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Wilkinson, S.W., Pastor, V., Paplauskas, S. et al. (2 more authors) (2018) Long-lasting β-aminobutyric acid-induced resistance protects tomato fruit against Botrytis cinerea. Plant Pathology, 67 (1). pp. 30-41. ISSN 0032-0862

https://doi.org/10.1111/ppa.12725

Long-lasting β-aminobutyric acid-induced resistance protects tomato fruit against *Botrytis cinerea*

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Running Title: Induced resistance in tomato fruit

Key Words

Botrytis cinerea; Induced resistance; β-aminobutyric acid (BABA); Post-harvest; Abscisic Acid (ABA); Tomato (*Solanum lycopersicum*).

Abstract

Minimising losses to pests and diseases is essential for producing sufficient food to feed our rapidly growing population. The necrotrophic fungus *Botrytis cinerea* triggers devastating pre- and post-harvest yield losses in tomato (*Solanum lycopersicum*). Current control methods are based on the pre-harvest use of fungicides, which are limited by strict legislation. Here, we have tested whether induction of resistance by β-aminobutyric acid (BABA) at different developmental stages, provides an alternative strategy to protect tomato fruit post-harvest against *B. cinerea*. Soil-drenching plants with BABA once fruit had already formed, had no impact on tomatoes susceptibility to *B. cinerea.* Whereas BABA application to seedlings was found to significantly reduce the post-harvest infection of fruit. This resistance response was not associated with a yield reduction, however there was a delay in fruit ripening. Untargeted metabolomics unravelled differences between fruit from water and BABA-treated plants, demonstrating that BABA triggered a defence-associated metabolomics profile that was long-lasting. Targeted analysis of defence hormones suggested a role of abscisic acid (ABA) in the resistance phenotype. Post-harvest application of ABA to the fruit of water-treated plants induced susceptibility to *B. cinerea*. This phenotype was absent from the ABA exposed fruit of BABA-treated plants, suggesting a complex role of ABA in the BABA-induced resistance phenotype. A final targeted metabolomic analysis detected trace residues of BABA accumulated in the red fruit. Overall, we have demonstrated that β-aminobutyric acid induces post-harvest resistance in tomato fruit against *B. cinerea* with no penalties in yield.

Introduction

With 163 million tonnes being produced annually, tomatoes (*Solanum lycopersicum*) are by weight the eleventh largest global commodity (FOASTAT, 2013). However, as with many crops, yields of tomato are significantly limited by losses to pests and diseases. One key pathogen that contributes to yield reductions in tomatoes is *Botrytis cinerea*, the species responsible for the grey mould disease. *B. cinerea* is a necrotrophic ascomycete with a host range of over 200 plant species, including a number of vegetables and soft fruits. In addition to its broad range of hosts, this pathogen produces large numbers of spores and is able to survive in a dormant state in soil. As a result the fungus is present in a wide range of environmental conditions (Hahn *et al.*, 2014). This includes the fridge where it is able to grow successfully, thus rendering cold storage an unsuitable strategy for combating the pathogen. *B. cinerea* is so prolific that out of all fungal pathogens infecting plants, in terms of scientific and economic importance, it was ranked second by the international fungal pathology community (Dean *et al.*, 2012).

In tomato, *B. cinerea* is particularly problematic as not only can it decimate green tissue, reducing yield potential, but it can also infect the fruit. Consequently, post-harvest losses in tomatoes are a significant problem, with as much as 50% of yield being lost in the developing world to pests, diseases and damage (FAO, 1989). With the world's population projected to increase to more than 9.7 billion by 2050, global crop production will need to be doubled in order to meet the increased demand for food. Reducing yield losses to pests and diseases will be an important step towards achieving this challenge (Godfray *et al.*, 2010).

Over the last 50 years, the most common strategy to combat pests and diseases has been the application of chemical pesticides. Furthermore, the primary method for reducing

post-harvest losses to *B. cinerea* in soft fruit and vegetables, including tomato, is pre-harvest fungicide application (Elad *et al.*, 2007). In recent years, there has been a decline in the volume of chemical pesticides used annually in Great Britain. The major reason for this reduction is not a decline in pest and disease outbreaks. Instead it is because research has highlighted the potential risks to the environment of applying pesticides, which has led to greater restrictions on their use (Elad *et al.*, 2007). Furthermore, pesticide resistance is a major problem. This particularly concerns species that produce large numbers of spores and are thus capable of rapid evolution, such as those belonging to the genus *Botrytis* (Leroch *et al.*, 2011). Consequently, these issues require the innovation of alternative control methods to successfully increase agricultural productivity and meet future food demands in a sustainable manner (Luna, 2016).

