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Article

Signalling mechanisms and agricultural applications of (Z)-3-hexenyl butyrate-mediated stomatal closure

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Abstract

Biotic and abiotic stresses can severely limit crop productivity. In response to drought, plants close stomata to prevent water loss. Furthermore, stomata are the main entry point for several pathogens. Therefore, the development of natural products to control stomata closure can be considered a sustainable strategy to cope with stresses in agriculture. Plants respond to different stresses by releasing volatile organic compounds. Green leaf volatiles, which are commonly produced across different plant species after tissue damage, comprise an important group within volatile organic compounds. Among them, (Z)-3-hexenyl butyrate (HB) was described as a natural inducer of stomatal closure, playing an important role in stomatal immunity, although its mechanism of action is still unknown. Through different genetic, pharmacological, and biochemical approaches, we here uncover that HB perception initiates various defence signalling events, such as activation of Ca^{2+} permeable channels, mitogen-activated protein kinases, and production of Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase-mediated reactive oxygen species. Furthermore, HB-mediated stomata closure was found to be independent of abscisic acid biosynthesis and signalling. Additionally, exogenous treatments with HB alleviate water stress and improve fruit productivity in tomato plants. The efficacy of HB was also tested under open field conditions, leading to enhanced resistance against *Phytophthora* spp. and *Pseudomonas syringae* infection in potato and tomato plants, respectively. Taken together, our results provide insights into the HB signalling transduction pathway, confirming its role in stomatal closure and plant immune system activation, and propose HB as a new phytoprotectant for the sustainable control of biotic and abiotic stresses in agriculture.

Introduction

Plants have evolved a complex and efficient innate immune system to cope with pathogen attacks. Two different defensive layers are involved in plant immune responses [1]. The first line of plant innate immunity is established upon the recognition of conserved microbial features, called pathogen- and microbe-associated molecular patterns (PAMPs and MAMPs), by receptors on the plasma membrane (pathogen recognition receptors; PRRs), activating the PAMP-triggered immunity (PTI) [2,3]. At this level, through different PRRs, plants are also able to perceive molecules, known as damage-associated molecular patterns (DAMPs), released from plant cells upon pathogen attack. This specific recognition also triggers a PTI response known as DAMP-triggered immunity (DTI) [4]. The second layer is started by the recognition of virulence factors, also called effectors, by intracellular receptors (nucleotide-binding, leucine-rich repeat receptors), activating the effector-triggered immunity (ETI) [5,6]. Although both types of plant immune responses involve different activation mechanisms, PTI and ETI share many downstream components and responses, such as production of reactive oxygen species (ROS), increases in cytosolic Ca^{2+} levels, or activation of mitogen-activated protein kinase (MPK) cascades. The amplitudes and dynamics differ at each defensive level, postulating that ETI is an accelerated and amplified PTI [7].

Many foliar pathogens, including bacteria, are unable to directly penetrate plant tissues, and they use natural openings such as stomata. As a countermeasure, plants have evolved a mechanism to rapidly close their stomata upon PAMPs/MAMPs perception limiting bacterial entrance, a defensive response known as stomatal immunity [8]. The bacterial MAMP flg22, an immunogenic epitope of the bacterial flagellin, is recognized by the PRR receptor flagellin-sensitive 2 (FLS2) and the coreceptors BRI1-associated receptor kinase 1 (BAK1) and Botrytis-induced kinase 1 (BIK1) [9]. This recognition by the FLS2 complex triggers a cascade of signalling events, including increases of Ca^{2+} influx, ROS burst through the activation of plant Nicotinamide adenine dinucleotide phosphate (NADPH) oxidases, MAPK cascades, and activation/inhibition of ion channels, all leading to stomatal immunity [10].

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The phytohormone abscisic acid (ABA) is essential in stomatal defence during plant immunity [11]. In fact, ABA-induced stomatal closure shares a common signalling pathway with PAMP-induced stomatal closure, including ROS burst, nitric oxide (NO) intermediate accumulation, activation of S-type anion channels, or inhibition of K^+ channels [10]. Despite this, there are some differences in the canonical stomatal immunity signalling components in both the PTI and ABA signalling pathways. ABA-induced ROS production depends on the kinase OST1, which phosphorylates and activates the NADPH oxidases RBOHF/RBOHD. However, during PTI stomatal closure, ROS are produced by the activation of RBOHD, which is directly phosphorylated by the plasma membrane-associated kinase BIK1 [9,10]. Furthermore, MPKs are known to play a specific role in stomatal immunity. MPK3 and MPK6 positively regulate flg22-triggered stomatal closure in a partially redundant manner, but they are not involved in ABA-mediated stomatal closure [12,13].

Volatile organic compounds (VOCs) act as fast signalling molecules that activate plant defensive response pathways between distant organs, and they even allow communication between plants [14–16]. Several studies have demonstrated that VOCs are able to induce defences against herbivorous insects, pathogens, and even environmental stresses [15,17–19]. Defence priming against pathogens induced by VOCs has been considered a sort of "green vaccination" [20].

A non-targeted gas chromatography-mass spectrometry (GC–MS) metabolomics analysis revealed the VOC profile associated with the immune response of tomato cv. Rio Grande plants upon infection with either virulent or avirulent strains of the model bacterial pathogen *Pseudomonas syringae* pv. tomato (*Pst*) DC3000. In the case of the avirulent infection leading to the establishment of ETI, the VOC profile of immunized plants was characterized by esters of (*Z*)-3-hexenol with acetic, propionic, isobutyric, or butyric acids, and several monoterpenoids such as linalool or α-terpineol [21]. The defensive role of these compounds was tested through exogenous application in tomato plants, and among all these compounds, treatments with (*Z*)-3-hexenyl butyrate (HB) resulted in the transcriptional upregulation of defensive genes, stomatal closure, and enhanced resistance to the bacterial infection with *Pst* DC3000, confirming the role of HB as a natural defence elicitor [22]. Additionally, HB-mediated stomatal closure was effective in different plant species, which also led to accelerated ripening in *Vitis vinifera* [22,23].

In this study, we investigated the signalling mechanisms underlying HB-mediated stomatal closure and plant immunity. Furthermore, we tested the efficacy of this compound under water stress, and its effectiveness was also assessed against both biotic and abiotic stresses under field conditions. Considering all our results together, we propose a new role for HB as a natural elicitor against biotic and abiotic stresses.

Results

Treatments with HB induce transcriptomic changes related to plant immunity and photosynthesis

To provide insight into the molecular mechanisms underlying the tomato plant response to HB, an RNA-seq analysis was performed on leaf tissues from mock and HB-treated tomato plants (see HB treatments in chambers in Material and Methods). After trimming and filtering the data, a total of 1456 genes were found to be differentially expressed (DEGs) between HB- and control-treated plants (DEG threshold = 2), indicating that HB treatments exert an outstanding effect at the transcriptional level. To classify DEGs, a Gene Ontology (GO) enrichment analysis for biological processes (BPs), cellular components (CCs), and molecular functions (MFs) was performed for upregulated and downregulated DEGs, independently.

Among the upregulated genes, 1122 DEGs were annotated, making the BP category the most enriched one (Fig. 1A). In this regard, most of the DEGs were putatively involved in organonitrogen compound metabolic processes (GO:1901564), chitin metabolic processes (GO:0006030), or amino glycan and amino sugar metabolic processes (GO:0006040, GO:0006022). The MF category included chitinase activity (GO:0004568), chitin binding (GO:00080761), or endopeptidase activity (GO:0004175), which are mainly related to pathogen defensive responses. On the other hand, only 334 downregulated DEGs were annotated, and most of them were associated with the CC category, mainly related to plastoglobule (GO:0010287), photosystem (GO:0009521), and chloroplast (GO:0009507) (Fig. 1B). Indeed, when BPs were assessed, the most enriched groups were related to photosynthesis (GO:0015979), and the only enriched MF term was chlorophyll binding (GO:0016168).

Gene expression of some defensive genes was confirmed byReverse transcription-quantitative polymerase chain reaction (RT-qPCR) (Fig. S1), observing a statistically significant induction for the pathogenesis-related genes *PR1* (X68738) and *PR5* (X70787) classic markers. Moreover, several defensive genes found in the RNA-seq analyses were validated by RT-qPCR, including a gene coding for a putative PRR immune leucine-rich repeat receptor-like kinase (Solyc12g036793) and a gene involved in heavy metal resistance and detoxification (Solyc06g066590). Besides, the reduction in the expression of genes found in the RNA-seq analysis, which encodes chlorophyll-binding proteins (Solyc06g069730; Solyc06g069000; Solyc12g011280), was also validated. Therefore, RNA-seq transcriptomic analysis revealed that HB treatments induced plant defensive responses against pathogens as well as photosynthesis reprogramming.

