day periods was lower than at the day periods before.

Measurement of priming-inducting activity

The screening for PrimACs was performed as illustrated in **Figure 2**. *A. thaliana* seedlings were dived in six groups and, where appropriate, treated at -28 h before elicitation with flg22. Immediately after elicitation, the OTR measurement was started (**Figure 4**). In the first hour of recording, all measurements show significantly increased OTR values. These are various running-in phenomena of the µRAMOS device, for example the MTP needs to first reach the temperature of the system before a stable OTR value

can be measured. This period of the first hour is, therefore, irrelevant for the screening of PrimACs and is not further discussed. Twelve wells of the MTP were neither pretreated, nor elicited to obtain a negative control (**Figure 4)**. The course of the OTR of the negative control remained at a low level (~1 mmol/L/h). Seedlings in 5 wells were treated with 100 µM SA and seedlings in 3 wells supplemented with 25 µM Tyr020 at -28 h. These 8 wells were not supplied with 50 pM flg22. This we did to record the course of the OTR of pretreated, but unelicited plants (**Figure 4**).

The course of the OTR of the negative control remained at a low level (~1 mmol/L/h). Seedlings in 5 wells of the MTP were treated with 100 µM SA and seedlings in 3 wells supplemented with 25 µM Tyr020 at the -28 h time point. These 8 wells were not supplied with 50 pM flg22. This we did to record the course of the OTR of pretreated, but unelicited plants (**Figure 4**). The course of the OTR (red and blue dotted) curves of these seedlings were like those of the negative control (black graph**, Figure 4**). Furthermore, seedlings in 5 wells were not pretreated but elicited with 50 pM flg22 (green curve). After elicitation for 8 h, the OTR of the seedlings was significantly higher than that of the negative control. The OTR of the unprimed, but elicited seedlings remained high for 40 h. Seedlings in 3 wells were treated with 100 µM SA (red curve, positive control) or 25 µM Tyr020 (blue curve) at 28 h before the OTR measurement was being started. These seedlings were elicited with 50 pM flg22 at the 0 h time point. The OTR values of SA-pretreated and later flg22-elicited seedlings were much higher than those of unprimed, but elicited seedlings. The course of the OTR of Tyr020-treated and elicited seedlings was like the course of the OTR of the positive control. Thus, Tyr020 was identified as promising PrimAC candidate in *A. thaliana*.

To investigate whether Tyr020 would affect the infection of *A. thaliana* by the oomycete pathogen *Hyaloperonospora arabidopsidis* (*Hpa*), *A. thaliana* (Col-0) plants were sprayed with a wettable powder (WP) formulation of Tyr020 before they were inoculated with *Hpa*. The Noco race of *Hpa* causes downy mildew disease on *A. thaliana* Col-0 plants (Coates and Beynon, 2010). **Figure 5 B** shows that pretreatment with Tyr020 seems to reduce the susceptibility of *A. thaliana* to downy mildew disease, as obvious by reduced *Hpa* sporulation on Tyr020-pretreated plants. This is suggesting that Tyr020 activates defense priming and reduces the susceptibility to *Hpa* infection in *A. thaliana*.

To further investigate Tyr020's mode of action, the accumulation of phosphorylated mitogen-activated protein kinases (MPKs) was analyzed in *A. thaliana* seedlings upon adequate treatments. Beckers et al. (2009) demonstrated that in this plant defense priming is associated with enhanced levels of latent MPK3 and MPK6. However, these enzymes remained catalytically inactive until further stimulation of the plants (Beckers et al. 2009). Once challenged, more MPK molecules became phosphorylation-activated in primed compared to unprimed plants (Beckers et al., 2009). To investigate the phosphorylation of MPKs in Tyr020-treated *A. thaliana* seedlings, appropriately treated seedlings were subjected to western blotting analysis and immunodetection with phospho-motif-specific antibodies (**Figure 5 C**). Two untreated seedlings were used as a negative control, whereas two other seedlings were treated with 50 µM SA (positive control) to activate defense priming. Ten seedlings were treated with Tyr020 at concentrations from 1 to 25 µM. One seedling of each condition was later elicited with flg22. As shown in **Figure 5 C**, MPK phosphorylation was detected in all seedlings elicited with flg22. For the unpretreated but elicited

seedling, the phosphorylation signal was very low, compared to the elicited positive control. No or only weak MPK phosphorylation was found in all the unelicited samples. Except for 1 µM, Tyr020 with increasing concentration strongly enhanced the flg22-induced MPK phosphorylation signal that, at 25 µM, was almost as strong as the phosphorylation signal in seedlings that were primed with 50 µM SA and later challenged with flg22 (**Figure5 C**). This result supports the above conclusions from the µRAMOS and gene expression studies, claiming Tyr020 as a previously unknown PrimAC.