One possible control method is the augmentation of the plants' innate defence mechanisms. Natural stimuli such as localized pathogen attack (systemic acquired resistance) and colonisation of plant roots by beneficial soil microbes such as *Pseudomonas putida* (induced systemic resistance) can result in systemic resistance against future attack by biotrophic and necrotrophic pathogens, respectively (Ton *et al.*, 2002). Induced resistance is not achieved through a costly constitutive expression of defence mechanisms, but instead it is most likely explained by an energy efficient sensitisation of these defence mechanisms known as priming (van Hulten *et al.*, 2006; Martinez-Medina *et al.*, 2016; Mauch- Mani *et al.*, 2017). Under benign conditions, the expression of defence mechanisms in primed plants is weak. When primed plants are challenged, their basal defence response is faster upregulated and stronger than unprimed plants and thus more likely to provide resistance (Conrath *et al.*, 2006). The sensitisation of plant defences provides a viable alternative or powerful complement, as part of an integrated disease management (IDM) strategy, to pesticide use (Conrath *et al.*, 2015; Luna, 2016).

Priming of defence is not only induced by biotic stimuli but also by abiotic agents including a variety of chemicals (Conrath *et al.*, 2015). For instance, application of the phytohormones salicylic acid (SA) and jasmonic acid (JA) can prime plant defence (Pastor *et al.*, 2013). Also, treatment with β-aminobutyric acid (BABA), a non-protein amino acid, has been demonstrated to induce resistance via priming of defence, in multiple plant species against a variety of biotic (Jakab *et al.*, 2001) and also abiotic (Jakab *et al.*, 2005) stresses. In *Arabidopsis thaliana* (referred to as *Arabidopsis* hereafter), this outstanding performance is the result of BABA priming both SA-dependent and independent defences (Zimmerli *et al.*, 2000; Ton *et al.*, 2005). This occurs following the binding of the active enantiomer, (R)-BABA, to the identified BABA receptor in *Arabidopsis,* an aspartyl-tRNA synthetase (AspRS; Luna *et al.*, 2014). Binding of (R)-BABA blocks the AspRS's canonical function, which results in the accumulation of aspartate and uncharged tRNA. Moreover, it is known that BABA, at relatively high concentration, supresses plant growth (Wu *et al.*, 2010). Luna *et al.* (2014) demonstrated that this stress response is dependent on the accumulation of uncharged tRNA and therefore that BABA-induced resistance (BABA-IR) and BABA-induced stress responses are controlled by different signalling pathways.

In tomatoes, BABA-IR has been shown to protect green tissue against *B. cinerea,* when BABA is applied by spray (Cohen, 2000) or by soil drench (Luna *et al.*, 2016). In addition, BABA-IR has been shown to be long-lasting following application at the seed or seedling stage (Worrall *et al.*, 2012; Luna *et al.*, 2016). However, the effect of BABA on the post-harvest defence response is not understood. Here we investigated whether BABA-IR can persist post-harvest, making tomato fruit more resistant to *B. cinerea,* following treatment with BABA at the seedling (Experiment 1) or fruiting stages (Experiment 2). As treatment with BABA can result in growth reductions and fitness costs (van Hulten *et al.*, 2006; Wu *et al.*, 2010), we have determined the effect on the economically important yield and fitness

parameters of tomato. To unravel the mechanisms by which BABA enhances resistance, an untargeted metabolomics analysis was carried out. This was followed by a targeted analysis of phytohormones associated with defence responses against *B. cinerea* (Audenaert *et al.*, 2002; Asselbergh & Höfte, 2007). Based on the findings of this targeted analysis, the impact of exogenous application of the phytohormone abscisic acid (ABA) on the induced resistance phenotype was assessed. Finally, we tested whether BABA is accumulated in the fruit.

Materials and Methods

Plant materials and growth conditions

Seeds of the tomato cultivar micro-tom (*Solanum lycopersicum* L. C.V. micro-tom, originally distributed by A Levy, Israel, and kindly provided by Dr. Victor Flors) were maintained at 28^{o}C in damp and humid conditions for four days to stimulate germination. Germinated seeds were transferred to individual pots containing Scott's Levington M3 soil (Everris) and grown under 14 hours/10 hours day/night cycles, 25^{o}C/20^{o}C day/night temperatures, 60% humidity and 160 µmol m^{-2} s^{-1} irradiance for 12 weeks.