ROS production via NADPH oxidases and Ca^{2+} signalling, but not ABA, is required for HB-mediated stomatal closure

To decipher the signalling pathway by means of which HB mediates stomatal closure, a method suitable for stomatal measurement analysis was developed, as described in Materials and Methods. In these experiments, we studied the effect of HB in comparison with flg22 and ABA, the positive controls for stomatal closure, considering both dose–response and time-course assays of these compounds to optimize the different treatments (Fig. S2). Once set up, we studied whether ABA was necessary for HB-dependent stomata closure. For this purpose, we took advantage of both *flacca* (*flc*) tomato mutants that are impaired in ABA biosynthesis and their corresponding parental Lukullus wild-type (WT) plants [24]. As expected, HB, flg22, and ABA treatments promoted stomatal closure in WT plants. In *flc* mutants, the stomatal aperture ratio in mock conditions was significantly higher compared to WT plants, probably due to a reduction in ABA levels. However, ABA treatments in *flacca* mutants closed the stomata to the same extent as flg22 and HB, suggesting that ABA biosynthesis is not required for flg22- and HB-mediated stomatal closure (Fig. 2A).

To analyse the role of Ca^{2+} and ROS in the HB-mediated stomatal closure, we performed pre-treatments with the calcium ion chelator EGTA, and the ROS production inhibitors DPI (Diphenyleneiodonium, which inhibits NADPH oxidase) or SHAM (salicylhydroxamic acid, acting on peroxidases) before

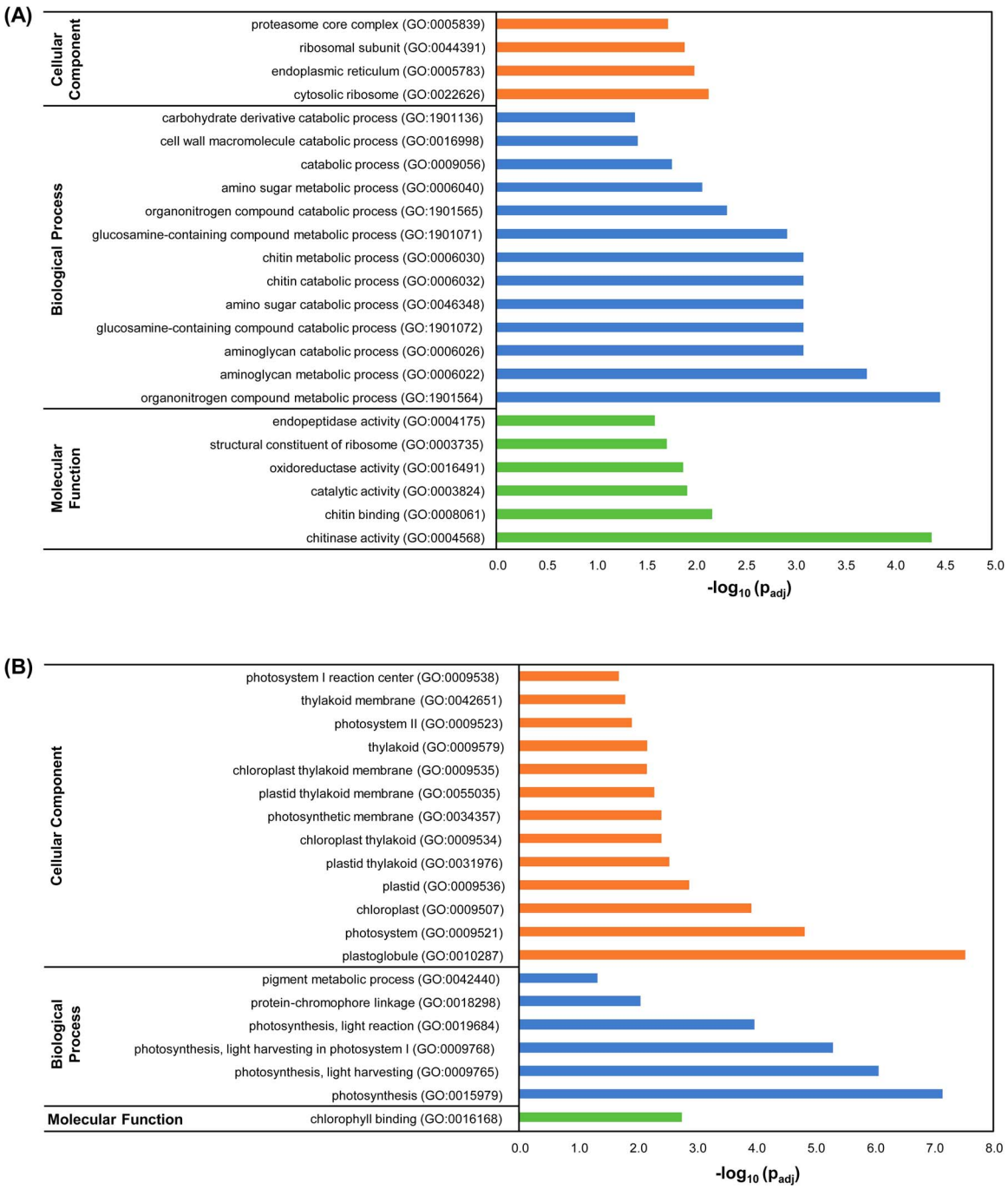

Figure 1. Transcriptomic analysis suggests a role of HB in plant immunity. Functional profiling analysis of upregulated (A) and downregulated (B) DEGs between HB- and control-treated plants.

triggering the stomatal closure with HB, flg22, or ABA. Thereby, EGTA pretreatments completely abolished the stomatal closure mediated by all HB, flg22, and ABA treatments, indicating that Ca^{2+} signalling is essential for stomatal closure (Fig. 2B). Interestingly, pretreatments with DPI partially abrogated the stomatal closure induced by HB, flg22, and ABA, suggesting that NADPH-dependent ROS production is somewhat necessary for this process (Fig. 2C). However, unlike flg22 but similar to ABA treatments, SHAM did not inhibit HB-induced stomatal closure, indicating that this effect promoted by HB is independent of ROS accumulation mediated by SHAM-sensitive peroxidases (Fig. 2D).

Our results appear to indicate that HB-mediated stomatal closure requires Ca^{2+} fluxes and is partially dependent on ROS generation by NADPH oxidases but is nevertheless independent of ABA biosynthesis and ROS generated through peroxidases.

Activation of MPK3 and MPK6 is essential for HB-mediated stomatal immunity

To test whether HB intracellular signalling occurs through activation of MPK-cascades, we analysed the phosphorylation of MPK3 and MPK6 in tomato and *Arabidopsis thaliana* leaf discs after treatments with flg22, ABA, and HB. Regarding tomato samples, HB induced MPK3 phosphorylation 15 minutes after treatments, and this activation persisted until 60 minutes after treatments (Fig. 3A). Nevertheless, in *A. thaliana* tissues, MPK3/6 phosphorylation was observed at 60 minutes after HB treatments, indicating

