



The local and systemic accumulation of ethylene determines the rapid defence responses induced by flg22 in tomato (*Solanum lycopersicum* L.)

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ABSTRACT

Plant defence responses induced by the bacterial elicitor flg22 are highly dependent on phytohormones, including gaseous ethylene (ET). While the regulatory role of ET in local defence responses to flg22 exposure has been demonstrated, its contribution to the induction of systemic responses is not clearly understood. For this consideration, we examined the effects of different ET modulators on the flg22-induced local and systemic defence progression. In our experiments, ET biosynthesis inhibitor aminoethoxyvinyl glycine (AVG) or ET receptor blocker silver thiosulphate (STS) were applied 1 h before flg22 treatments and 1 h later the rapid local and systemic responses were detected in the leaves of intact tomato plants (*Solanum lycopersicum* L.). Based on our results, AVG not only diminished the flg22-induced ET accumulation locally, but also in the younger leaves confirming the role of ET in the whole-plant expanding defence progression. This increase in ET emission was accompanied by increased local expression of *SLACO1*, which was reduced by AVG and STS. Local ET biosynthesis upon flg22 treatment was shown to positively regulate local and systemic superoxide (O_2^-) and hydrogen peroxide (H_2O_2) production, which in turn could contribute to ET accumulation in younger leaves. Confirming the role of ET in flg22-induced rapid defence responses, application of AVG reduced local and systemic ET, O_2^- and H_2O_2 production, whereas STS reduced it primarily in the younger leaves. Interestingly, in addition to flg22, AVG and STS induced stomatal closure alone at whole-plant level, however in the case of combined treatments together with flg22 both ET modulators reduced the rate of stomatal closure in the older- and younger leaves as well. These results demonstrate that both local and systemic ET production in sufficient amounts and active ET signalling are essential for the development of flg22-induced rapid local and systemic defence responses.

1. Introduction

Plants being sessile organisms are especially exposed to adverse environmental conditions thus, their ability to respond to these changes or pathogen attacks in a short time has crucial importance for successful defence (Kollist et al., 2019). To cope with pathogen challenges, host plants have developed specialized pattern recognition receptors (PRRs) to recognize the evolutionary-conserved microbe- or pathogen-associated molecular patterns (MAMPs or PAMPs) after pathogen infection resulting in PAMP- or effector-triggered immunity (PTI and ETI) (Jones and Dangl, 2006). Integration of these localized stimuli is indispensable for the activation of rapid systemic signalling that reaching the distal, uninfected tissues in a short time (within 6 h)

can elicit whole-plant expanding responses termed systemic acquired resistance (SAR) (Fichman and Mittler, 2021; Johns et al., 2021; Kachroo and Robin, 2013).

In the last years, numerous molecules have been identified as potent long-distance signal transducers upon primary pathogen infection such as reactive oxygen- and nitrogen species (ROS and RNS), methyl-salicylate (MeSA), azelaic acid (AZA), ethylene (ET), jasmonic acid (JA), pipecolic acid (Pip) and monoterpenes (Fichman and Mittler, 2021; Gao et al., 2021; Shah and Zeier, 2013; Wang et al., 2014). Among them, ROS and calcium waves have been assigned to be the first signals activated in rapid systemic responses, moreover, these transducers integrate other long-distance signals on the whole plant level (Fichman and Mittler, 2021; Klessig et al., 2018). Slower responses activated even

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after hours also can be modulated by rapid systemic signals leading to an overall acclimated state to withstand pathogen challenges (Kollist et al., 2019).

Earlier it was supposed, that long-distance signals are primarily generated and transported via the phloem, however, the novel studies are attaching much more importance to volatiles in orchestrating systemic responses (Gao et al., 2021; Hammerbacher et al., 2019; Vlot et al., 2021). The gaseous ET can easily diffuse from the site of production to nearby cells, regulating germination, nutrient acquisition, senescence, ripening, physiological and molecular mechanisms under optimal or stressful conditions (Fatma et al., 2022; Baharudin and Osman, 2023). It has a predominant role in modulating JA- and SA-dependent responses rather than provoking them, by activating the mitogen-activated protein kinase (MAPK) cascade as well as by ET response transcription factors (ERFs) during the signalling processes (Broekgaarden et al., 2015). Enhanced ET production was observed during the establishment of hypersensitive response (HR) in *Arabidopsis* showing a maximum after 2 h upon avirulent *Pseudomonas syringae* pv. *tomato* infection (Mur et al., 2009). In tobacco leaves, ET production showed a biphasic pattern following *Pseudomonas syringae* infection, in which the first rise was observable within 1–4 h post-inoculation whereas the second one in 6 h (Mur et al., 2008). Although ET was thought not to play a crucial role in the establishment of SAR, by positively regulating the accumulation of ROS and other volatiles as well as acting as an airborne signal in itself, this issue needs to be clarified (Fu and Dong, 2013; Pierik et al., 2014). Diffusing freely from the producing cells primarily steers local responses, however, it can be transported through the aerenchyma, as well as its immediate precursor (1-aminocyclopropane 1-carboxylic acid; ACC) can be transported in the phloem to distal tissues, therefore it can function as a potential long-distance signal (McManus, 2012; Polko and Kieber, 2019).

One of the best-characterized elicitors triggering plant defence responses is the 22-amino acid epitope of the bacterial flagellum, flg22, which binding to its cell-surface located receptor FLS2 can induce rapid defence responses (Felix et al., 1999; Macho et al., 2012; Spoel and Dong, 2012). Flg22 perception triggers proton and calcium influx in minutes, as well as the generation of ROS even in 30 min (Chi et al., 2021; Yuan et al., 2021). In *Arabidopsis* leaves flg22 induced fast stomatal closure within 15 min by the activation of SLAC1 and SLAH3 anion channels (Guzel Deger et al., 2015). Rapid changes in the transcription profile were also observed after 30- or 60-min-long flg22 treatment in *Arabidopsis* seedlings, which primarily involved genes encoding components of signal transduction and defence regulation such as ET biosynthesis and response factors (Navarro et al., 2004). The contribution of ET signalling to flg22-induced local defence regulation was confirmed by ET insensitive (*etr1*, *ein 1*) *Arabidopsis* mutants which showed diminished oxidative burst (Mersmann et al., 2010). BIK1, a receptor-like cytoplasmic kinase involved in flg22 perception also mediates ET signal transduction, moreover, its activity increased following ACC treatment whereas decreased upon the blockage of ET perception suggesting a primary role of ET in flg22-induced responses (Laluk et al., 2011; Lu et al., 2010). ET production following flg22 perception was increased already after 1–2 h in contrast to SA, confirming its pivotal role in early defence responses (Felix et al., 1999; Mersmann et al., 2010) but its systemic effects remained uninvestigated.

Although many of the signalling components of systemic defence responses have been identified regarding their interaction and hierarchy, several questions remained unanswered (Fichman and Mittler, 2021). An additional difficulty in the field of investigating fast, systemic responses is that most of the experiments are carried out on detached organs (Czékus et al., 2020). Connected to this issue, whereas the function of ET in flg22-induced local defence responses has been well-described, much less is known about its potential role as a volatile compound in regulating fast systemic responses, moreover, its contribution to systemic defence development has still been contradictory.

In this work, our aim was to examine the potential role of ET in the

rapid local- and systemic defence progression upon flg22 treatments. By applying ET biosynthesis inhibitor aminoethoxyvinyl glycine (AVG) as well as silver thiosulphate (STS) an inhibitor of ET perception, both the role of ET biosynthesis as well as signalling were tested locally and systemically in rapid defence responses triggered by flg22 in intact tomato plants.

2. Materials and methods

2.1. Plant growth and experiment conditions

Seeds of tomato plants (*Solanum lycopersicum* L. cv. Ailsa Craig) were germinated in the dark at 27 °C for three days, then seedlings were placed and grown in perlite for the next two weeks before transferring them into pots (500 ml) in hydroponic culture. The nutrient solution used for experimental plant cultivation was described earlier by Poór et al. (2011) [2 mM Ca(NO₃)₂, 1 mM MgSO₄, 0.5 mM KH₂PO₄, 0.5 mM Na₂HPO₄, 0.5 mM KCl, 0.02 mM Fe(III)-EDTA and micronutrients (0.001 mM MnSO₄, 0.005 mM ZnSO₄, 0.0001 mM CuSO₄, 0.0001 mM (NH₄)₆Mo₇O₂₄, 0.0001 mM AlCl₃, 0.0001 mM CoCl₂, 0.01 mM ₃BO₃] and it was changed three times a week. For growing plants, a controlled environment was established based on Czékus et al. (2021a) with a photosynthetic photon flux density of 200 μmol m⁻² s⁻¹ [PPFD; White LED (5700 K) illumination supplemented with FAR LEDs; PSI, Drásov, Czech Republic], 12–12 h of light/dark period, day-night temperatures of 24/22 °C and a relative humidity approximately between 55 and 60%. All of the experiments were conducted by using 7- to 8-week-old intact plants with 8–9 developed leaves.