Discussion

An LED module was developed that allows equal light exposure of plant seedlings in each well of a 48-well MTP. Development of a similar illumination device has been published previously (Morschett et al., 2017). The described resulting improvements of individually illuminated plant cultivation could be reproduced with the introduced LED module. The addition of fresh medium to *A. thaliana* in the LED-µRAMOS lead to an increased OTR during the following night cycle, showing the increased metabolic activity of the plant seedlings due to the newly added nutrients. The MS medium supplemented with sucrose seems to satisfy the seedlings' carbon demand and, therefore, the photorespiration might have become lower. In the second- and third-night cycle after addition of fresh medium, the OTR was still higher than before addition. In summary, the addition of sterile medium was successful, so that in the future longer cultivation of plant seedlings is also conceivable. This result suggested that metabolic changes of the plant seedlings seem to be more promoted during the night cycle. Hence, the evaluation of candidates of PrimACs is best executed during an elongated night period. To mimic field conditions, the growth was performed applying a 8 h day/16 h night cycle, using the LED module. The beginning of the elongated night period was only associated with the addition of flg22. At the same time, the µRAMOS device was mounted on top of the MTP and the OTR measurement started. After elicitation, primed plant seedlings showed much higher OTR values than unprimed, but elicited, or only elicited plant seedlings (Fig. 4). Thus, PrimAC enhance the capacity of respiratory activity of intact plants. As this enhanced capacity of respiratory activity is just measured at this stage of experimental protocol, the µRAMOS device needs to be applied, while the germination, growth and priming can be performed separately in a microtiter plate. Through time-shifted initiation of cultivations in microtiter plates, the screening of 48 plant seedlings every 40 h per µRAMOS device becomes possible. The results of the model screening (Fig. 4**)** indicate, that it might be possible to reduce this measurement period to 8 h, since the difference between successfully primed plant seedlings and the corresponding negative control is most evident in the first 8 h after elicitation. A shortened measurement period would further enhance the throughput of the screening method to up to 48 plant seedlings per 8 h per µRAMOS device.

Conclusions

In this paper, the use of the µRAMOS for intact plant seedlings in 48-well MTP was demonstrated. The major advantage of using intact plants rather than cell suspension cultures is the differentiation status and to overcome the limitations to parsley and some other plant species. µRAMOS is likely compatible

with essentially any crop and, thus, closer to application. However, in contrast to soil-grown plants, measurements can be done at early growth stages in controlled conditions, as parameters like nutrition and light become adjustable. By switching from the RAMOS device, which allows simultaneous screenings in eight shake flasks, to the µRAMOS device, which enables 48 simultaneous screenings, the throughput per experiment was drastically increased. Based on the increased OTR after elicitation (Fig. 4), Tyr020 has been identified as previously unknownPrimAC.

The newly introduced LED-µRAMOS combination enables individual light intensities and irradiation periods for each well, while the OTR is independently measured online. This allows for different irradiation intensities, which might be required by different plant species within one experiment. In the future, candidates for PrimACs and combinations thereof could simultaneously be tested for their priming-inducing activity in different crops.

Methods

Chemicals

SA and BHT are commercially available and Tyr020 has been synthesized according to Krug et al.(2018). Krug et al. published this compound under the name compound 8a (Krug et al. 2018)

LED module

The LED module is custom-made by Lumitronix® and consists of 48 individual dimmable LEDs **(Figure 1**). The plate can be mounted underneath a transluminescent-bottomed 48-well MTP resulting in one LED beneath each well. To allow a light gradient within the plate, each LED is individually adjustable. Optical isolators prevent stray light from LEDs into neighboring wells. A black microtiter platebody needs to be used to eliminate stray light between wells.

Determination of the OTR using µRAMOS device

The OTR was determined using RAMOS (Anderlei et al. 2001, Anderlei et al. 2004) for MTPs (µRAMOS), which was developed and built in house (Flitsch et al. 2016, Schilling et al. 2015). Measurements were performed using black, clear bottom microtiter plates. The µRAMOS device was mounted on top of the MTP. The measurement was performed at 20°C with a shaking frequency of 600 rpm and a shaking diameter of 3 mm. The filling volume was 1.6 mL per well.

A. thaliana growth

A. thaliana (accession Col-0) seeds were washed in 70% (v/v) ethanol for 30 min and then twice in 100% (v/v) ethanol for 1 min, according to **Figure 2**. Dried seeds were transferred to a MTP using a seed dispenser to a number of 15 ± 5 seeds per well. The seeds were provided with 1.5 mL (MS) medium. MS medium, including vitamins (M0222; Duchefa Biochemie B.V.) was prepared as recommended by the

manufacturer and supplemented with 2.5 g/L sucrose. The pH value was adjusted to 5.7 using 0.01 M potassium hydroxide, before the medium was autoclaved (121°C, 20 min).

For stratification the seed-loaded MTPs were stored at 4 °C overnight to ensure synchronous and efficient germination. On the next days, the plates were cultivated on the LED module at 20 °C. After 3 weeks the µRAMOS device was mounted atop.