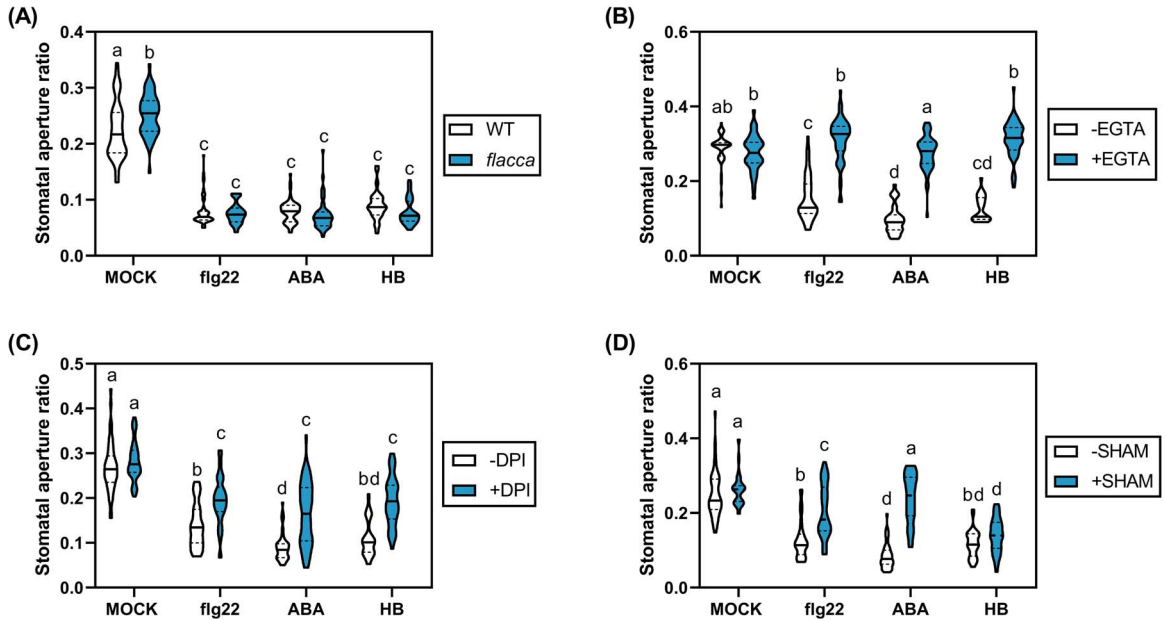

Figure 2. HB-mediated stomatal closure requires Ca2+ signalling and ROS production by NADPH oxidases, but not ABA biosynthesis. (A) Lukullus (WT) and *flacca* tomato leaf discs were floated on liquid MS for 3 hours under light. Then, 1 μM flg22, 10 μM ABA, or 50 μM HB were applied, and stomatal aperture ratio was determined 2 hours after treatments. In the case of chemical inhibitors experiments, 2 mM EGTA (B), 20 μM DPI (C), or 2 mM SHAM (D) were added in the liquid medium for MoneyMaker tomato leaf discs before elicitor treatments. Violin plots represent the stomatal aperture ratio of 50 stomata for each treatment. Different letters indicate statistically significant differences for each genotype and treatment ($P < 0.05$, two-way ANOVA with Tukey HSD test).

that MPKs activation phenomenon occurs earlier in tomato than in *A. thaliana* plants (Fig. 3B).

To confirm the importance of MPK3/6 activation in HB-mediated stomatal immunity, a pre-treatment with the MPKs inhibitor PD98059 on tomato leaf discs was performed. After PD98059 application, treatments with flg22, ABA, and HB diminished their ability to induce stomatal closure, confirming that MAPKs play a pivotal role in all the elicited stomatal responses (Fig. 3C). Furthermore, treatments with PD98059 were also carried out *in planta* (see Materials and Methods). In this regard, PD98059 application diminished the HB enhanced resistance to bacterial infection (Fig. 3D). These results indicate that HB induces stomatal immunity partially via activation of the MPK3/6 signalling cascades in tomato plants.

To better characterize this, a genetic approach was performed in *A. thaliana mpk3* and *mpk6* mutants analysing the stomatal behaviour as described in Materials and Methods. As expected, ABA treatments dramatically reduced stomatal aperture in both *Arabidopsis* mutants and WT plants. In contrast, and in accordance with the chemical inhibitor experiments performed in tomato plants, stomata aperture ratio was identical to that observed in mock-treated plants not only in flg22- but also in HB- treated *mpk3* and *mpk6* mutants, while treatments were efficient in the corresponding WT *Arabidopsis* plants (Fig. 3E). The incapacity of HB in closing stomata in *mpk3* and *mpk6* mutants resulted in a lack of enhanced resistance to bacterial infection with *Pst* after HB treatments (Fig. 3F).

Additionally, the expression of MPK3/6 target genes was also explored upon HB treatment. As shown in Fig. S3A and B, HB treatments of tomato plants significantly induced *SlWRKY33A* and *SlWRKY33B* transcription factors, which correspond to tomato orthologues of *AtWRKY33*. This gene has been described to work downstream of MPK3/MPK6 in the reprogramming of the expression of genes involved in camalexin biosynthesis and pipecolic acid in *Arabidopsis* [25,26]. Since MAPK3/6 play a central role in the regulation of the ethylene response pathway [27], ethylene signalling-related genes like the ethylene receptor *ETR4* and an ethylene responsive factor (*ERF*) were also analysed, observing a significant induction of the expression of both genes (Fig. S3C and D). The expression of these four genes was checked in the RNA-seq analysis (Fig. S3E), confirming the HB-mediated induction of the MPK3/6 target genes. Taken together, these results indicate that MPK3/6 are involved in HB-mediated stomatal immunity.

HB treatments confer drought tolerance in tomato

The robust effect of HB in promoting the closure of stomata prompted us to study the effect of HB on drought tolerance. To that purpose, well-watered as well as drought-stressed tomato plants were periodically treated with HB, and stomatal opening and closing dynamics were monitored. Three days after drought exposure (orange violins, Fig. 4A; Fig. S4), stomatal aperture ratio in HB-treated plants was lower than in control-treated plants, confirming the role of HB as stomatal closure inducer in tomato plants. It should be noted that HB treatments in well-watered conditions (blue violin) caused similar stomatal closure to those control-treated plants subjected to drought (orange violin). Moreover, at 6 DAD, the stomatal behaviour in all treatments and conditions was similar to that observed at 3 days after drought exposure (DAD), except in the control plants exposed to drought, in which it was not possible to take leaf samples due to the wilting phenotype of the plants.

To correlate the HB-mediated stomatal closure upon drought conditions with the water content of tomato plants, the fresh weight at 3 and 6 DAD was also measured. At 6 DAD, control-treated plants under drought conditions showed an approximately 50% reduction in the fresh weight. Nevertheless, in the case of HB-treated plants, no statistically significant differences were observed between well-watered plants and those