2.2. Treatments

To examine the role of ET in flg22-induced fast local and systemic defence responses in detail, plants were co-treated with flg22 (5 μM) and ET biosynthesis inhibitor aminoethoxyvinyl glycine (AVG; 10 μM) or silver thiosulphate (STS; 20 μM) which inhibits the ET perception or signalling. STS stock solution was prepared according to Poór et al. (2013) by mixing 0.1 M sodium thiosulphate and 0.1 M silver thiosulphate in 1:4 M ratio. The treatments were applied with a squirrel hair brush to the abaxial side of all leaves at the selected leaf levels, as this is where most of the stomata in tomato plants are located. While AVG treatments were applied on the 6th leaf level from the apex (older leaves), STS treatments were carried out on the upper leaves of the 5th leaf levels (younger leaves) at 7:00 a.m. One hour later, at 8:00 a.m., plants were treated on the 6th leaf level from the apex with 5 μM flg22 (Genscript Biotech Corporation, Piscataway, NJ, USA) dissolved in sterile distilled water (Czékus et al., 2021b). As a control treatment, sterile distilled water was applied on the 6th or 5th leaves from the apex. As a positive control of ET response, ACC (10 μM) was applied on the 6th leaves from the apex at 8:00 a.m. Defence responses of plants were examined 1 h later of flg22 or ACC treatments using both leaf levels (9:00 a.m.) Local defence responses were detected using leaves from the treated older (ACC, AVG, flg22, water) 6th leaf levels from the apex, while the induction of whole-plant expanding, systemic defence progression was investigated using the upper, untreated younger leaves. In the case of STS, we treated leaves on the 5th leaf levels from the apex, to study the importance of ET signalling in the flg22-mediated systemic defence mechanisms (Fig. 1).

2.3. Measurement of ET production

Emission of ET by plant leaves was measured with a Hewlett-Packard 5890 Series II gas chromatograph (GC) equipped with a flame ionization detector and a column packed with activated alumina according to Poór et al. (2015). Leaf sample (0.5 g) was collected into gas-tight flasks, which contained 0.5 ml of deionized water to prevent its dehydration, then incubated for 1 h at dark. After that, with a gas-tight syringe, 2.5 ml

Experimental setup

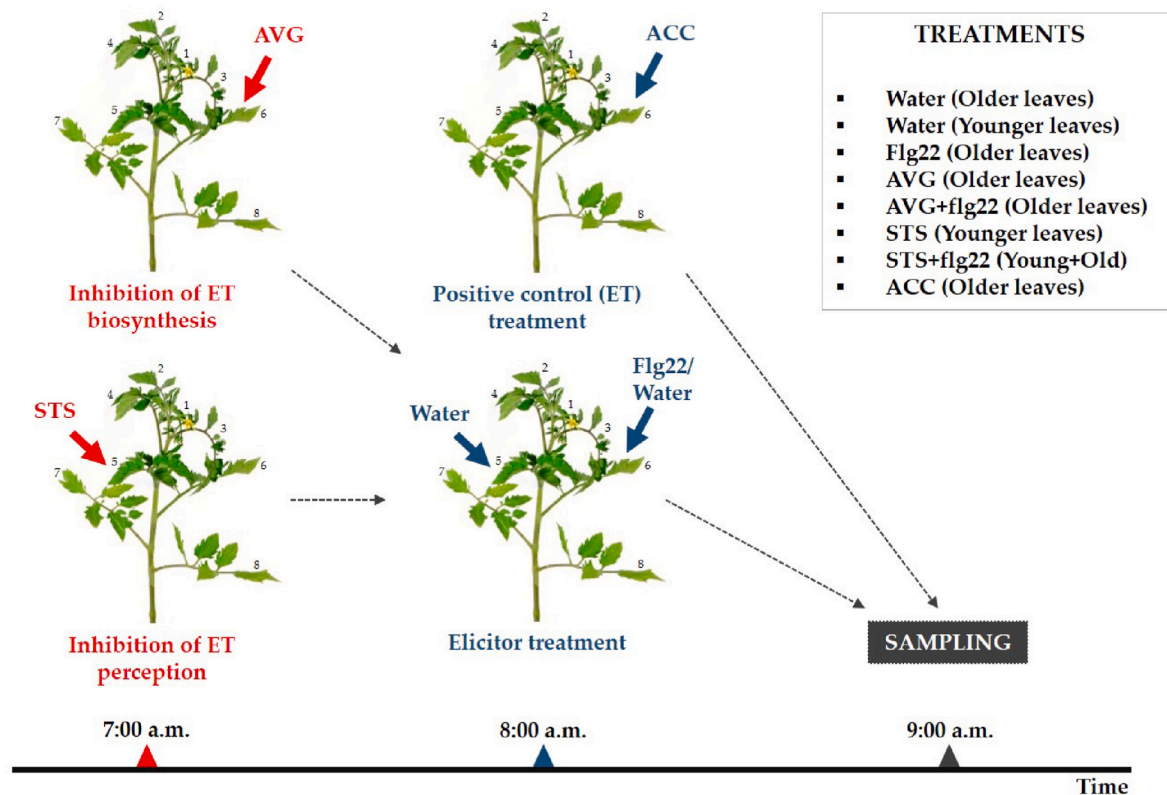


Fig. 1. The experimental setup of ethylene (ET) modulators and flg22 treatments. The abaxial side of leaves of intact tomato plants on the 6th leaf level (older leaves) from the apex was treated with ET biosynthesis inhibitor aminoethoxyvinyl glycine (AVG) to inhibit local ET production or with ET perception blocker silver thiosulphate (STS) on the 5th leaf level (younger leaves) from the apex at 7:00 a.m. to inhibit the ET sensing in the systemic leaves. One hour later, at 8:00 a.m., plants were treated on the 6th leaf level from the apex with 5 μ M flg22 dissolved in sterile distilled water. Sterile distilled water was applied on the 6th or 5th leaf levels from the apex as a control treatment. As a positive control of ET response, ACC was applied on the 6th leaf level from the apex at 8:00 a.m. Sampling was performed 1 h later after flg22 or ACC treatments by using both leaf levels (9:00 a.m.).

of gas was removed from the flasks and injected into the GC. The content of ET generated by the leaves was determined using a set of ET standards.

2.4. RNA extraction, gene expression analyses by quantitative real-time PCR

Total RNA from tomato leaves was extracted with TRI reagent (Chomczynski and Sacchi, 1987). Leaves from at least three different plants were collected and mixed in the case of each sample. Following extraction, genomic DNA was digested by DNase I enzyme (Fermentas UAB, Vilnius, Lithuania). The RNA content of samples was determined by NanoDrop™ 1000 Spectrophotometer (NanoDrop Technologies, Washington, DC, USA). Thereafter, cDNA was synthesized by MMLV reverse transcriptase (Fermentas UAB, Vilnius, Lithuania) using random hexamer primer (Fermentas UAB, Vilnius, Lithuania). The relative transcript level of the selected tomato genes [*SLACS6* (Solyc08g008100): F: 5'-AGGGTTTCCTGGATTAGGG-3', R: 5'-GACAACGGCATCATGTG-TACG-3'; *SLACO1* (Solyc07g049530): F: 5'-ATGTCCTAAGCCCG ATTGA-3', R: 5'-CCTCCTGCGTCTGTATGAGC-3'), mined from Sol Genomics Network (SGN; <http://solgenomics.net/>) database] was examined by quantitative real-time reverse transcription-PCR (qRT-PCR; qTOWER Real-Time qPCR System, Analytik Jena, Jena, Germany) based on Takács et al. (2016). The PCR reaction mixture contained 5 μ L of Maxima SYBR Green qPCR Master Mix (2 \times) (Thermo Scientific,

Waltham, MA, USA), 3 μ L of Molecular Biology Water (AccuGENE®, Lonza Group Ltd, Basel, Switzerland), 400 nM forward and 400 nM reverse primers and of 10 ng of cDNA template at a final volume of 10 μ L. Following an initial denaturation step at 95 °C (7 min), the qPCR programme consisted of 40 cycles of denaturation (95 °C, 15 s) and annealing extension (60 °C, 60 s). Data were analysed by calculating with $2^{(-\Delta\Delta Ct)}$ formula (Livak and Schmittgen, 2001), where the *Elongation factor-1 α* subunit served as a reference (qTOWER Software 2.2; Analytik Jena, Jena, Germany). Data were normalized to the transcript levels of the reference gene, and to the transcript levels of untreated control leaves.