Cultivation, priming and elicitation of soil-grown *A. thaliana* seedlings

A. thaliana seedlings were grown on soil with a 16 h day/ 8 h night cycle applied in a pests-free room. When needed, the soil was irrigated. After 4 - 6 weeks, seedlings were used for conventional priming experiments. The leaves of *A. thaliana* seedlings were sprayed with Tyr020 dilutions in ascending concentrations between 1 µM and 100 µM. As positive control, the leaves of another plant seedling were sprayed with 100 µM BTH. As negative control leaves of plant seedlings were sprayed with 21 % (w/v) wettable powder in water. Every condition was applied on six plant seedlings each. The plant seedlings were further grown for 48 h, before three plants of each condition were infiltrated with 1 nM flg22 solution.

Determination of *WRKY6, WRKY53* and *Actin*2 expression

Five hours after elicitation, leaves were harvested and homogenized. RNA was isolated from frozen leaves using the TRIZOL method, as published by Chomczynski, 1993. The RT-qPCR reactions were performed in a 10 µL volume. The PCR mixture was consisting of 2.7 µL nuclease free water, 5 µl Takyon TM No Rox SYBR MasterMix dTTP Blue (Eurogentec), 0.15 µL forward primer, 0.15 µL reverse primer, and 2.5 µL template. The according primers are listed in **Table 1**. The amplification was performed using a PCR machine (Applied Biosystems). The PCR was performed for 3 min at 95°C as initial denaturation, followed by 40 cycles of 15 sec at 95 °C for denaturation, 60 sec at 60°C as annealing, 15 sec at 95°C for extension. The final extension was set to 60°C for 1 min.

Table 1: Primers used for RT-qPCR.

Primer	Sequence 5´-3´
WRKY6	ACTTCACGGTCATTATCTCCAGC
	TGAATTTAGGTTTCCGGTGAGTC
WRKY53	CTCCATCGGCAAACTCTTCAC
	CCGAGCGTACAACTTATTCCG
Actin2	GGTAACATTGTGCTCAGTGGTGG
	GGTGCAACGACCTTAATCTTCAT

SDS-PAGE, western-blotting analysis and immunodetection

To detect MPK phosphorylation sites, leaves of the soil-grown seedlings were harvested and homogenized at 5 h after elicitation. Frozen tissue was either ground using the Precellys 24 homogenizer (Bertin Instruments). All tubes and buffers were kept cold during the procedure. Ground plant material was washed twice with 100 % acetone and centrifuged at
16,100 *g* at 4 °C for 5 min. The pellet was resuspended in 10 % (w/v) TCA in acetone and were transferred to an ultrasound ice-water bath for 10 min followed by another centrifugation step (16,100 *g*, 4 °C, 5 min). Then, the pellet was washed once with 10 % (w/v) TCA in acetone, once with 10 % TCA (w/v) and once with 80 % (v/v) acetone. The supernatant was discarded and the pellet was resuspended at RT in freshly prepared dense SDS buffer (100 mM Tris-HCl pH 8.0, 30 % (w/v) sucrose, 2 % (w/v) SDS, 5 % (v/v) β-mercaptoethanol). An equal volume of phenol/Tris-HCl pH 8.0 (Applichem) was added to each tube. Samples were centrifuged at 16,100 *g* at RT for 20 min. The upper phase of each tube was split into two new tubes, 5 volumes of 100 mM ammonium acetate in methanol were added and total protein was precipitated at -20 °C for 60 min. Proteins were collected by centrifugation at 16,100 *g* at 4 °C for 5 min. Pellets were washed once with 100 mM ammonium acetate in methanol and once with 80 % (v/v) acetone and the pellets were dried. To determine total protein concentration, pellets were resuspended in buffer (7.7 M urea, 2 M thiourea, 300 mM NaCl, 0.25 % (w/v) CHAPS, 50 mM NaH_2PO_4 pH 8, 50 mM Tris pH 8, 20 mM imidazole) and incubated at RT for 1 h before Bradford protein assay (Quick-Start Bradford; BioRad) was performed. Protein samples were resuspended in loading buffer (10X Sample Reducing Agent, 4X LDS Sample Buffer; NuPAGE) to equal concentrations and heated at 95 °C for 10 min. The denatured samples were applied to a two parted polyacrylamide gel, consisting of a 4% collection gel and a 12% separation gel. The pockets were loaded with 5 µL sample and gel electrophoresis was performed at 175 V for 60 min. MES, pH 7.3, was used as running buffer.

For the subsequent western blot, the proteins were electrophoretically transferred from the SDS gel to a nitrocellulosemembrane (Carl Roth) applying 60 min at 250 mA. After blotting, the membrane was washed 2 times for 5 min each in TBST buffer (20 mM tris; 150 mM NaCl; 0.1 % (v/v) Tween 20). The blocking was performed for 60 min in a 5% (w/v) skimmed milk powder (Nutricia Protifar) in TBST buffer solution. After blocking, the membrane was washed 2 times for 5 min each in TBST buffer. The primary antibody anti p44/42 (Cell SignalingTechnologyR) was diluted 1:1000 in a 5% (w/v) Bovine Serum Albumin (Panreac AppliChem) in TBST buffer solution. The membrane was incubated for 12 h at 10 °C. After incubation, the membrane was washed twice for 5 min in TBST buffer.