β-aminobutyric acid (BABA)

BABA was sourced from Sigma Aldrich (catalogue number: A44207). Solutions of BABA were made up fresh each time in distilled water (dH_20) to the specified concentrations. Concentrations were selected based on previously described work by the authors Luna *et al*, (2016).

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153 *Experiment 1 - Treatment of tomato seedlings with BABA*

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155 A total of 16 micro-tom seeds were planted in individual pot propagators (approximate
156 volume 80 mL) containing M3 soil. After two weeks, eight seedlings ("BABA Seedling"
157 treatment) were soil-drenched with 8 mL per pot of 5 mM BABA solution, so to generate a
158 final concentration of 0.5 mM in the soil. The other eight seedlings ("Water Seedling") were
159 soil-drenched with 8 mL per pot of distilled water (dH_2O). One week post treatment, roots
160 from the 16 seedlings were carefully washed under running tap water and then the plants
161 were transplanted into individual 2.2 L pots containing untreated M3 soil. The plants were
162 allowed to grow for nine more weeks until the fruit turned red, at which point they were
163 harvested and infected with *B. cinerea*. This experiment was repeated twice with similar
164 results.

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166 *Experiment 2 - Treatment of mature tomato plants with BABA*

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168 A total of 24 micro-tom tomato plants were grown under identical conditions in individual
169 2.2 L pots containing M3 soil. At seven weeks post planting, when green tomatoes had begun
170 to be produced, eight plants were treated with BABA ("BABA Green" treatment). This was
171 achieved by soil-drenching each pot with 220 mL of 10 mM BABA solution, resulting in an
172 approximate concentration of 1 mM BABA in the soil of each pot. The other sixteen plants
173 were identically soil-drenched with distilled water (dH_2O). For the four weeks following the
174 "BABA Green" treatment, all plants received the same amount of water per pot to insure the
175 maintenance of the BABA concentration and the osmotic balance of the plants.

At 11 weeks post planting, when the plants had started to ripen their tomatoes, eight out of the 16 plants previously treated with water were each soil-drenched with 220 mL of 10 mM BABA ("BABA Red" treatment), taking the BABA concentration in the soil of each pot to 1 mM. The other 16 plants ("Water" and "BABA Green" treatments) were soil-drenched with an identical volume of dH_2O. Subsequently, when plants were watered the same volume of water was used. One week after the "BABA Red" treatment, "Water", "BABA Green" and "BABA Red" red tomatoes were harvested and then infected with *B. cinerea*.

Fitness Parameters

Fruit number and fruit ripening were assessed by counting the number of red fruit at different times during the 12 weeks of growth. In addition, the tomatoes harvested for infection (see below) were photographed and the diameters calculated digitally using Photoshop CS5 (Adobe Systems Incorporated). Finally, the average percentage water content of tomatoes from different treatments was measured. Four red tomatoes were harvested from each of the plant and weighed to measure their combined fresh weight (FW). The tomatoes were then dried for two days at $100^{o}C$ in individual tinfoil cases (one per plant). Following drying the combined dry weight of the four tomatoes was measured, with the difference between FW and DW corresponding to the water content.

Botrytis cinerea cultivation and inoculation method

B. cinerea cultivation and infection was performed as previously described in Luna *et al.*, (2016) with modifications. Inoculum was prepared by combining 3 mL of spore suspension containing $1.4x10^5$ spores per mL, 3.3 mL of 100 mM glucose and 2.2 mL of 100 mM

KH_2PO_4, obtaining a final spore concentration in the inoculum of $5x10^4$ spores per mL. At 12 weeks post planting, four red tomatoes were harvested from each plant and placed with the tip pointing upwards on plastic frames laid out in a tray containing wet absorbent paper. A needle was used to create an approximately 2 mm deep wound at the tip of the tomato. To each wound, a 5 µL drop of $5x10^4$ spore per mL inoculum was added. The tomatoes were then incubated in the dark at 100% humidity and 23°C.

Disease scoring in tomatoes

At three days post innoculation (dpi), the diameter of the visible necrosis on the top of each infected tomato was measured using Vernier calliper's. Four dpi, the same infected tomatoes were classified into one of four classes based on their visible external necrosis characteristics (**Figure 1c**): Class I (white) - No external mycelium or signs of necrosis, healthy tomato; Class II (pink) - external mycelium + necrosis diameter <10mm; Class III (dark pink) - external mycelium + necrosis diameter >10mm; Class IV (red) - tissue collapse, whole tomato necrotic, lesion diameter = tomato diameter.