2.5. Quantitative and qualitative determination of superoxide radical (O_2^-) content

Quantitative determination of superoxide radical (O_2^-) levels was carried out spectrophotometrically according to Chaitanya and Naithani (1994). 100 mg of leaf tissue was homogenized with 1 ml of 0.1 M sodium phosphate buffer (pH 7.2) containing superoxide dismutase inhibitor [1 mM sodium diethyldithiocarbamate trihydrate (SDDT; Sigma-Aldrich, St. Louis MO, USA)]. Following the 15-min-long centrifugation at 13,000 RPM and 4 °C, 0.3 ml of supernatant was added into a reaction mixture containing 0.65 ml of 0.1 M sodium phosphate buffer (pH 7.2) and 50 μ L of 12 mM nitroblue tetrazolium (NBT; Sigma-Aldrich, St. Louis MO, USA). The absorbance of the samples was

measured in the 2nd (A0) and 7th (AS) min of the incubation at 540 nm with spectrophotometer (KONTRON, Milano, Italy). Production of O_2^- was determined using $\Delta A540 = AS - A0$ formula and expressed as $\Delta A540 \text{ (min}^{-1} \text{ g}^{-1} \text{ fresh mass)}$.

For the qualitative detection of O_2^- accumulation NBT staining assay was performed (blue colour development) based on Overmyer et al. (2000). Leaves of tomato plants were incubated in 0.1% (w/v) NBT prepared with 10 mM potassium phosphate buffer (pH 7.0) for 1 h subsequently after sampling. To eliminate pigments from the leaves, samples were boiled at 96 °C in 96% (v/v) ethanol for 30 min, then washed with 96% (v/v) ethanol. The leaf samples were stored in glycerine:distilled water:ethanol (45:45:10) solution and photographed with a Canon EOS 700D camera (Tokyo, Japan) under white transillumination (Dark Hood DH-50, Biostep, Germany).

2.6. Quantitative and qualitative determination of hydrogen peroxide (H_2O_2) levels

Leaf tissue (200 mg) was ground with 1 ml of 0.1% (w/v) ice-cold trichloroacetic acid (TCA), then centrifuged (12,000 RPM, 4 °C) for 10 min. 0.25 ml of supernatant was added into a reaction mixture containing 0.25 ml of 50 mM potassium phosphate buffer (pH 7.0) and 0.5 ml of 1 M potassium iodide (KI). Samples were placed in the dark for 10 min, then the absorbance of samples was measured at 390 nm spectrophotometrically (KONTRON, Milano, Italy) (Velikova et al., 2000). The H_2O_2 content of samples was determined by standard H_2O_2 curves. All chemicals originated from Sigma-Aldrich (St. Louis, MO, USA).

The production of H_2O_2 in leaf tissues was also visualized histochemically with 3,3'-diaminobenzidine tetrahydrochloride hydrate (DAB) staining (brown colour development) according to Thordal-Christensen et al. (1997). Detached leaves were incubated in 1 mg/ml DAB (Fluka, USA) solution prepared with 10 mM potassium phosphate buffer (pH 7.0) for 2 h, then pigments were eliminated from the leaf tissues by boiling them at 96 °C in 96% (v/v) ethanol for 30 min. At the end of the boiling, leaves were rinsed with 96% (v/v) ethanol. The leaf samples were stored in glycerine:distilled water:ethanol (45:45:10) solution and photographed with a Canon EOS 700D camera (Tokyo, Japan) under white transillumination (Dark Hood DH-50, Biostep, Germany).

2.7. Measurement of stomatal apertures

Epidermal strips from the abaxial surface of the leaves were prepared subsequently after sampling from the 6th as well as 5th leaf levels and placed into incubation buffer (10 mM KCl, 5 mM MES; pH 6.15) (Melotto et al., 2006). Stomatal apertures of the strips were observed by Nikon Eclipse TS-100 Inverted Routine Microscope (Nikon Instruments, Tokyo, Japan) and immediately photographed (Czékus et al., 2020). The width of at least 60–90 stomatal pores from three different plants was measured in case of all of the treatments with Image-Pro Plus 5.1 software (Media Cybernetics, Inc., Rockville, MD, USA).

2.8. Statistical analysis

All of the experiments were repeated at least three times. The data presented in graphs are means \pm SE. Sigma Plot 11 software (Systat Software Inc. Erkrath, Germany) was used for statistical analyses where significant differences were analysed after one-way ANOVA with Duncan's multiple range comparison test. Mean values were considered to be significantly different if $p \leq 0.05$.

3. Results

3.1. Flg22 triggers local and systemic ET accumulation which is inhibited by AVG and STS

Despite the importance of ET in flg22-induced defence responses have been described, its possible role in the regulation of rapid systemic responses has still been less investigated. Our work focused on how ET modulates rapid responses triggered by flg22 using different ET modulators in intact tomato plants providing the opportunity to check the systemic responses of plants as well.

To get more information about the ET-dependent regulation of flg22-induced rapid local and systemic defence responses, firstly the emission of ET was determined in the older (6th leaf level from the apex) and younger leaf levels (5th leaf level from the apex) of intact tomato plants. Treatment with the bacterial elicitor flg22 not only elevated the ET emission locally by more than 370% in the older leaves but also significantly increased it by 200% in the distal, younger leaves. Under control condition AVG alone did not have an effect on the endogenous ET levels, however, applying as a pre-treatment before flg22 application significantly moderated its local (160%) and systemic accumulation (170%) triggered by the bacterial elicitor. Treatment with STS in the younger leaves alone only elevated slightly the ET emission from the younger leaves, whereas did not influence it in the older leaves. However, when it was used in combination with flg22, it slightly inhibited the flg22-induced ET accumulation both locally and systemically. As a control, exogenous ACC treatment was applied in the older leaf level which induced significant ET production locally by 330% but not in the distal leaves (Fig. 2).

3.2. AVG inhibits the flg22-induced local expression of *SIACO1*

The expression of ET biosynthesis genes was examined after combined treatments with flg22 and ET biosynthesis- and signalling modulators. While the relative transcript levels of *SIACS6* were neither affected by flg22 nor by the examined ET modulators (Fig. 3A), *SIACO1* showed enhanced expression in the older leaves upon flg22 treatment. Although neither AVG nor STS had a significant effect alone on the expression of *SIACO1*, AVG alleviated the flg22-induced transcript accumulation in the older leaves. Treatment with ACC also caused a significant induction locally only in the case of *SIACO1* (Fig. 3B).

3.3. AVG decreases the flg22-induced local and systemic O_2^- accumulation, however STS only systemically inhibits it

Accumulation of ROS is one of the earliest responses observable after pathogen challenge or elicitor treatments. O_2^- levels were found to be basically higher in the younger leaf levels even under control conditions based on our photometric results. Flg22 induced significant O_2^- generation both locally (216%) and systemically (151%) which levels were higher in the younger leaf levels. The accumulation of O_2^- was unaffected by the application of AVG alone however it significantly moderated the increase triggered by flg22 in both leaf levels. STS alone caused a non-significant increase in O_2^- levels of the younger leaves as compared to the control whereas significantly reduced the flg22-induced elevation in the same leaf level. ACC treatment also significantly elevated the O_2^- production by 152% in the leaves (Fig. 4).

NBT staining of tomato leaves confirmed the results obtained from spectrophotometric determination of O_2^- levels. Basically O_2^- level was higher in the younger, distal leaves, however, it showed the highest accumulation following flg22, AVG, and ACC treatments based on extensive formazan deposition (blue colour). Flg22 treatment alone induced a significant increase of O_2^- in both leaf levels, however, it was significantly decreased locally and systemically by AVG, and systemically upon the combination with STS (Fig. 5).