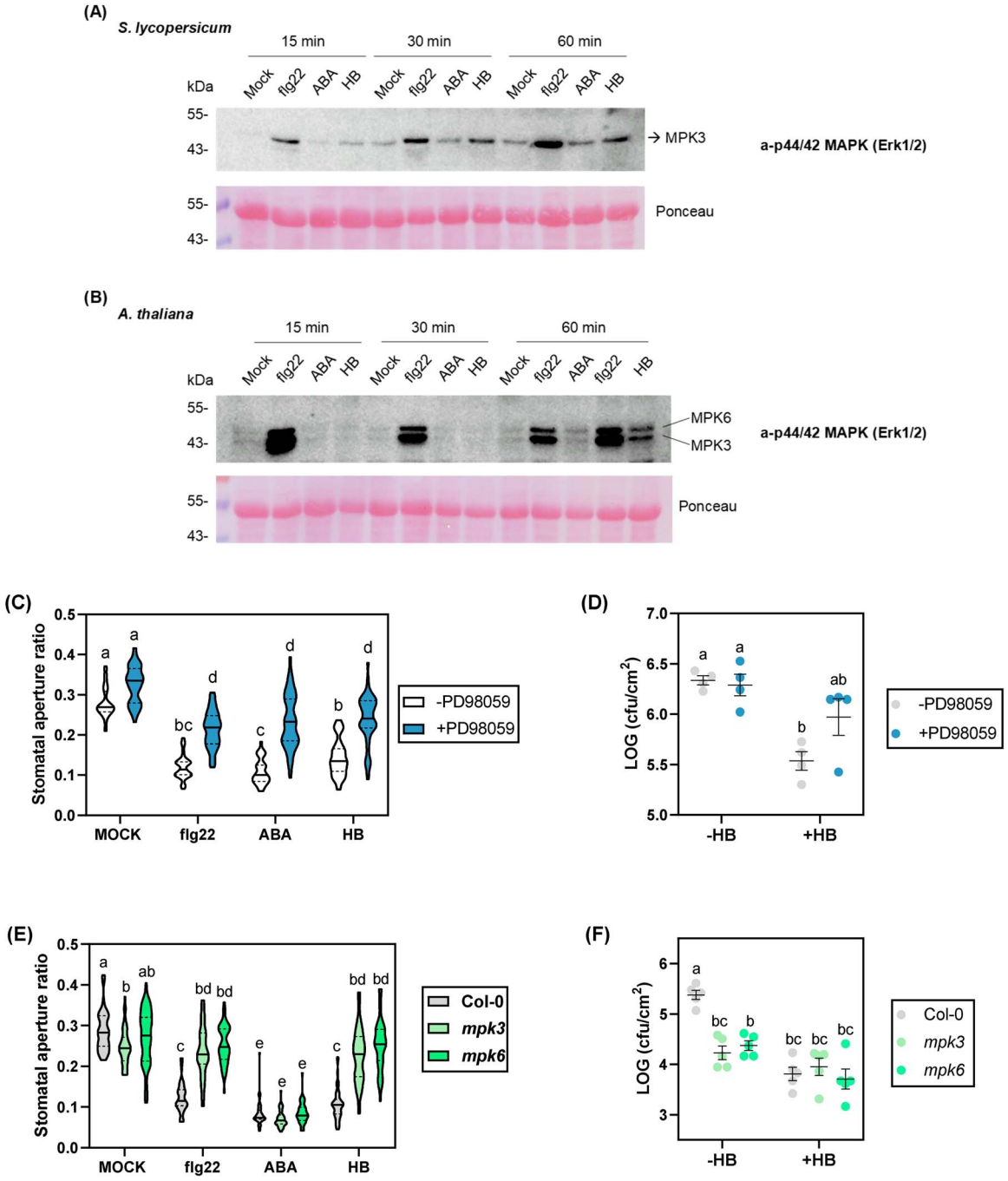

Figure 3. Activation of MPK3 and MPK6 is involved in HB-mediated stomatal immunity. MPK activation assay in tomato (A) and *Arabidopsis thaliana* (B) leaf discs 15, 30 and 60 minutes after treatments with 1 μM flg22, 10 μM ABA and 50 μM HB. MPK activation was detected by immunoblot analysis using the Phospho-p44/42 MAPK (Erk1/2; Thr-202/Tyr204) rabbit monoclonal antibody. Western blot experiments were performed three times and yielded similar results. (C) Stomatal aperture ratio was measured in tomato leaf discs floated on MS liquid for 3 hours under light, pre- treated with the MPKs inhibitor PD98059 20 μM and followed by treatments with 1 μM flg22, 10 μM ABA and 50 μM HB for 2 hours. (D) Growth of *Pst* on tomato leaves of control (-HB) and HB-treated (+HB) after treatments with the MPKs inhibitor PD98059. Plants were sprayed with PD98059 100 μM for 3 hours, subsequently treated with HB 5 μM or water for 24 h into methacrylate chambers, and then dip inoculated with *Pst*. Bacterial growth measurements were done 24 hours after inoculation. (E) Stomatal aperture ratio of *Arabidopsis* Col-0, *mpk3*, and *mpk6* mutants leaf discs 2 hours after treatments with flg22, ABA, and HB. (F) Growth of *Pst* on *A. thaliana* leaves of control (−HB) and HB-treated (+HB) 3 days after inoculation. Plants were treated with HB 5 μM or water for 24 hours into methacrylate chambers, and then infected by spray with *Pst*. In stomatal aperture experiments, 50 stomata were measured per each treatment and condition (violin plots). Data correspond to at least four independent plants ± SEM of a representative experiment. Statistically significant differences are represented by different letters ($p < 0.05$, two-way ANOVA with Tukey HSD).

subjected to water deficit conditions (Fig. 4B), thus indicating HB confers an outstanding drought tolerance.

Finally, plasma membrane damage was also evaluated by measuring electrolyte leakage in tomato leaves. In severe drought conditions (6 DAD), no significant differences were observed between HB-treated plants. However, control-treated plants subjected to drought displayed a higher percentage of ion leakage, suggesting the role of HB in cell membrane protection and stabilization (Fig. 4C). Nonetheless, no differences in chlorophyll content were found between treatments and conditions,

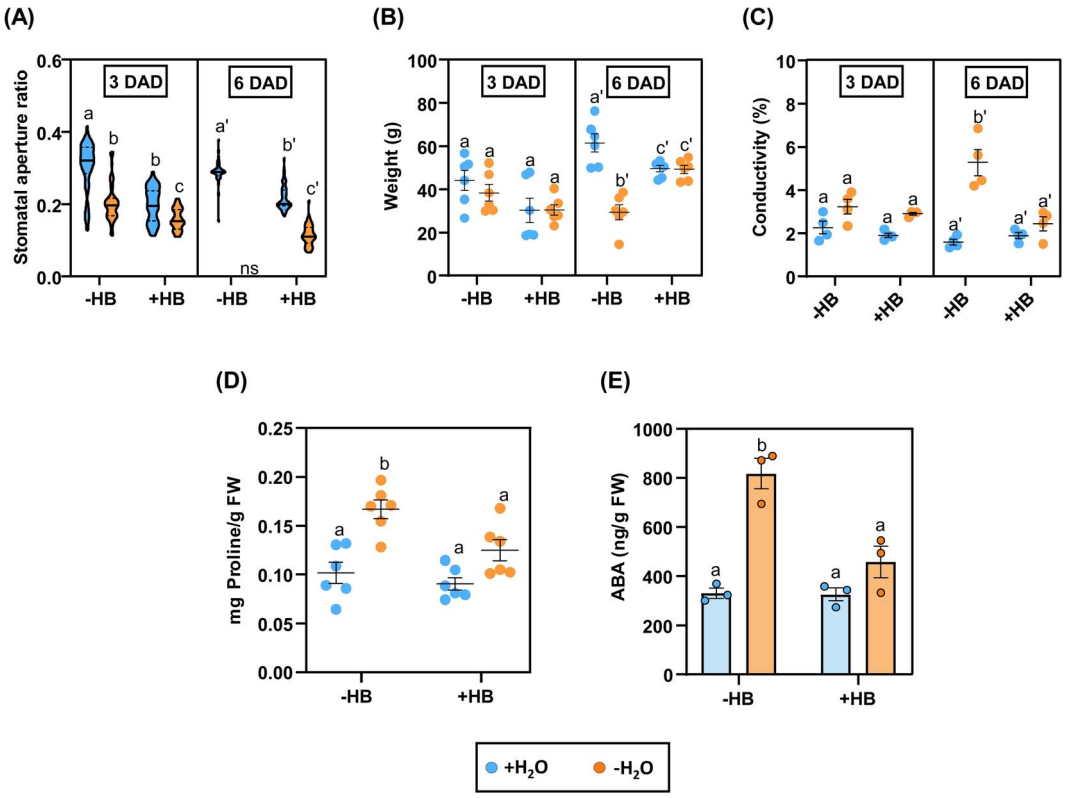

Figure 4. HB treatments induce tolerance to drought in tomato plants and reduce proline and ABA contents. Differences related to stomatal aperture ratio (A), weight (B), ion leakage (C), proline content (D) and ABA content (E) in tomato plants treated (+HB) or not (−HB) with HB, in normal (+$H_2$0) or water stressed conditions (−$H_2$0). Samples were taken 3 or 6 days after drought exposure (DAD). Data in (A) corresponds to 50 stomata per each treatment and condition (violin plots). Data in (B), (C) and (D) correspond to six independent plants ± SEM of a representative experiment. Data in (E) correspond to the averages of three independent plants ± SEM of a representative experiment. Different letters indicate statistically significant differences for each genotype and treatment ($p < 0.05$, two-way ANOVA with Tukey HSD).

indicating that water-stressed plants had a withered appearance, but they were not completely collapsed (Fig. S5).

Tomato plants treated with HB show lower proline and ABA levels under drought conditions

To better understand the role of HB in drought tolerance, levels of both proline, a well-known osmoprotectant, and ABA were analysed in HB-treated or non-treated, and well-watered or non-watered tomato plants.

As expected, non-treated tomato plants subjected to water deprivation accumulated higher proline levels than well-watered plants. However, when HB-treated plants were analysed, no statistical differences were found between non-stressed and water-stressed plants (Fig. 4D). Accordingly, a similar trend was observed regarding proline biosynthesis, in which Δ^1-pyrroline-5-carboxylate synthetase 1 (P5CS1) is the rate-limiting enzyme [28]. We observed that tomato *P5CS1* gene (*Solyc06g01970*) expression was significantly induced under drought conditions in both HB and non-treated plants. Nevertheless, *P5CS1* expression was lower in the case of HB-treated plants and the expression level was statistically similar to watered, non-treated plants (Fig. S6A).