As secondary antibody, the anti rabbit IgG, HRP-linked Antibody #7074 (Cell SignalingTechnologyR) was applied. It was diluted 1:2000 in a solution of 5 % (w/v) skimmed milk powder in TBST buffer solution. The membrane was incubated for 1 h at room temperature in the solution, before being washed twice in TBST for each 5 minutes.

For protein detection, the ChemiDocTMMP Imagine System (BioRadR) with white light irradiation was used.

Ponceau staining

To check for equal protein loading the membrane was stained with Ponceau S. For staining the Ponceau S Solution (Panreac AppliChem) was used, and the producer's protocol was followed precisely.

Determining *A. thaliana* susceptibility to *Hyaloperonospora arabidopsidis* (Hpa)

A. thaliana Col-0 plants were grown in short day (8 h light of 120 µmol/m^2/s^1, 22 °C, 65% relative humidity). Two-week-old plants were sprayed with wettable powder (WP) (control) or a WP formulation of Tyr020 (final concentration 25 µM). 24 h after treatment, plants were inoculated by spraying with a conidiospore suspension of Hpa (race Noco; 4 x 10^4 spores per mL of water). Inoculated plants were covered with a transparent lid to ensure high humidity and kept in short day condition. After 7 d, the number of spores released by Hpa was determined as described by Schmitz et al. (2010).

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

All authors agree that the present work will be published.

Availability of data and materials

The raw data cannot be published as this service of the university is closed to unknown date due to Covid-19. All data are shown in the article, the raw data are available on request as they are too large for supplementary material.

The datasets used and/or analysed in the current study are available on reasonable request from the corresponding author.

Competing interest

The authors declare that they have no competing interest.

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Authors' contributions

All authors contributed to the design of the research; A.M., S.W., A.B. K.S., and R.K. performed the research; all authors analyzed and discussed the data; J.L. drafted the manuscript. J.B. U.C., U.S., and J.P. read, commented, and revised the manuscript. J.B. initiated the study, assisted with data analysis and manuscript preparation. All authors read and approved the final manuscript.