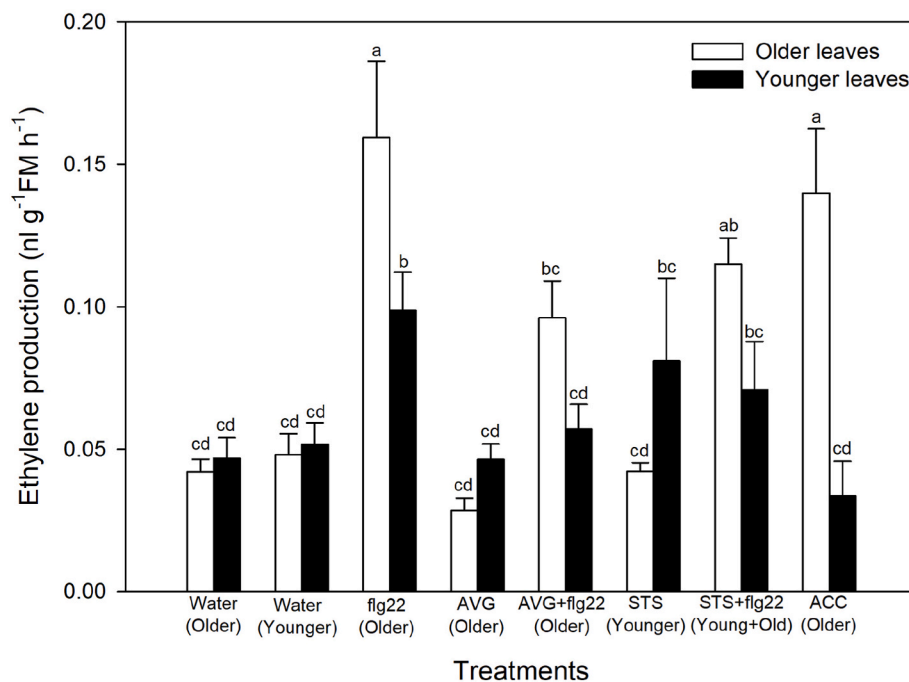


Fig. 2. Changes in the ethylene (ET) production in the leaves of intact tomato plants treated foliar with 5 μ M flagellin (flg22), 10 μ M aminoethoxyvinyl glycine (AVG), 20 μ M silver thiosulphate (STS) or 10 μ M 1-aminocyclopropane 1-carboxylic acid (ACC). Plants were pre-treated with AVG (older leaves) or STS (younger leaves) at 7:00 a.m. before treatment with flg22 (older leaves) at 8:00 a.m. ACC treatments (older leaves) were carried out at 8:00 a.m. Sampling was conducted at 9:00 a.m. Graphs indicate means \pm SE ($n = 3$). Analyses of means were carried out with one-way ANOVA where significant differences were determined with Duncan's multiple range test. Values considered to be significantly different were marked with distinct letters if $P < 0.05$.

3.4. Flg22-triggered rapid local and systemic H_2O_2 generation is inhibited by AVG, but STS primarily decreases it in the distal leaves

In contrast to O_2 , the H_2O_2 levels did not differ significantly between the older and younger leaves under control conditions at this time point based on our photometric measurement results. Flg22 generated a significant H_2O_2 increase both locally (150%) and systemically (146%). Whereas AVG on its own did not influence the production of H_2O_2 , significantly restrained its elevation provoked by the bacterial elicitor treatment in both leaf levels. Similar to AVG, STS also caused only a slight increase in H_2O_2 levels, however significantly diminished its accumulation systemically triggered by flg22. The application of ACC did not influence H_2O_2 contents at this time point (Fig. 6).

Histochemical H_2O_2 determination results from DAB staining basically showed a strong correlation with our quantitative, spectrophotometric data. Flg22 alone caused significant H_2O_2 accumulation (brown colour) both in the older and younger leaves based on the appearance of brown areas, however, in the presence of AVG or STS, it was significantly reduced in both leaf levels. While AVG and ACC did not influence the production of H_2O_2 , STS alone caused a slight increase in H_2O_2 content in the distal leaves (Fig. 7).

3.5. Flg22-induced local and systemic stomatal closure is diminished by AVG, while STS impairs only the systemic stomatal response

Stomata functioning as the first line of plant immunity have pivotal importance in plant defence reactions. Flg22 treatment triggered significant stomatal closure in 1 h (38%) which was observable in the distal leaves (26%) as well. The ET biosynthesis inhibitor AVG also closed stomata both locally (17%) and systemically (13%), however, in plants co-treated with AVG and flg22, the elicitor-triggered closure was greatly inhibited in both leaf levels. Upon STS treatment, significant stomatal closure was observable primarily in the younger leaf levels (29%) however applying together with bacterial elicitor flg22 significantly impaired its potential to close stomata in the younger leaves. ACC treatment already resulted in significant stomatal closure both in the older (16%) and younger leaves (16%) confirming the crucial role of ET in defence progression (Fig. 8).

4. Discussion

Under stress conditions, the outcome of rapid defence responses is highly dependent on the time in ET emission upon flg22 exposure both in the older and younger leaves of intact tomato plants, which is strongly inhibited by ET biosynthesis inhibitor AVG that further negatively affects the ET-regulated defence reactions such as stomatal closure. In addition to rapid stomatal opening, it is well known that ET induces other defence-related signalling processes, such as ROS generation or induction of response genes such as *PR-3-type basic chitinase*, *PR-4-type hevein-like protein* and *PR-12 defensin PDF1.2* (van Loon et al. 2006). It is also well known that ROS can be generated and transported in the local and distal tissues of plants after stress stimuli (Devireddy et al., 2018). Rapid local, as well as systemic defence responses, can be induced even in mins in which generation of ROS and stomatal closure have crucial importance (Daudi et al., 2012; Devireddy et al., 2018). However, not all signal molecules are transported through vascular connections. While the transport of signal transmitters playing a crucial role in systemic signal transduction takes place primarily through the xylem, such as in the case of ET precursor ACC, various gaseous molecules can function as volatile signal such as ET (Pierik et al., 2014; Anfang and Shani, 2021). Volatile phytohormones due to their state are capable of rapid transport indispensable for local as well as systemic defence progression (Gao et al., 2021). Although ET has been known to regulate the flg22-induced immune responses locally, its exact role is still debated today. It was reported that early defence responses upon flg22 exposure were proved to be dependent primarily on ET, whereas late responses were under the regulation of SA (Mersmann et al., 2010). At the same time, flg22-induced resistance was also shown to be independent of both ET and SA signalling in *Arabidopsis thaliana* (Wang et al., 2018). In particular, our knowledge about the role of ET in mediating rapid systemic defence responses is quite contradictory, furthermore, we have limited information regarding its contribution to the establishment of systemic resistance in flg22-induced rapid plant responses (Czékus et al., 2021b; Fichman and Mittler, 2021). Applying modulators of ET biosynthesis or perception simultaneously with flg22 provides the opportunity to study the exact role of ET in the flg22-induced local and systemic defence processes in detail on intact plants. AVG, by inhibiting the ACC synthase (ACS), is one of the most commonly used ET biosynthesis inhibitors