Metabolites extraction

At 12 weeks post planting, one red tomato was harvested from each of four plants from each of the five treatments (experiment 1 and 2). For each tomato 0.5 g of pericarp was crushed to a fine powder with a liquid nitrogen-cooled pestle and mortar. The powder was suspended in 1 mL of extraction buffer (Methanol:dH_2O:formic acid 95:4.9:0.1, v:v:v) following which it was vortexed for 2 seconds and then centrifuged at 19,000 g and 4°C for 10 minutes. A total of 900 µL of supernatant was removed and the pellet was re-suspended in 500 µL of

extraction buffer. The pellet and extraction buffer was vortexed for 10 seconds and then centrifuged for 10 minutes at 13,000 rpm and 4°C. A total of 400 µL of supernatant was removed and pooled with the first 900 µL of supernatant. The pooled supernatant was vortexed for 2 seconds and then centrifuged at 13,000 rpm and 3°C for 10 minutes. The 1200 µL of supernatant was split equally between three aliquots and then placed overnight in a speed-vacuum concentrator (SpeedVac Plus SC210A, Savant, UK) coupled to a refrigerated vapour trap (RVT100, Savant, UK) to remove all moisture. To preserve the samples between extraction and analysis, dried aliquots were stored at -80°C.

Metabolomics by Ultra-Performance Liquid Chromatography coupled to quadrupole-orthogonal Time-Of-Flight mass spectrometry (UPLC-qTOF-MS)

Dried samples were resuspended in 100 µL of Methanol:dH_2O:formic acid 50:49.9:0.1, v:v:v), sonicated in cold water for 20 minutes, vortexed and then centrifuged for 15 minutes at 4°C. The resulting supernatants (80 µL) were transferred into glass vials prior to UPLC-qTOF-MS. Mass spectra of the tomato pericarp extractions were recorded in positive (ESI^+) and negative (ESI^-) electrospray ionisation modes using an ACQUITY UPLC system interfaced to a SYNAPT G2 qTOF mass spectrometer with an electrospray source (Waters, UK). Metabolites separation was achieved with an ACQUITY UPLC BEH C18 column (2.1 × 50 mm, 1.7 µm, Waters) protected by a pre-column (VanGuard, 2.1 x 5 mm, 1.7 µm, Waters) at a solvent flow rate of 0.6 mL min^{-1}. The Solvent A (water with formic acid 0.05 %, v/v) and solvent B (acetonitrile with formic acid 0.05 %, v/v) gradient was as followed: 0 – 3 min 5 – 35 % B, 3 – 6 min 35 – 100 % B, 6 – 7.5 min 100 % B, 7.5 – 7.6 min, 100 – 5% B. The injection volume was 10 µL and the column was kept at 40°C. Blank samples (MeOH:dH_2O, 50:50, v:v) were injected between each treatment condition. Detection by

SYNAPT G2 was performed with a scan time of 0.2 s for full scan (MS) and at elevated energy mode (5 to 45 eV, MS^E), over a mass range of 50 - 1200 Da. The following conditions were used for ESI^-: capillary voltage - 3 kV, sampling cone voltage - 60 V, extraction cone voltage - 3.5 V, source temperature 120°C, desolvation temperature 350°C, desolvation gas flow 800 L h^{-1}, cone gas flow 60 L h^{-1}; for ESI^+: capillary voltage + 3.5 kV, sampling cone voltage + 60 V, extraction cone voltage + 3.5 V, source temperature 120°C, desolvation temperature 350°C, desolvation gas flow 800 L h^{-1}, cone gas flow 60 L h^{-1}. Accurate mass measurements for each run were ensured by using the lockmass leucine enkephalin as the internal reference. MassLynx v 4.1 (Waters) was used to operate the system.

XCMS in R v 3.1.3 was used to integrate metabolic signals with a correction for total ion current and median fold change. Resulting *m/z* intensities were corrected for FW of each sample. Metabolic similarities/ trends between biological treatment were visualised by un-supervised 3D Principle component analyses (3D-PCA) using MetaboAnalyst v 3.0 (http://www.metaboanalyst.ca/). MarVis v 2.0 ((http://marvis.gobics.de) was used to filter metabolic markers (Student T-test $P < 0.01$) and correct for adducts and/or isotopes. The resulting 289 significant markers were clustered using MeV (http://www.tm4.org/mev.html) and their intensities displayed as a heatmap. Subsequent Volcano Plots were performed in MetaboAnalyst in order to select markers that were significantly up/down regulated (Student T-test, $P < 0.01$) by more than 2-fold. As described (Pétriacq *et al.*, 2016b), the putative identification for each marker was based on the accurate mass spectral data screened in MarVis (tolerance: m/z = 0.1 Da, RT = 10 s) and the METLIN online chemical database (https://metlin.scripps.edu/index.php). PubChem was used to validate the putative pathways (https://pubchem.ncbi.nlm.nih.gov/).