Regarding the levels of ABA, no significant differences between untreated and HB-treated plants under well-watered conditions were observed. Remarkably, water-stressed plants treated with HB accumulated significantly less ABA than the stressed untreated plants, with levels comparable to those observed in non-stressed plants (Fig. 4E). The pattern of expression of genes that were involved in different critical steps of ABA biosynthesis, like 9-cis-epoxycarotenoid dioxygenase (NCED; Solyc07g056570), zeaxanthin epoxidase (Solyc02g090890), and ABA 8′-hydroxylase (Solyc04g078900) was also analysed by RT-qPCR, observing a tendency to down-regulation upon HB treatments in irrigated plants (Fig. S6B–D). Besides, we analysed the expression of marker genes of ABA signalling [29], such as Responsive to ABA 18 (*RAB18*), the transcription factor *MYB44*, and a late embryogenesis abundant (*LEA*) protein in tomato plants at 6 DAD (Fig. S7). Drought treatment induced the expression levels of *RAB18* and *LEA* in untreated and HB-treated plants. Interestingly, *LEA* gene induction in HB-treated plants appeared to be greater than in untreated plants under both irrigation and drought stress conditions. The induction of *LEA* in HB-treated, water-stressed plants (Fig. S7C), which accumulate lower levels of ABA (Fig. 4E), appears to reinforce that HB induces the abiotic response in an ABA-independent manner.

Our results indicate that the effect of exogenous HB appears to alleviate drought stress, since the mechanisms that counteract water deprivation, including ABA and proline accumulation, are less activated in HB-treated plants.

Transcriptomic changes in HB treatments under water stress revealed a higher induction of the abiotic responses and a repression of the water transport

To provide insight into the molecular responses of HB-exposed plants subjected to water deprivation, a further RNA-seq analysis was performed. Similar to above, a GO enrichment analysis was performed for upregulated and downregulated DEGs under water stress.

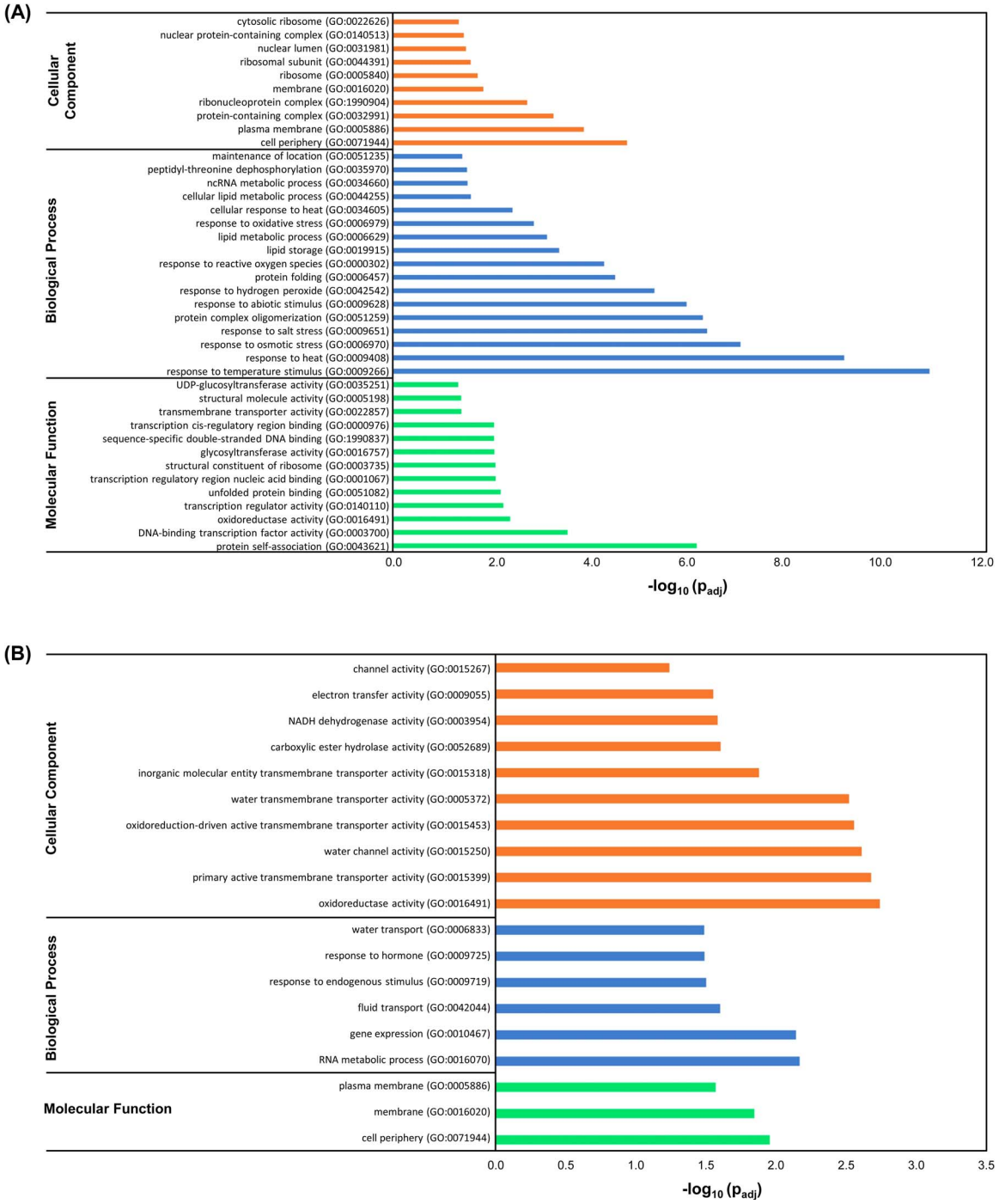

Figure 5. Transcriptomic analysis of HB-treated tomato plants under drought stress. Functional profiling analysis of upregulated (A) and downregulated (B) DEGs between HB- and control-treated plants under water deprivation.

Among the upregulated genes, most of the DEGs were putatively involved in response to temperature stimulus and heat (GO:0009266 and GO:0009408), response to osmotic and salt stress (GO:0006970 and 0009651), response to abiotic stimulus (GO:0009628) or response to hydrogen peroxide and reactive oxygen species (GO:00042542 and 000302), which are mainly related to abiotic stress responses (Fig. 5A). On the other hand, the annotated downregulated DEGs were associated with the fluid and water transport (GO:0042044 and 0006833) or with the response to hormone (GO:0009725) (Fig. 5B).

Moreover, a gene clustering analysis with DEGS from both RNA-seq (DEG threshold = 2), was performed to discover groups of correlated genes between different treatments (non-treated and HB-treated) and conditions (well-watered and non-watered plants). Following this approach, 16 clusters were generated (Fig. S8). According to cluster trends, we grouped them into three groups of interest: first DEGS that share the same response to HB in well-watered and non-watered conditions (clusters 7, 10, 12, and 16), second DEGS that only respond to HB in non-abiotic stress conditions (clusters 5 and 6) and third DEGS that only respond to HB in water deficit conditions (clusters 2 and 3). When a GO analysis with these new three groups was performed, only the third group, formed by clusters 2 and 3, showed enrichment in the same categories as in the previous GO analysis, including as a