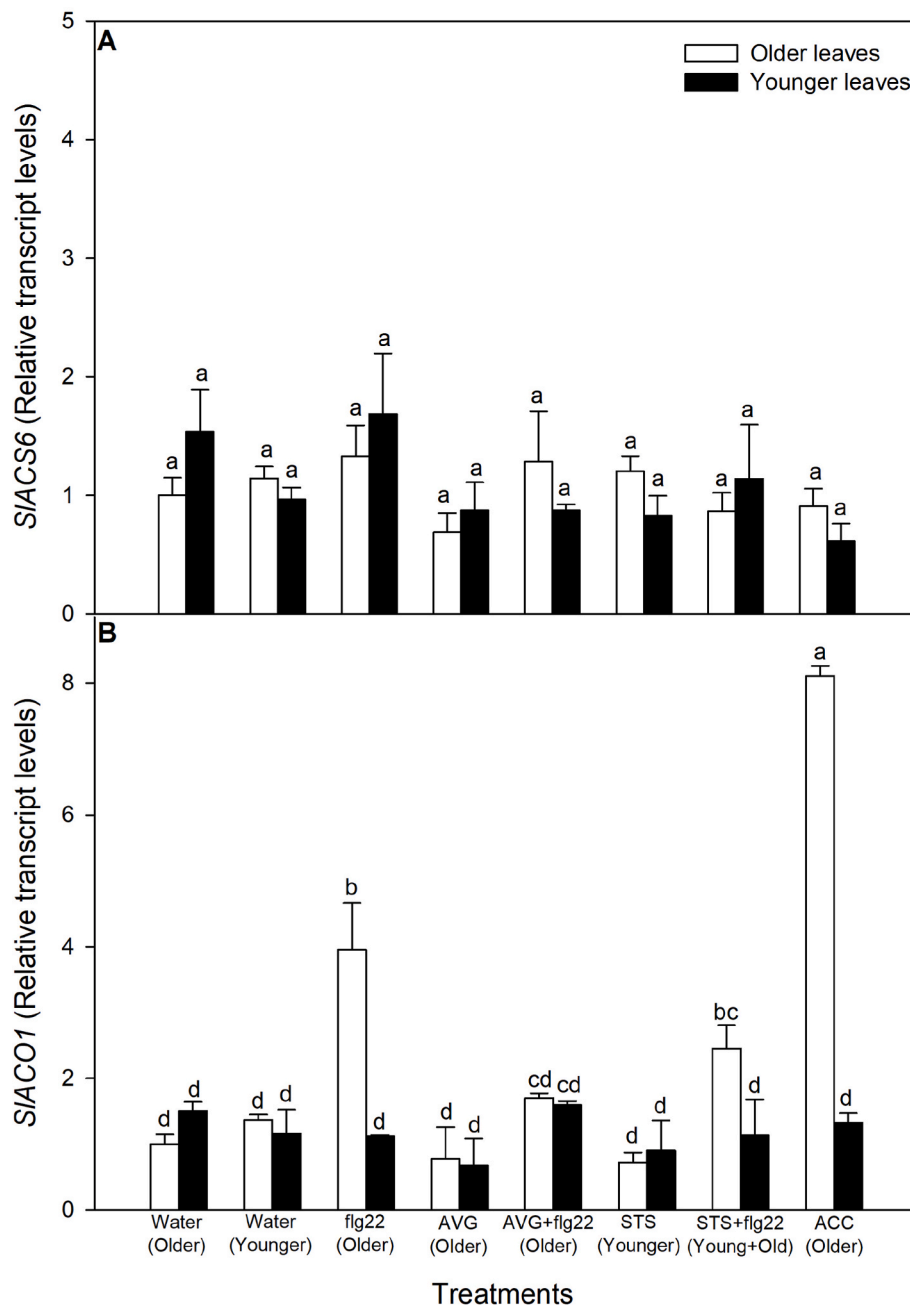


Fig. 3. Changes in the relative transcript accumulation of ethylene (ET) biosynthesis genes *SIACS6* (A) and *SIACO1* (B) in the leaves of intact tomato plants treated foliar with 5 μ M flagellin (flg22), 10 μ M aminoethoxyvinyl glycine (AVG), 20 μ M silver thio-sulphate (STS) or 10 μ M 1-aminocyclopropane 1-carboxylic acid (ACC). Plants were pre-treated with AVG (older leaves) or STS (younger leaves) at 7:00 a. m. before treatment with flg22 (older leaves) at 8:00 a.m. ACC treatments (older leaves) were carried out at 8:00 a.m. Sampling was conducted at 9:00 a.m. Graphs indicate means \pm SE ($n = 3$). Analyses of means were carried out with one-way ANOVA where significant differences were determined with Duncan's multiple range test. Values considered to be significantly different were marked with distinct letters if $P < 0.05$.

(Ickeson and Apelbaum, 1983). In this study, the biosynthesis of ET was inhibited locally with AVG in the older (flg22-treated) leaves, however, we were also detecting its systemic effects. At the same time, the possible effects of AVG on the biosynthesis of auxin cannot be totally ruled out (Vanderstraeten and Van Der Straeten, 2017) but these effects appear only 3–5 days later after the application of the ET biosynthesis inhibitor (Lewis et al., 2011) but plant volatiles induced by pathogen showed a maximum within 1 h (Aguirre et al., 2023). Besides applying AVG, the contribution of ET signalling to flg22-induced defence progression was examined by STS in younger leaves, as silver competing for the Cu-binding sites of ET receptors effectively inhibits the downstream signalling, and therefore ET response, providing a more specific approach for investigating the regulator role of ET (Poór et al., 2013; Rodriguez et al., 1999). In addition, to examine how the lack of ET in different levels influences flg22-induced responses, and to confirm the positive role of ET in the defence regulation, ET biosynthesis precursor ACC was also applied to the leaves of intact tomato plants. At the same

time, only two leaf levels of intact plants were analysed in this study (ET sender and ET receiver), but the gaseous ET produced by flg22 may also affect other leaves nearby.

Earlier enhanced emission of ET was observed locally in tobacco and tomato leaves upon flg22 treatment showing a peak 1–2 h later (Felix et al., 1999; Mur et al., 2008). We have recently described that early ET production subsequently after flg22 application is not only observable locally, but also in the distal leaves of tomato plants, where stomata were also closed (Czékus et al., 2021b). Our present results supported the direct effect of flg22 on the rapid local and systemic ET accumulation induced within 1 h. ACS has been considered for a long time as an enzyme catalyzing the rate-limiting step of ET biosynthesis converting *S*-adenosyl-*L*-methionine (SAM) into ACC, however, nowadays more and more research results confirm the predominance of ACO, an enzyme catalyzing the formation of ET from ACC (Houben and Van de Poel, 2019). Based on our previous research, *SIACS6* and *SIACO1* were selected as ET biosynthesis genes whose expression was showing a

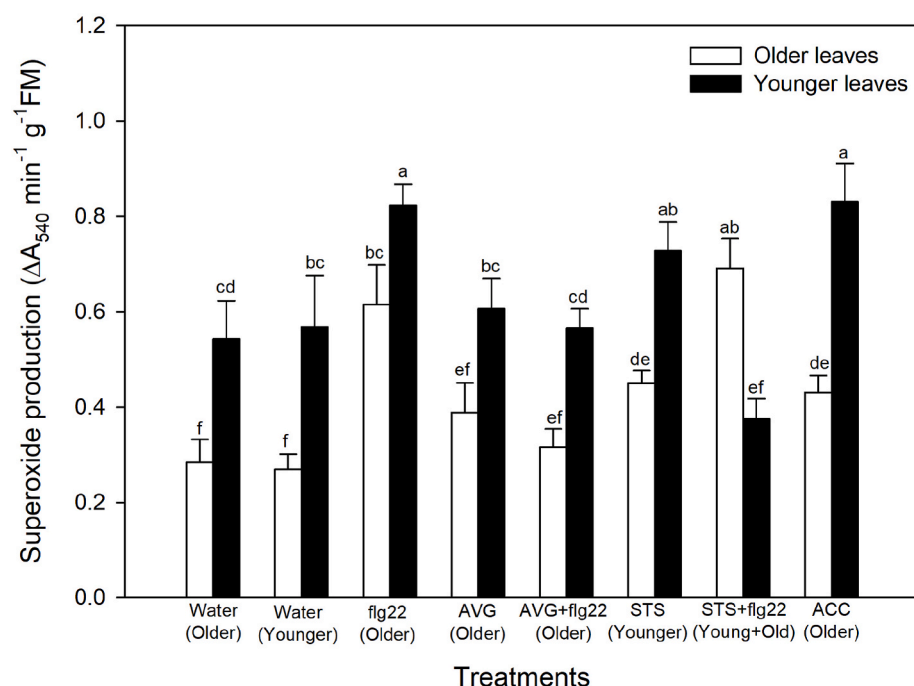


Fig. 4. Changes in the superoxide radical (O_2^-) production in the leaves of intact tomato plants treated foliar with 5 μ M flagellin (flg22), 10 μ M aminoethoxyvinyl glycine (AVG), 20 μ M silver thiosulphate (STS) or 10 μ M 1-aminocyclopropane 1-carboxylic acid (ACC). Plants were pre-treated with AVG (older leaves) or STS (younger leaves) at 7:00 a.m. before treatment with flg22 (older leaves) at 8:00 a.m. ACC treatments (older leaves) were carried out at 8:00 a.m. Sampling was conducted at 9:00 a.m. Graphs indicate means \pm SE ($n = 3$). Analyses of means were carried out with one-way ANOVA where significant differences were determined with Duncan's multiple range test. Values considered to be significantly different were marked with distinct letters if $P < 0.05$.