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Figures

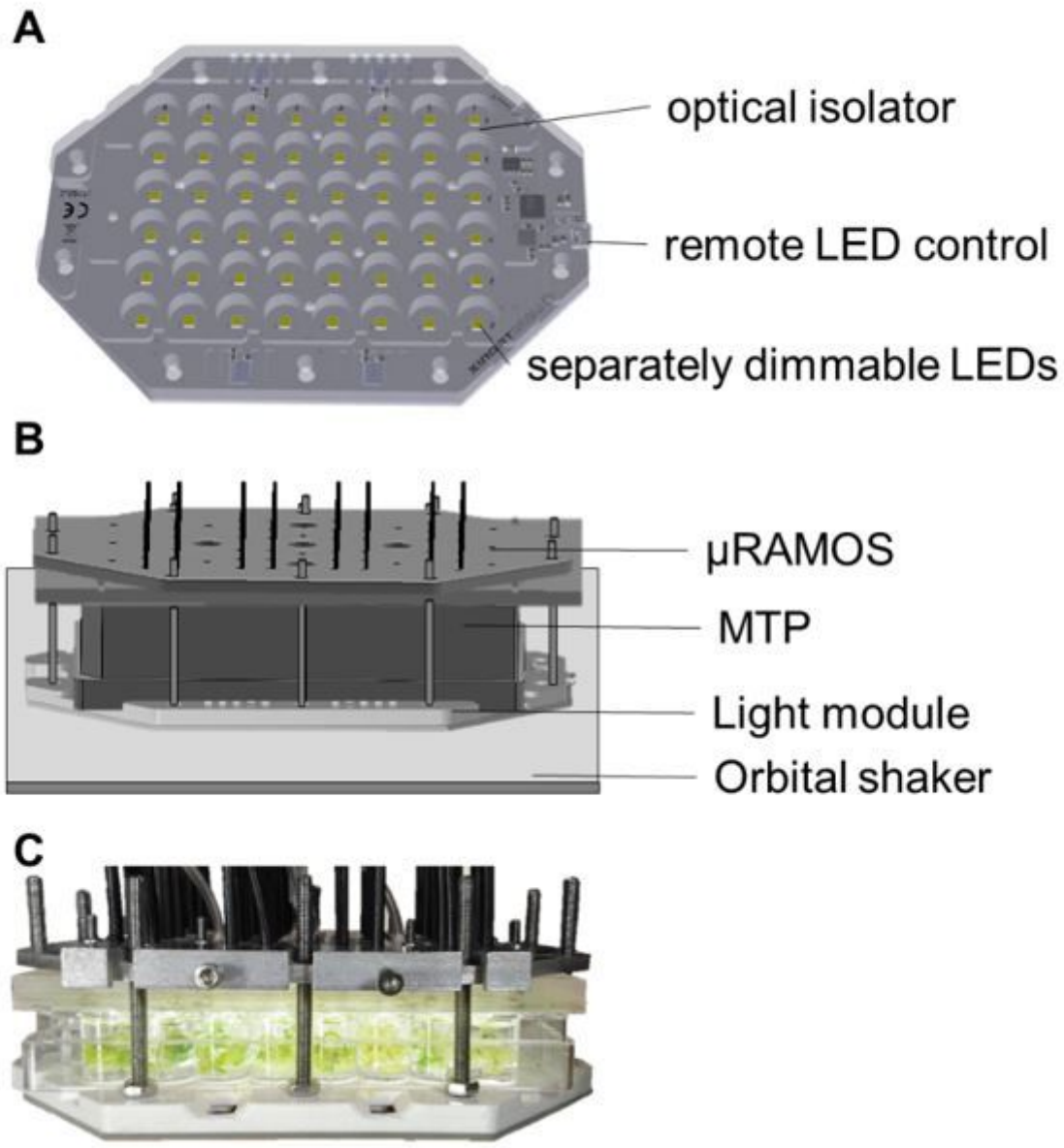

Figure 1

(A) Custom-made LED module. 48 individually dimmable LEDs for each well of a MTP. An optical isolator prevents interference between individual wells of the MTP. (B) Combination of the LED module with a µRAMOS device. The LED module is mounted below the MTP. µRAMOS measures the partial pressure of oxygen in each well and calculates the resulting OTR. (C) Photograph of the assembled device with A. thaliana plant seedlings. Translucent MTPs were used for illustration purposes. For online monitoring, black MTPs with transparent bottoms were used.