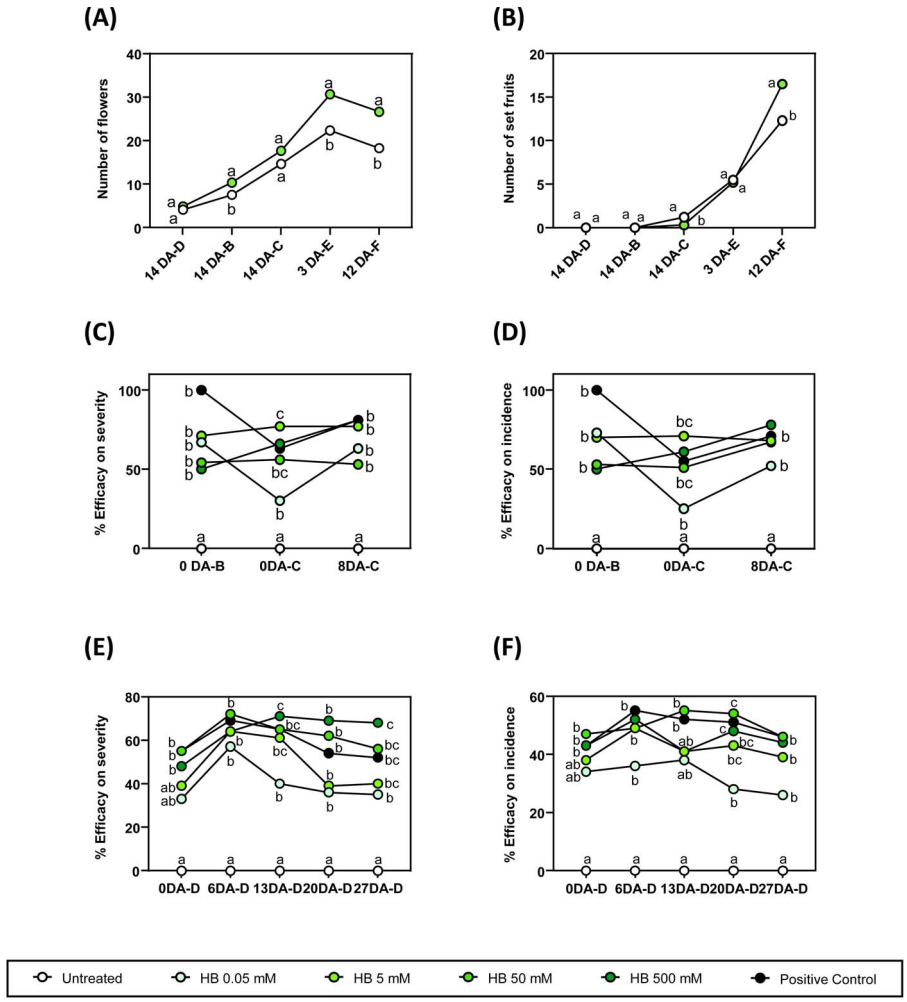

Figure 6. Agricultural applications of HB treatments under open field experiments. Number of flowers (A) and number of set fruits (B) of tomato plants under limited water availability conditions (50%) and treated (+HB) or not (−HB) with 5 mM HB. Time units are referred as follows: X DA Z, being X day (0–18); DA days after; Z day of treatment (A–H). Points represent mean values. Different letters indicate significant differences at each time point ($P < 0.05$, two-way ANOVA with Tukey HSD). Efficacy on severity (C) and efficacy on incidence (D) of tomato plants naturally infected by *Pseudomonas syringae* and either untreated, HB weekly treated at different concentrations (0.05, 5, 50, 500 mM) or treated with the positive control OXICOOP (0.5%). Time units are referred as follows: X DA Z, being X day (0–8); DA days after; Z day of treatment (A–C). An ANOVA test was performed, and different letters indicate statistical significances with a *P*-value <0.05. Efficacy on severity, (E) and efficacy on incidence (F) of potato plants naturally infected by *Phytophthora infestans* and either untreated, HB weekly treated at different concentrations (0.05, 5, 50, 500 mM) or treated with the positive control OROCOBRE (0.3%). Time units are referred as follows: X DA Z, being X day (0–27); DA Days After; Z day of treatment (A–D). An ANOVA test was performed, and different letters indicate statistical significances with a $p < 0.05$.

new GO term GO:0009737, response to ABA. Focusing on the genes contributing to that GO term, we observed that, under water stress conditions, HB clearly affects the ABA response since a differential expression of those genes was observed (Fig. S9), correlating with the lower ABA levels observed (Fig. 4E). Those genes correspond to some dehydrin or dehydrin-like proteins (Solyc01g109920, Solyc02g084850, Solyc02g093255, or Solyc02g062390) some Ninja-family proteins (Solyc05g012210 or Solyc04g005380), and a BZIP transcription factor (Solyc04g078840).

HB application improves productivity under water-limiting conditions

To study whether the drought tolerance conferred by HB treatments in tomato plants could contribute to improve productivity, we carried out treatments in an experimental field under limited water availability (Fig. S10).

On the one hand, we examined tomato fruit set throughout the experimental trial. As shown in Fig. 6A, plants treated with HB under water stress developed significantly more flowers than non-treated plants during almost the entire experiment. In addition, the greater number of flowers translated into a higher fruit set yield at 12 DA-F (Days After treatment F, See Materials and Methods) (Fig. 6B). On the other hand, we assessed the number of fruits and total productivity at different time points. At the beginning of the harvest period, the number of fruits on HB-treated plants was lower than on non-treated plants. However, from the fifth harvest (14 DA-H), the number of fruits on HB-treated plants became similar to that of untreated tomato plants. At the end of the harvesting period, HB-treated plants produced a higher yield (23%) than non-treated plants (Fig. S11A). Furthermore, the same trend was observed regarding fruit weight (Fig. S11B), thus indicating that periodic HB treatments can improve tomato fruit size and productivity under drought conditions. The harvested fruits were also classified depending on their size into extra, class I, class II, or class III. Both fruit weight and fruit number were higher in the extra and class I groups, in HB-treated tomato

plants subjected to drought conditions (Figs S10E and S11C and D). Our results indicate that HB treatments increase productivity and quality of the fruits in water-stressed tomato plants.

HB treatments lead to pathogen resistance

Since HB application activates defence-related genes and confers resistance to the bacteria *Pst* DC3000 under greenhouse conditions with controlled inoculations [22], experimental trials were carried out to assess whether HB defensive effects have potential uses in agriculture (Fig. S12).

The first trial was performed to confirm the agricultural applications of HB in tomato plants against *Pst* DC3000. For this purpose, tomato plants were treated periodically with different HB concentrations (0.05, 5, 10, and 50 mM), using the commercial pesticide OXICOOP 50 (0.5%) as a positive control. Applications began as preventive and were performed as a broadcast foliar application on the tomato plants prior to the appearance of bacterial disease symptoms (see Materials and Methods). On each assessment date, the percentage of leaves presenting symptoms per plot (incidence) and the leaf area infected (severity) were evaluated on 10 plants per plot, and the efficacy of incidence and severity were calculated. From the beginning of the experiment, HB-treated plants showed differences with respect to untreated plants in the percentage of efficacy of severity or incidence of the bacterial infection. At 0 DA-C and 8 DA-C, intermediate and high concentrations of HB acted similarly to the commercial pesticide OXICOOP 50, the efficacy of incidence ranging between 68 and 78% for high dose HB treatments and between 77 and 81% at last assessment considering the percentage of infected leaf (Fig. 6C and D; Fig. S12C and D).

Furthermore, we tested the efficacy of HB in a different plant-pathogen interaction in which stomatal immunity is crucial, such as potato defence against *Phytophthora infestans* (Fig. S13). For this end, the same approach was followed as in the tomato field trial against *Pst* but employing OROCOBRE (0.3%) as a commercial positive control treatment. Regarding the percentage of damaged leaf area, all plots showed statistical differences compared to the untreated control since 6 DA-D (Fig. 6E, Fig. S13D and E). The best results were obtained with the 500 mM HB treatment, reaching efficacies close to 70% and exceeding the performance of the reference OROCOBRE whose efficacy was around 52% at the end of the trial. Regarding symptoms in potato plants, HB treatments showed statistical significances from 6 DA-D, obtaining similar efficacy on the incidence of downy mildew as the treatments with the commercial fungicide (Fig. 6F).

Therefore, our results indicate that HB can be efficiently used to prevent diseases in agriculture caused by pathogens whose entry is through stomata.

Discussion

In previous studies, we identified different green leaf volatiles (GLVs) that were emitted when tomato plants efficiently resisted bacterial infection with *Pst*. Particularly, the volatile HB has been patented as a universal stomatal closure compound [30], but the detailed mechanisms of HB action in stomatal immunity remain undeciphered [22]. In this work, a transcriptomic analysis revealed that HB treatments triggered the activation of different processes that were related to plant defence mechanisms such as chitin catabolism, glucosamine, and aminoglycan metabolism or endopeptidase activity, reinforcing the role of HB in the defensive response activation (Fig. 1A; Fig. S1).