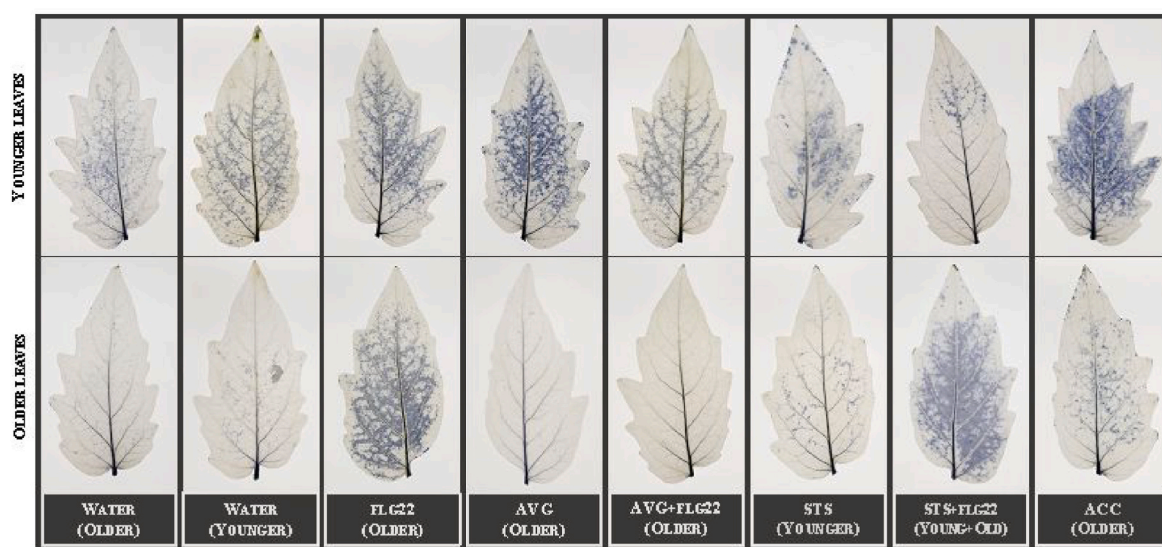


Fig. 5. Detection of superoxide radical (O_2^-) accumulation by nitroblue tetrazolium (NBT) staining of the leaves of intact tomato plants treated foliar with 5 μ M flagellin (flg22), 10 μ M aminoethoxyvinyl glycine (AVG), 20 μ M silver thiosulphate (STS) or 10 μ M 1-aminocyclopropane 1-carboxylic acid (ACC). Plants were pre-treated with AVG (older leaves) or STS (younger leaves) at 7:00 a.m. before treatment with flg22 (older leaves) at 8:00 a.m. ACC treatments (older leaves) were carried out at 8:00 a.m. Sampling was conducted at 9:00 a.m. Representative results are shown.

strong correlation with elevated ET accumulation upon fungal elicitor chitosan (CHT) treatments in the leaves of tomato plants (Czékus et al., 2021a). Others have also confirmed that flg22 increased the expression of ACS2/6/7/8 and ACO4 genes within 3–7 h in Arabidopsis, of which ACS genes showed the strongest induction, which were up-regulated by flg22 only in 1 h (Denoux et al., 2008; Park et al., 2015). Interestingly, in our experiments, where we tested only two selected ET biosynthesis-related marker genes, neither flg22, nor ACC triggered the expression of tomato *SIACS6* within 1 h, however, significantly elevated the transcript levels of *SIACO1* locally in the older leaves suggesting that flg22 induces ET accumulation mainly by promoting the ACC oxidizing step of ET synthesis in the lower leaves of tomato plants. This observation is in accordance with the most recent studies, which emphasize the

predominance of ACO as the key biosynthetic enzyme for ET production rather than ACS (Houben and Van de Poel, 2019). Our results suggest, that *de novo* ET synthesis in gene expression level has not been induced systemically in this short time period neither upon flg22 nor by ACC treatments, however, significant ET accumulation was observable in the distal leaves following the bacterial elicitor treatments, suggesting the ET biosynthesis-related enzymes were rapidly activated on protein level. While AVG alone only resulted in a non-significant decrease of ET accumulation in the older leaves, inhibited the flg22-induced *SIACO1* expression, which could further be manifested in reduced local ET accumulation. In parallel, this effect of AVG confirms the participation of ET in flg22-induced local defence responses. The ET perception inhibitor STS alone did not influence significantly the local ET

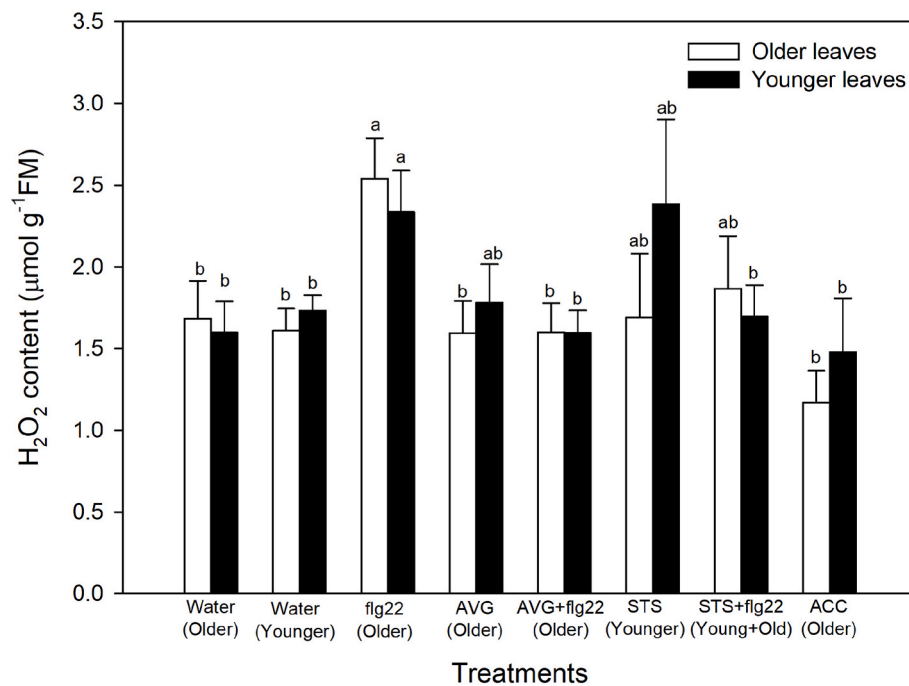


Fig. 6. Changes in the hydrogen peroxide (H₂O₂) production in the leaves of intact tomato plants treated foliar with 5 μM flagellin (flg22), 10 μM aminoethoxyvinyl glycine (AVG), 20 μM silver thio-sulphate (STS) or 10 μM 1-aminocyclopropane 1-carboxylic acid (ACC). Plants were pre-treated with AVG (older leaves) or STS (younger leaves) at 7:00 a. m. before treatment with flg22 (older leaves) at 8:00 a.m. ACC treatments (older leaves) were carried out at 8:00 a.m. Sampling was conducted at 9:00 a.m. Graphs indicate means ± SE (n = 3). Analyses of means were carried out with one-way ANOVA where significant differences were determined with Duncan's multiple range test. Values considered to be significantly different were marked with distinct letters if P < 0.05.

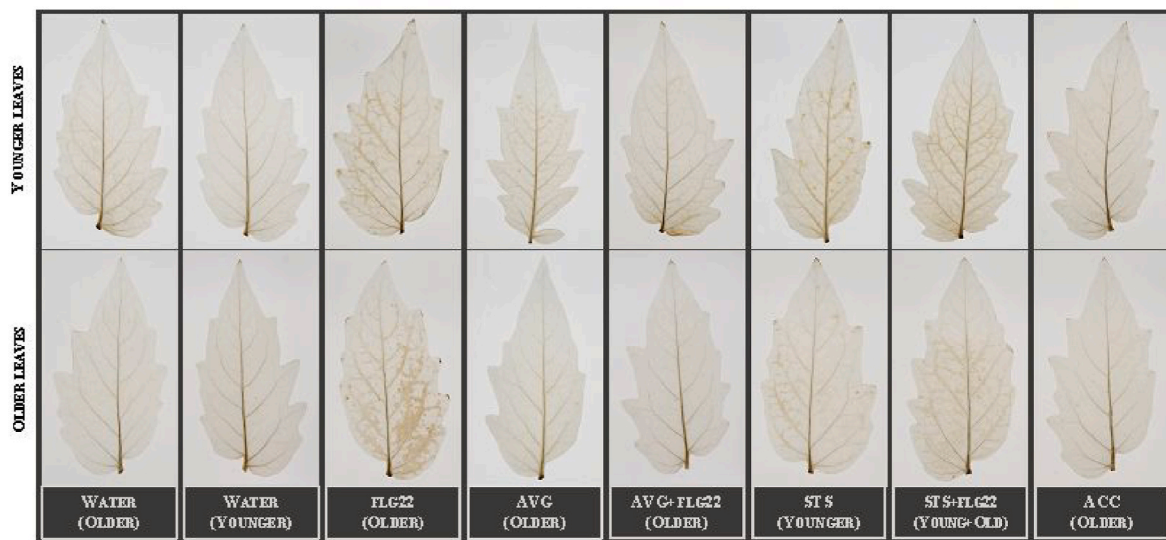


Fig. 7. Detection of hydrogen peroxide (H₂O₂) accumulation by 3,3'-diaminobenzidine tetrahydrochloride hydrate (DAB) staining of the leaves of intact tomato plants treated foliar with 5 μM flagellin (flg22), 10 μM aminoethoxyvinyl glycine (AVG), 20 μM silver thiosulphate (STS) or 10 μM 1-aminocyclopropane 1-carboxylic acid (ACC). Plants were pre-treated with AVG (older leaves) or STS (younger leaves) at 7:00 a.m. before treatment with flg22 (older leaves) at 8:00 a.m. ACC treatments (older leaves) were carried out at 8:00 a.m. Sampling was conducted at 9:00 a.m. Representative results are shown.

accumulation, while in the younger leaves a slight increase was observable which could be the result of negative ET feedback response in these tissues observed earlier in ET receptor mutant *Never ripe* tomato plants where, due to the lack of ET sensing, mutant plants were able to synthesize ET even in a higher amount as compared to wild type plants (Borbély et al., 2020; Czékus et al., 2021a). STS applied together with flg22 did not induce any significant changes in ET production as compared to flg22 treatment alone. These results also suggest, that the rapid systemic ET accumulation triggered by flg22 presumably was not only originated from systemic ET spread but can be regulated by ROS. On the one hand, this is confirmed by the fact, that the application of ACC induced ET emission only locally at this time point but not in the

distal leaves where, however, the superoxide accumulation was induced. This suggests and confirms the activation of other signalling compounds and phytohormones induced by flg22 in addition to ET (Navarro et al., 2004; Mersmann et al., 2010; Wang et al., 2018; Chi et al., 2021).