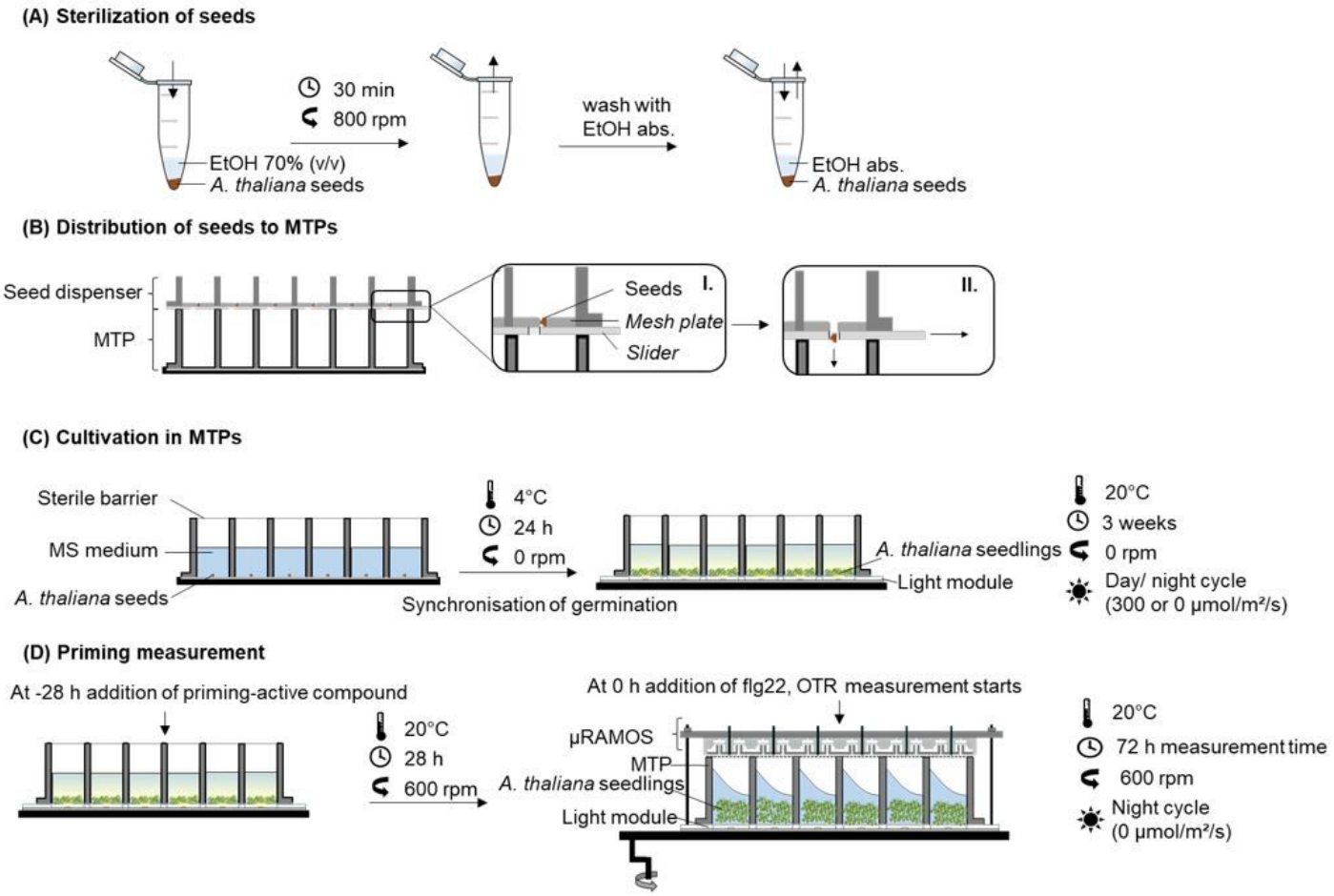

Figure 2

Scheme of the setup for PrimAC identification using A. thaliana seedlings in the LED-µRAMOS device. (A) Seeds were sterilized by washing with 70% (v/v) ethanol and ethanol absolute (EtOH abs.) (B) A seed dispenser was used to transfer sterilized seeds to the wells of a MTP. (C) Cultivation in MTPs for the effective and synchronized germination of seeds. A. thaliana was grown on 1.5 mL MS medium for 3 weeks at a 16-h day/8-h night cycle. (D) The respiratory activity was measured in the absence of light at 20 °C and 600 rpm shaking frequency with a shaking diameter of 3 mm. Treatment with compounds was done 28 h before the OTR measurement were started. Seedlings were elicited using 50 pM flg22 at time zero (0 h).

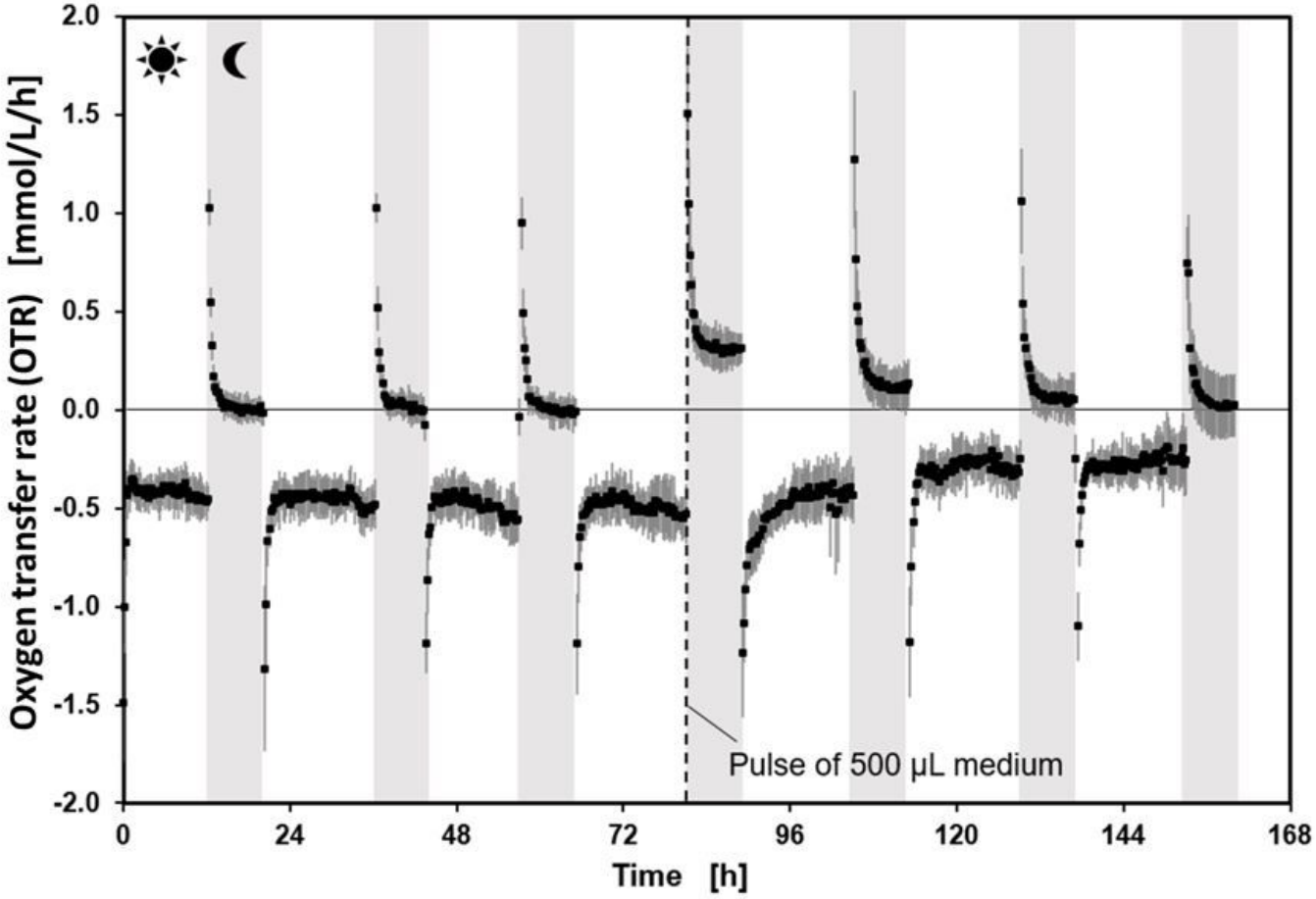

Figure 3

OTR of A. thaliana seedlings during x-h day/y-h night cycle in the LED-µRAMOS combination. The white and grey background illustrate day and night periods, respectively, and are indicated by sun or moon. Three-week old A. thaliana seedlings, grown as in Fig. 2 C, were transferred to the LED-µRAMOS combination and kept at 400 rpm shaking frequency, 3 mm shaking diameter, 20 °C, in 1.5 mL MS medium with 2.5 g/L sucrose, initial pH 5.7, light module irradiation intensity 300 µmol/m²/s at day time, 0 µmol/m²/s at night time. The standard deviation of 12 replicates is represented by the grey shadow around the data points.