The role of stomata as active defensive barriers in plant immunity has been extensively studied. Our results showed that HB treatments induce stomatal closure as effectively as ABA and flg22, (Fig. S2). Nonetheless, HB treatments in ABA-deficient mutants triggered stomatal closure (Fig. 2A), demonstrating that HB-mediated stomatal closure is ABA-independent. Moreover, chemical approaches also indicated that, similarly to flg22, HB treatments induced stomatal closure and plant defence signalling through NADPH-oxidase ROS production and Ca^{2+} ion permeable channels (Fig. 2B–D). Furthermore, we have also observed that MPK3 and MPK6 activation is essential for HB-triggered stomatal immunity (Fig. 3). Our results correlate with other studies that have shown that GLV exposure induced the activation of these downstream responses. For instance, exposure of *Arabidopsis* plants to (*E*)-2-hexenal and (*E*)-2-hexenol involved membrane potential depolarization through an increase in cytosolic Ca^{2+} via ROS-activated calcium channels [31]. Besides, maize and *Arabidopsis* plants exposed to (*Z*)-3-hexenol and (*E*)-2-hexenal, respectively, increased the transcript abundance of different genes involved in defence signalling [32,33]. The fact that GLVs are formed from endogenous plant components, are emitted upon pathogen infection, and that their exogenous application activates defence signalling mechanisms suggests that GLVs, and particularly HB, could act as DAMPs [34]. DAMPs are danger signals released by plants upon pathogen attack that have a relevant role in amplifying the immune responses.

Drought is considered one of the most important factors limiting crop productivity, and new strategies are needed to cope with this global threat. In response to drought stress conditions, the first option for plants is to partially close stomata to prevent water loss through transpiration [35]. In this study, we have shown that the stomatal aperture ratio of HB-treated tomato plants was similar to the stomatal aperture ratio of plants that were subjected to water stress (Fig. 4A), a phenomenon that led to minimize weight loss and ion leakage after 6 days of water deprivation (Fig. 4B and C), indicating that HB-mediated stomatal closure was effective in drought and alleviated its effects. Interestingly, levels of both proline and ABA were lower in HB-treated tomato plants subjected to water deprivation (Fig. 4D and E), while the expression of a *LEA* gene, which is thought to participate in membrane protein stability, osmotic adjustment and macromolecular stabilization [36], is highly induced (Fig. S7C). The transcriptomic analysis of HB-treated plants under drought conditions (Fig. 5) appear to indicate that HB-mediated stomata closure could be producing a higher induction of the abiotic responses and a limitation of the water transport, therefore alleviating the drought effect in tomato plants. Besides, the downregulation of the hormone response (Fig. 5B) could be associated with the observed ABA repression (Fig. 4E), thus reinforcing the idea that HB acts in an ABA-independent manner (Fig. 2A). In fact, when we compared both RNA-seq analyses and we focused on clusters 2 and 3, which only respond to HB in water deficit conditions, an enrichment on the GO corresponding to ABA response was observed, correlating the repression of ABA-related genes (Fig. S9) with the lower levels of ABA observed in those plants (Fig. 4E). Our results suggest that HB is conferring tolerance to drought in tomato plants through ABA-independent mechanisms, which appear to be non-essential but replaced by the HB-mediated response.

The application of osmoprotectants and biostimulants as elicitors to confer plant tolerance to different biotic and abiotic stresses through priming is a promising alternative to conventional techniques. The priming mechanism enables plants to respond more robustly and rapidly, which results in an

enhanced resistance or tolerance to a stress condition [37]. Most-know priming compounds are synthetic SA analogues, such as 2,1,3-benzothiadiazole (BTH) or 2,6-dichloroisonicotinic acid and its methyl ester (both referred to as INA) which trigger the activation of systemic acquired resistance (SAR), and the non-protein amino acid β-aminobutyric acid (BABA) also protects plants against various biotic and abiotic stresses in a plethora of plant species [38]. Many VOCs have also been described as elicitors and priming compounds. Hexanoic acid can activate broad-spectrum defences by inducing callose deposition and activating SA and JA signalling pathways. Besides, its efficiency has been demonstrated in a wide range of host plants and pathogens [39,40]. Another example of VOCs as priming agents against abiotic factors is the GLV (Z)-3-hexenyl acetate, since maize seedlings primed with this volatile exhibited cold tolerance [19].

In greenhouse conditions, our laboratory demonstrated that HB induces resistance against bacterial infection through stomata closure [22]. In this study, the enhanced resistance to pathogens and tolerance to drought conditions caused by HB has also been demonstrated in field conditions. Regular exogenous HB treatments in tomato plants under limited water availability conditions resulted in increased number of flowers, which lead to enhanced number and size of tomato fruits compared to non-treated plants (Fig. 6A; Fig. S11). When conditions are unfavourable, plants tend to alter flowering processes to ensure that seed is produced for the next generation [41].The observed higher induction of the abiotic responses in HB- treated water-stressed plants (Fig. 5) could be affecting the flowering development, therefore explaining the greater number of flowers observed (Fig. 6A). An enrichment of GO terms in flowering was not observed in HB-treated plants under drought stress, probably due to the sampling upon HB treatment, containing mostly leaf material and little meristems. Nevertheless, alterations in the expression of flowering-related genes were specifically detected indicating that HB may influence flower production (data not shown). Further studies will help us better understand and exploit the effect of HB on flowering and fruit set upon water stress conditions.

HB efficacy in field was also demonstrated in the previously analysed tomato-bacteria interaction and also against potato late blight caused by *P. infestans*, the efficacies obtained in both cases being higher than those of commercial agrochemicals (Fig. 6C–F) with the added advantage of being natural and non-toxic unlike most conventional pesticides. According to these results, HB emerges as a promising natural elicitor compound against biotic and abiotic stress.

In summary, the results obtained in this work provide evidence for the role of HB in stomatal immunity in tomato and *Arabidopsis* plants, being ROS generation by NADPH oxidases, Ca^{2+} fluxes, MPK3/6 activation, but not ABA, essential for HB-mediated stomatal immunity. Furthermore, HB-induced stomatal closure resulted in enhanced resistance and tolerance to pathogen infection and drought in field conditions, therefore proposing HB as a novel natural elicitor candidate and a promising strategy for sustainable agriculture.

Materials and methods

Plant material and growth conditions

In this study, tomato (*Solanum lycopersicum*) cv. MoneyMaker was used. Tomato ABA-deficient mutant *flacca (flc)* [42] and its corresponding parental Lukullus plants were kindly provided by Dr Jorge Lozano (IBMCP, Valencia, Spain). Tomato plants were grown as described in Ref. [22].

For greenhouse drought experiments, water stress was simulated by quitting irrigation for 6 days in the case of stressed plants, while control plants were normally watered. HB treatments were performed every 2 days until the end of the experiments.

Arabidopsis plants were in the Columbia (Col-0) ecotype background. *mpk3* and *mpk6* mutants were kindly provided by Dr Borja Belda-Palazón (IBMCP, Valencia, Spain). Plants were grown in a chamber under short-day conditions (10/14 hours; 19/23°C light/darkness), with a relative humidity ranging from 50 to 60%.

HB treatments under greenhouse conditions

Treatments were carried out on 4-week-old tomato plants either in closed chambers or by spray. Five tomato plants were placed into 121 l (50 × 50 × 50 cm exterior lengths) transparent methacrylate boxes containing hydrophilic cotton swabs soaked with HB (Sigma-Aldrich, Saint Louis, MO, USA) or distilled water in the case of control plants. Specifically, a total of 123 μl of HB were distributed in six cotton swabs, which were placed homogeneously among the plants to reach a HB final concentration of 5 μM in the chamber. Methacrylate boxes were airtight sealed during the 24-hour treatment. In the case of spray treatments, tomato plants were pre-treated by spray with HB at a concentration of 2 mM or distilled water (mock), containing 0.05% Tween 20 as a wetting agent. The solution was applied uniformly as a fine mist with a hand-held sprayer until the suspension ran off the leaf surfaces, and plants were placed on greenhouse benches during the HB treatment.

Bacterial inoculation assays

The bacterial strain used in this study was *P. syringae* pv. tomato (*Pst*) DC3000 (kindly provided by Dr Selena Gimenez, Centro Nacional de Biotecnología, Madrid, Spain). Bacterial inoculations were performed as described by López-Gresa *et al.* [22]. For spray inoculation assays on *Arabidopsis* plants, a *Pst* DC3000 culture (OD_{600} = 0.2) was resuspended in 10 mM $MgCl_2$ + 0.05% Silwet L-77 and sprayed onto 4-week-old plants. The bacterial growth experiments were carried out by sampling three leaf discs (1 cm^2 each) from each plant at 72 hours post inoculation. Tissue samples were ground in 10 mM $MgCl_2$. Serial dilutions were done and plated on Petri dishes containing King's B medium supplemented with rifampicin. Bacterial colonies were counted 48 hours after serial dilutions.

Stomatal aperture measurement

To measure the stomatal aperture in tomato plants, clear nail polish was applied in the abaxial part of five leaves from three independent plants. Once the film was dry, it was carefully peeled off and leaf impressions were obtained.