Many studies have revealed, that flg22 induces a rapid increase of ROS even in min, however, most of these experiments were carried out by treating detached leaves or leaf disks making it impossible to detect systemic responses of the plants (Janda et al., 2019; Kimura et al., 2020; Ma et al., 2021). Earlier, it was observed that flg22-induced defence responses were accompanied by locally enhanced O₂⁻ accumulation after 20 min in Arabidopsis plants, which was diminished in *fls2* mutants (Qi

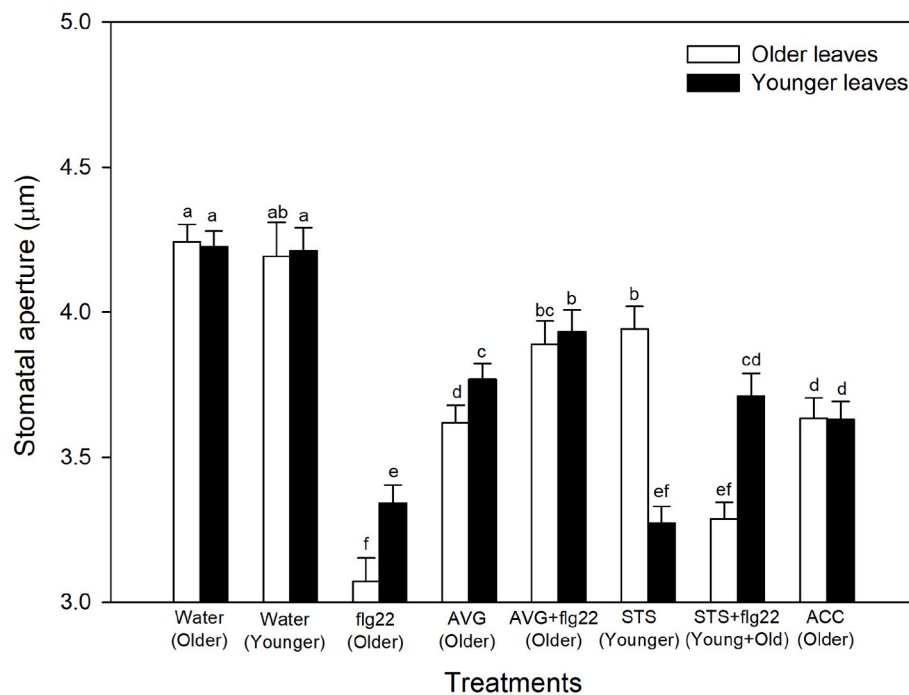


Fig. 8. Changes in the size of stomatal apertures in the leaves of intact tomato plants treated foliar with 5 μ M flagellin (flg22), 10 μ M aminoethoxyvinyl glycine (AVG), 20 μ M silver thiosulphate (STS) or 10 μ M 1-aminocyclopropane 1-carboxylic acid (ACC). Plants were pre-treated with AVG (older leaves) or STS (younger leaves) at 7:00 a.m. before treatment with flg22 (older leaves) at 8:00 a.m. ACC treatments (older leaves) were carried out at 8:00 a.m. Sampling was conducted at 9:00 a.m. Graphs indicate means \pm SE ($n = 3$). Analyses of means were carried out with one-way ANOVA where significant differences were determined with Duncan's multiple range test. Values considered to be significantly different were marked with distinct letters if $P < 0.05$.

et al., 2018). In intact tomato plants, we observed earlier, that 1 h after flg22 treatment the production of O_2^- was increased in the distal leaves in which the O_2^- content was basically higher as compared to lower leaves, presumably due to the relatively higher light intensity to which the upper leaves were exposed (Czékus et al., 2021a, 2021b). Here, we found similar changes, flg22 significantly elevated the O_2^- accumulation both locally and systemically. This increment in O_2^- level is probably due to the activation of NADPH oxidase as flg22 induced the phosphorylation of Arabidopsis RbohD pS39 within mins both in the local and distal leaves, however, it was diminished in *rbohD* mutants (Ranf et al., 2011; Dubiella et al., 2013). Flg22-triggered rapid cytosolic Ca^{2+} responses were also reduced or altered by the application of Diphenyleneiodonium chloride (DPI), an inhibitor of NADPH oxidase activity (Nomura et al., 2012; Thor and Peiter, 2014). In addition, the activation of NADPH oxidase plays a crucial role in the rapid spread of systemic signals by O_2^- into distal leaves from the stressed ones (Devireddy et al., 2018). Our results of ACC treatment suggest that ET produced in the older leaves could significantly enhance the accumulation of O_2^- in the younger leaves, as neither ET biosynthesis nor ET accumulation increased significantly in these leaf levels after 1 h, however O_2^- content was elevated in a great extent. In addition, the contribution of ET to flg22-induced local and systemic O_2^- accumulation was further supported by the application of the ET biosynthesis inhibitor AVG, which significantly reduced the O_2^- burst triggered by flg22 in both leaf levels. The requirement of the local ET emission for flg22-induced rapid oxidative burst was also demonstrated in ET-insensitive Arabidopsis *etr1* and *ein2* mutant plants as it was abolished upon flg22 exposure (Mersmann et al., 2010). Based on our previous results with ET-insensitive *Never ripe* tomato plants we suggested that ET has a positive regulatory role on the systemic production of O_2^- and stomatal closure (Czékus et al., 2021b). In accordance with this observation, ET receptor inhibitor STS also inhibited the flg22-induced systemic O_2^- production, as well as stomatal closure, confirming further the indispensable role of ET in systemic defence progression.

H_2O_2 generation by the dismutation of O_2^- is also involved in long-distance defence signalling upon pathogen challenge (Mittler et al., 2011). Based on the findings of Noshi et al. (2016), H_2O_2 production upon flg22 exposure was dependent on RbohD. In contrast to O_2^- , the H_2O_2 contents under normal conditions did not differ significantly in the

different leaf levels. At the same time, H_2O_2 production was triggered by flg22 both locally and systemically as part of the early defence progression. It is well-known that ET promotes ROS accumulation under stress conditions contributing to the development of defence responses (Riyazuddin et al., 2020; Xia et al., 2015). It was reported that, exogenous application of ACC induced ROS and H_2O_2 production in Arabidopsis (Wi et al., 2010) and tomato (Poór et al., 2013), respectively. Similarly, systemic ROS accumulation also can trigger ET biosynthesis. Namely, previous studies have described the positive effect of ROS on the biosynthesis of ET in transgenic potato plants via inducing ACO expression within 3 h (Kim et al., 2008). Treatment with H_2O_2 also resulted in increased ET emission in rice epidermal cells by the up-regulation of ACO1 which in turn induced H_2O_2 accumulation by the activity of NADPH oxidase (Steffens and Sauter, 2009). The ROS-induced ET production was also described upon Cd stress in Arabidopsis (Schellingen et al., 2015) and in wounded *Dalbergia odorifera* plants (Cui et al., 2019). Elevated ROS production parallelly with ET emission could act in a positive feedback loop that contributes to the manifestation of hypersensitive response during plant-pathogen interactions (Bouchez et al., 2007). Based on these results, we suppose that flg22 induces ET emission locally and systemically, triggered by ROS accumulation in distal leaves. Combined treatment of flg22 with STS also supported our hypothesis, as it reduced systemic superoxide production in parallel with a slight decrease in ET levels in systemic tissues. Similarly, the inhibition of flg22-induced systemic ET production by AVG was presumably due to a decrease in systemic ROS production. Thus, based on these results, we suggest that the rapid systemic ET production following flg22 treatment is most likely triggered by ROS accumulated in these leaves.