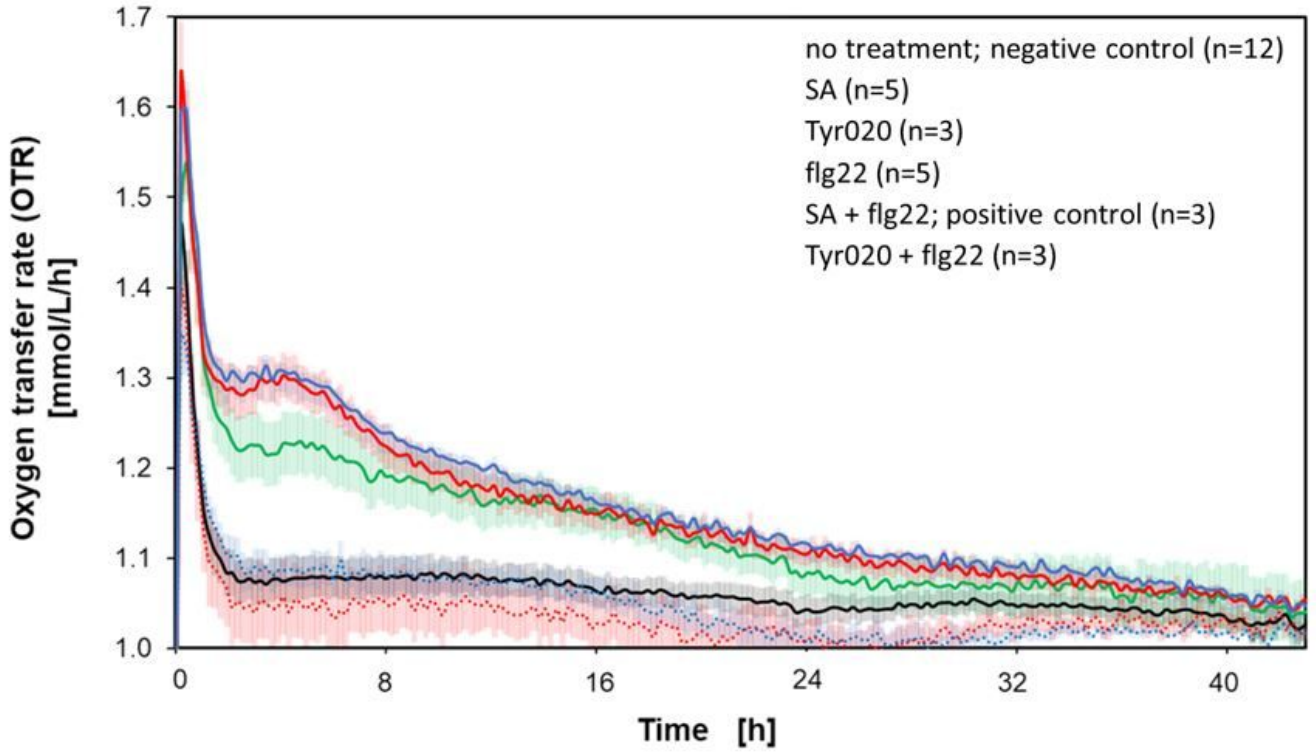

Figure 4

Respiratory activity of A. thaliana seedlings after treatment with priming compounds with and without flg22 elicitation, and reference cultivation without treatment with chemicals. OTR as a function of time of A. thaliana seedlings primed with 100 µM SA and 50 pM flg22 (red) or 25 µM Tyr020 and 50 pM flg22 (blue). Reference cultivations: without additives, negative control (black), addition of 100 µM SA only (red dotted line), 25 µM Tyr020 (blue dotted line), and with the addition of 50 pM flg22 only (green line). SA and Tyr020 were added at the -28 h time point relative to the time point of flg22 addition (0 h). Three-week-old A. thaliana seedlings were transferred to the µRAMOS device, according to Fig. 2 D, and cultivated at 600 rpm shaking frequency, 3 mm shaking diameter, filling volume 1.5 mL, 20 °C, in MS medium with 20 g/L sucrose, initial pH 5.7. The number of replica (n) is specified for each condition in the legend. The standard deviation of replicates is indicated by the shadow surrounding the data.