In the case of leaf discs, they were taken from 3- to 4-week-old plants and floated on Murashige and Skoog medium (MS) for 3 hours under light to induce stomatal opening. Then, stomata closing elicitors (1μM flg22, 10μM ABA, and 50μM HB) were added to the medium for 3 hours. For chemical inhibition, 2 mM salicylhydroxamic acid (SHAM), 20 μM diphenyliodonium chloride (DPI), 2 mM ethylene glycol-bis(β-aminoethyl)-N,N,N′,N′-tetraacetic acid (EGTA), and 20 μM 2-(2-Amino-3-methoxyphenyl)-4H-1-benzopyran-4-one (PD98059) were added 30 minutes before incubation with the elicitors.

Samples were then visualized under a Leica DC5000 microscope (Leica Microsystems GmbH, Wetzlar, Germany), and pictures of different regions were analysed with the *ImageJ*

software (https://imagej.net/ij/). Aperture ratio was measured as stomata width/length from at least 50 stomata per plant and/or treatment, considering a value of 1 as a totally opened stoma.

Ion leakage estimation

Five leaf discs (1 cm^2) were excised using a stainless steel cork borer, gently washed, and then immersed in tubes containing 40 mL of deionized water and shaken at 200 rpm for 2 hours at 28°C. The conductivity of the solution (L1) was measured with a conductivity meter (DDS-11A, Shanghai Leici Instrument Inc, Shanghai, China). After that, the solution containing the leaf discs was boiled for 15 minutes, cooled to room temperature, and the conductivity of the disrupted tissues (L2) was measured. Ion leakage was calculated as the ratio of L1 to L2.

RNA isolation and RT-qPCR analysis

The total RNA of tomato leaves was extracted as described by López-Gresa *et al.* [22]. Quantitative PCR was carried out as previously described [43]. The housekeeping gene transcript actin was used as the endogenous reference. The PCR primers were designed using the online service Primer3 (https://primer3.ut.ee) and are listed in Table S1.

RNA-seq and GO analyses

Six individual 4-week-old MoneyMaker tomato plants were placed into methacrylate chambers and treated with HB or distilled water as described above. Twenty-four hours post-treatment, leaf samples were collected, and total RNA was extracted and analysed using a 2100 Agilent Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA, USA) to check RNA integrity and quality. RNA-seq and bioinformatics analysis were performed by Genomics4All (Madrid, Spain). Samples were sequenced with ×50 genome coverage using 1 × 50 bp reads. Raw data were aligned to *S. lycopersicum* genome using HISAT2 v2.1.0 [44]. StringTie was used to obtain the differential expression [45]. Data and transcript-level expression analysis and functional enrichment analysis were performed following previously described protocols [46,47]. GO enrichment analysis was performed with the Panther Classification System (https://pantherdb.org) [48].

MPK phosphorylation assay

Leaf discs were excised from 3- to 4-week-old tomato and *A. thaliana* plants and floated on MS medium for 3 hours under light as described above. Then, 1 μM flg22, 10 μM ABA, or 50 μM HB treatments were performed, and samples were taken 15, 30, and 60 minutes after treatments and immediately frozen in liquid nitrogen. Frozen leaf samples were homogenized, and protein extracts were obtained by Laemmli extraction method. Briefly, leaf disc powder (100 mg) was mixed with 150 μl 2× Laemmli buffer and incubated on ice for 20 minutes, vortexing every 5 minutes. Samples were thereafter boiled for 10 minutes, cooled on ice for 5 minutes, and centrifuged at 12 000 × *g* for 5 minutes at 4°C to clarify homogenates. The resulting supernatants (10 μl) were analysed by western blot. Proteins were separated by 10% SDS-PAGE, transferred onto PVDF membranes for 90 min 110 V at 4°C using a Bio-Rad wet blotting transfer system (transfer buffer 192 mM glycine, 25 mM Tris, 0.1% SDS, 20% ethanol), and immunodetected by using Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (Cell Signaling, 1679101S; dilution 1:1000) primary antibodies, and peroxidase-conjugated goat anti-rabbit IgG (Jacksons, 111035144; dilution 1:20 000) secondary antibodies. Immunogenic bands of ~43 kDa were revealed by using the SuperSignal™ West Femto Maximum Sensitivity Substrate (Thermo Scientific) and a AMERSHAM ImageQuant 800 equipment.

Open-field HB treatments

Abiotic stress

This assay was carried out at Picanya, located in Valencia, Spain (39°26′08″N, 0°26′09″O). Due to the volatility of the compound, the field trial was performed in two separate greenhouses, one for HB treatments and the other for control treatments. The experimental design used was randomized blocks (9 m^2) with four biological replicates per treatment. Plants were sprayed with 5 mM HB every two weeks. To provoke water stress, irrigation was completely stopped. The soil capacity was continuously monitored, and once it reached 50%, the water regime was re-established. Different parameters, such as number of flowers, fruit set, and total fruits as well as fruit weight, were assessed. Moreover, total yield was analysed at harvest per category as follows: small size (25–50 mm diameter, 25–100 g weight), medium size (50–75 mm diameter, 100–200 g weight), big size (75–100 mm diameter, 200–300 g weight), and extra size (>100 mm diameter, >300 g weight).

In all the field trials, time units are referred to as follows: X DA Z, being X day (0–18); DA Days After; Z day of treatment (A–H).

Abiotic stress trials were performed under General Standards in accordance with EPPO Guidelines PP 1/153 (4), PP 1/181 (4), PP 1/135 (5), and PP 1/239 (2).

Biotic stress

The trial of tomato plants inoculated with *P. syringae* was performed under open field conditions in Torrellano (Alicante, Spain, 38°17′39″N, 0°35′11″O). The tomato plants were transplanted on 26 August 2020, and the commercial variety Muchamiel was used. The experiment comprised six plots (10 plants each) treated with HB at 0.05, 5, 50, and 500 mM, plus another plot that was treated with the commercial pesticide OXICOOP 50 (0.5%) as a positive control, and one plot was left untreated. Treatment applications were carried out using a motorized knapsack sprayer. Three applications were carried out: application A was preventive and subsequent applications (B and C) were performed within 6- to 8-day interval coinciding with the persistence of the effect of HB [22].

To determine the efficacy of HB for the control of *Downy mildew* in potatoes, an independent open field condition trial was performed in Borbotó (Valencia, Spain, 39°30′55″N, 0°23′27″O). The potato plants were transplanted on February 18 2019, and the variety used was Vivaldi. The experimental design used was a randomized block with six plots (one plot per treatment) and 60 plants in each one. Foliar treatments were performed following the same methodology and strategy as in the case of the tomato experiment, but in this case, ORO-COBRE ZINEB (0.3%) was used as a positive control. Four exogenous treatments (A, B, C and D) were carried out, being treatment A before disease appears, and following treatments (B–D) with 7 ± 1 day intervals.

In both experiments, the degree of infection was assessed as the percentage of leaves presenting symptoms (incidence) and the percentage of infected leaf area per diseased leaf (severity). The efficacy (E) of the treatments was calculated according to Abbott's formula [49], using the percentage of leaf area affected in the control (C) and treated (T) groups. The following formula was used: $E = [(C-T)/C] * 100$.

Tomato and potato pathogen inoculation trials were performed in accordance with EPPO (Efficacy evaluation of Plant protection Products) guidelines PP1/135 (4), PP1/152 (4), and PP1/181 (4) and specific EPPO PP1/2 (4).

Statistical analysis

The statistical analysis of two or more variables was carried out using Student's *t*-test or analysis of variance (ANOVA), respectively, employing GraphPad Prism 9 software. In all the analyses, a *P*-value <0.05 was considered statistically significant.

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Author Contributions

M.P.L.-G. and P.L. designed the research. C.P., B.B.-P., F.V., and J.P.P. performed research. L.J., F.V., and J.P.P. contributed RNA-Seq analytic tools. C.P., B.B.-P., F.V., J.P.P., L.J., I.R., J.M.B., M.P.L.-G., and P.L. analysed and discussed data. C.P., M.P.L.-G., and P.L. wrote the paper and incorporated the input of the rest of the authors.

Data Availability

The data underlying this article are available in https://www.ncbi.nlm.nih.gov/sra/ and can be accessed with PRJNA1009051.

Conflict of Interests

None declared.

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Supplementary Information

Supplementary data is available at *Horticulture Research* online.

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