Upon perception of flg22 by the FLS2 receptor complex, its BIK1 component directly activates the RbohD NADPH oxidase via site-specific serine phosphorylation, contributing to ROS production which can trigger rapid stomatal closure that is indispensable for preventing the colonization of pathogens (Li et al., 2014; Sierla et al., 2016; Toum et al., 2016). Stomatal closure triggered by flg22 is among the first immune responses of plants induced already within 20 min which is accompanied by the activation of the SLAC1 anion channel however its initial steps are different from the ABA-derived pathway (Guzel Deger et al., 2015). Unlike ROS, the role of ET in the regulation of stomatal closure is

rather contradictory, since it can trigger both stomatal opening and closure (Daszkowska-Golec and Szarejko, 2013). It was reported that treatment with exogenous ACC significantly closed stomata in *Arabidopsis* (Desikan et al., 2006). Additionally, stomatal closure was also inhibited in ET insensitive *etr1*, *ein2* *Arabidopsis* and *Never ripe* tomato mutant plants upon flg22 exposure suggesting its positive regulatory role in the stomatal closure and defence responses of plants (Czékus et al., 2021b; Mersmann et al., 2010). Ge et al. (2015) have demonstrated that ET induces stomatal closure in a dose-dependent manner by promoting H_2O_2 accumulation via NADPH oxidase activation mediated by heterotrimeric G protein α subunit. In accordance with our previous observation (Czékus et al., 2021b), flg22 induced significant, rapid stomatal closure both locally and systemically in the leaves of intact tomato plants where significantly higher ROS production was also measured. Based on our results, ET-induced ROS production has a prominent role in the regulation of flg22-induced local and systemic stomatal responses as the application of AVG significantly inhibited both the local and systemic stomatal closure as well as O_2^- and H_2O_2 accumulation induced by flg22. Blockage of ET signalling by STS alone closed the stomata, primarily in the distal leaves, presumably due to the ET-independent, systemic O_2^- production, while ACC treatment also reduced the size of stomatal apertures, most probably by increasing the systemic O_2^- production. Independent of the regulation of ET signalling, other effects of silver have been reported, such as the induction of ROS production (Bagherzadeh Homaei and Ehsanpour, 2016) and polyamine metabolism (Kumar et al., 2009), which may also contribute to changes in stomatal pore size. However, others also found that plants exposed to ET inhibitor silver nitrate failed to close stomata similarly to ET-insensitive *etr1*, *ein2*, *arr 2* *Arabidopsis*, and *Never ripe* tomato mutants, confirming the key regulator role of ET in stomatal responses (Czékus et al., 2021b; Desikan et al., 2006). Our experiments further support, that ET has an important regulator role in the rapid, flg22-induced local and systemic stomatal closure, as plants exposed to flg22 in the presence of STS were unable to close their stomata to the same extent as in the presence of active ET signalling. This also strengthens our belief about the rapid systemic role of ET as a key mediator component in flg22-induced defence progression.

Plant defence responses triggered by the bacterial elicitor flg22 are regulated by phytohormones such as ET to induce rapid stomatal closure to prevent pathogen penetration and to promote transcriptional changes such as activation of phytohormone biosynthesis or defence-related response genes. Local and systemic ROS production plays a crucial role in these processes following elicitor sensing by leaves. Our results suggest that ET biosynthesis and signalling play a critical role not only in the local responses to flg22 exposure, but also in the rapid systemic defence progression, where it exerts its effect mainly through systemic ROS production. Thus, maintaining adequate ET levels and ensuring low ET sensitivity could be critical to the success of defence responses.

5. Conclusions

Based on our results ET basically determines the flg22-induced rapid local and systemic defence responses in leaves of intact tomato plants. The local *SIACO1* expression and ET emission induced by flg22 are highly dependent on active ET signalling mediated by ROS (Fig. 9). Local emission of ET upon flg22 treatment triggers not only local but also systemic O_2^- and H_2O_2 production, which presumably contributed to ET accumulation in the distal leaves within 1 h. However, this systemic production of ROS is also ET-dependent, as both the inhibition of ET biosynthesis by AVG and the lack of active ET signalling upon STS treatment decreased the systemic ROS burst under flg22 exposure. The rapid stomatal closure in the distal leaves of tomato plants induced by flg22 is also regulated mainly by H_2O_2 and O_2^- accumulation, however in the absence of ET biosynthesis or lack of active ET signalling, all of these defence responses were inhibited. The positive role of ET in stomatal closure was supported by applying ACC which induced systemic

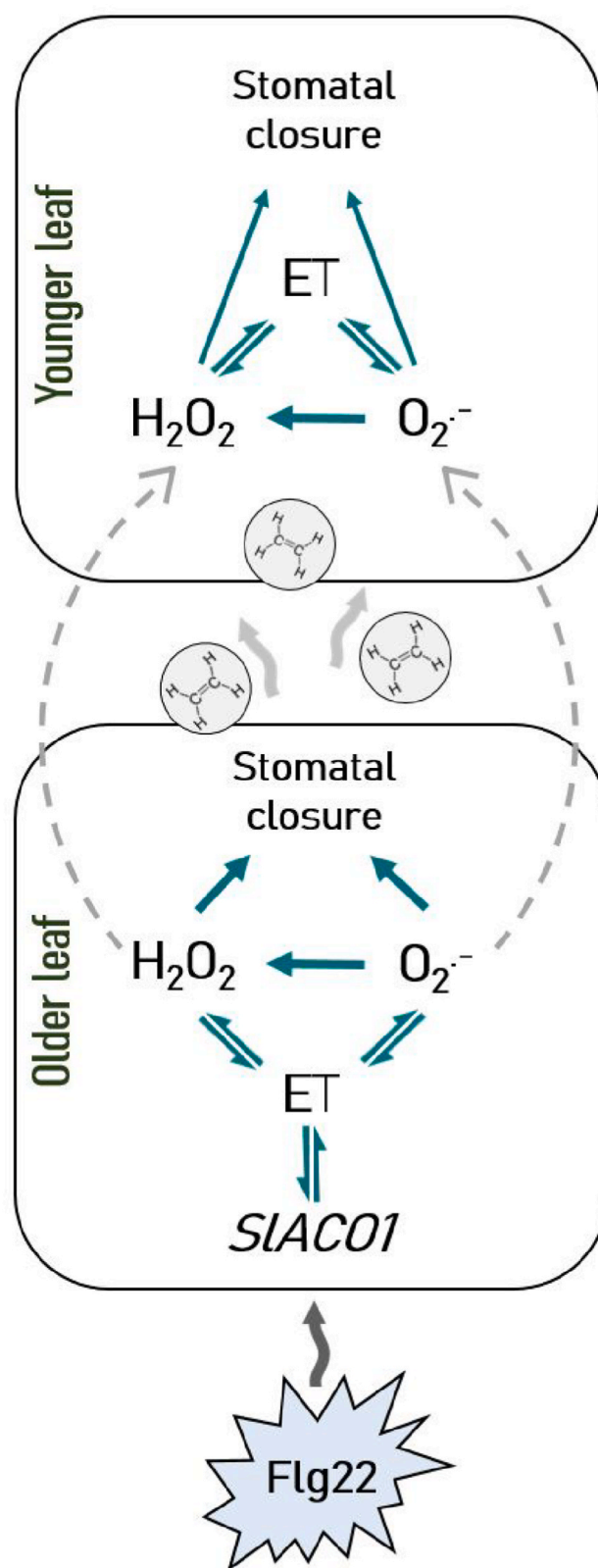


Fig. 9. Schematic model of the role of gaseous ET in flg22-induced rapid local and systemic defence responses in leaves of intact tomato plants. Flg22 induces rapid local ET accumulation and upregulates *SIACO1* expression mediated by ROS (superoxide and hydrogen peroxide). Local ET emission after flg22 treatment triggers not only local but also systemic ROS production and induces stomatal closure.

stomatal closure, moreover STS alone presumably due to negative ET feedback response induced slight ET production accompanied by O₂ production and stomatal closure. These results suggest a central role for ET in the rapid defence progression induced by flg22, which besides regulating local resistance also plays a pivotal role in the development of systemic responses, mainly through systemic O₂ and H₂O₂ production. Our study may help to understand the ET-dependent regulation of the rapid local and systemic defence responses to flg22 treatment, thereby increasing the effectiveness of bacterial pathogen control.

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CRediT authorship contribution statement

Zalán Czékus: Investigation, Writing – original draft, Writing – review & editing. **Atina Martics:** Investigation. **Boglárka Pollák:** Investigation. **András Kukri:** Investigation. **Irma Tari:** Writing – review & editing. **Attila Ördög:** Writing – review & editing. **Péter Poór:** Conceptualization, Investigation, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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