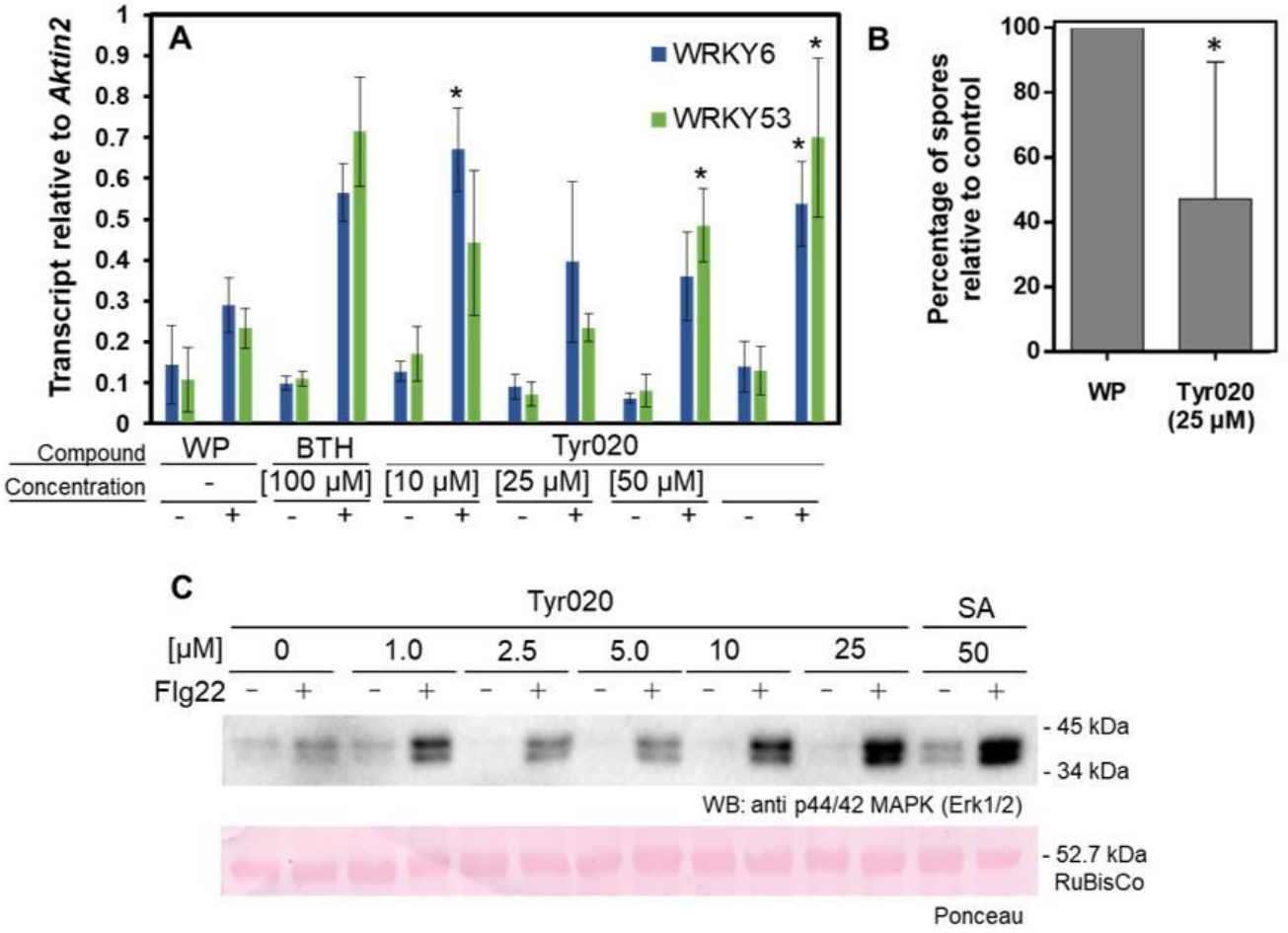

Figure 5

Verification of identified priming compound in soil grown A. thaliana plants. (A) Tyr020 primes A. thaliana plants for enhanced WRKY6 and WRKY53 defense gene activation. Six-week-old plants remained untreated (-) or sprayed with wettable powder (+) or the indicated concentration of Tyr020 in wettable powder (WP) (+) before they were elicited (+) or not (-) with flg22. As positive control 100 µM Benzothiadiozole (BTH) was used. The accumulation of WRKY6 and WRKY53 mRNA transcript was determined by qRT-PCR. Stars (*) indicate significant differences between control (WP; +) and sample ($p \leq 0.05$). (B) Sulforaphane (SFN) reduces downy mildew disease. Plants were treated with WP or a WP formulation of Tyr020 (25 µM). Twenty-four hours later, we spray-inoculated plants with a suspension of Hyaloperonospora arabidopsidis (Hpa) conidiospores (4 x 104 spores per mL of water). Inoculated plants were kept at high humidity in short day. After 7 d, we determined the number of spores released by Hpa. Data were analyzed by Student's t test. Asterisk denotes a statistically significant difference with 95% confidence. Data presented are means +/- SD. n > 5. (C) A. thaliana seedlings pretreated with Tyr020 have enhanced phosphorylation of mitogen-activated protein kinase (MPK) activation motifs upon flg22 challenge. This indicates stronger MPK activation in Tyr_020-pretreated plants. As positive control A. thaliana seedlings were treated with 50 µM salicylic acid (SA).