Quantification of defence hormones

The relative quantification of phytohormones was performed using the MS^E function in ESI^- as described by Pétriacq *et al* (2016). SAG and SGE has been provided by Victor Flors (Universitat Jaume I, Castellón, Spain).

Post-harvest treatment of tomatoes with Abscisic Acid (ABA)

A total of 24 micro-tom plants were grown and treated as described in Experiment 1 (seedling treatments). Fruit were harvested 11 weeks after treatment and treated with either freshly prepared solution of 100 µM ABA (Sigma Aldrich, A1049) or dH_2O. Both solutions were supplemented with 0.01% (v/v) Silwet L-77 (LEHLE SEEDS, VIS-30) to ensure even application across the fruit. Fruit were incubated at 23°C in the dark for one day before being infected with *B. cinerea* as described above. Infection was scored at 5 dpi. This experiment was repeated twice with similar results.

BABA Quantification

Liquid chromatography (LC) ESI tandem mass spectrometry coupled to a triple quadrupole (TQD, Waters) in positive mode, with external standardization, was used to quantify BABA. Dried samples were resuspended in 500 µL of 90:10 dH_2O:meOH, supplemented with perfluoroheptanoic acid (Sigma-Aldrich, 342041) at 1 mM as a final concentration and

301 filtrated through a 0.22 µm filter. The LC separation was performed by high-performance
302 liquid chromatography (HPLC) using a YMC-Pack ODS-AQ HPLC column (Waters, 5 µm
303 particle size, 12 nm pore size, 100 x 2.0 mm). BABA was eluted with a gradient of methanol
304 and water containing 0.1 mM perfluoroheptanoic acid, which started at 90:10 dH_2O:meOH
305 and linearly reached 10:90 in 5 minutes, and then returned to the initial concentration in 3
306 minutes. The column was allowed to equilibrate for 1 minute, giving a total time of 9 minutes
307 per sample. The solvent flow rate was 0.3 $mL.min^{-1}$. The retention time for BABA was 1.07
308 minutes and the transition in positive electrospray mode of the parent and daughter ions was
309 104 and 44, respectively.
310
311 *Statistical analyses*
312
313 For analysis of average lesion diameters and fitness parameters of Experiment 1 and 2,
314 normal distributions were confirmed by Shapiro-Wilk tests and equality of variances were
315 determined by Levene's test. If normal distributions and homogeneity of the variances could
316 be confirmed, differences in means were analysed using a one-way ANOVA or independent-
317 sample t-tests. Furthermore, if there was a significant result from the ANOVA, the means
318 were further analysed with the least significance difference (LSD) post-hoc test. If normal
319 distributions or variances homogeneity could not be confirmed, differences in means were
320 analysed using the non-parametric Kruskal-Wallis tests or non-parametric Mann-Whitney *U*-
321 test. A two-way ANOVA was used, following confirmation of normal distributions and
322 homogeneity of variances, to test the effect of seedling treatment, the exogenous application
323 of ABA and the interaction, on average lesion diameter. Differences in the infection class
324 distributions between treatments were analysed using Pearson's $\chi 2$ tests. All analyses were
325 conducted with IBM SPSS Statistics software (version 22.0).

Results

Impact of BABA treatment on post-harvest disease resistance

In order to investigate the long-lasting effect of chemical priming by BABA on tomato fruit, we assessed the resistance of fruit harvested from plants treated with BABA at different developmental stages. In experiment 1, tomatoes produced by plants which had been treated with BABA at the seedling stage ("BABA seedling" treatment), were more resistant to *B. cinerea* than those produced by the controls ("Water seedling" treatment; **Figure 1a**). At three days post inoculation (dpi), the tomatoes from BABA-treated plants had on average significantly smaller lesion diameters than those from the water-treated controls (**Figure 1b**). Furthermore, at four dpi, a greater percentage of tomatoes from BABA-treated plants compared to the water-treated plants were classified into the lower two external necrosis classes (**Figure 1c**). Thus BABA-IR is capable of protecting tomato fruit post-harvest even though it was induced many weeks before the first emergence of fruit. To establish whether BABA treatment could also induce resistance when applied at a later developmental stage, a second experiment was established with three treatments: "BABA Green", plants treated with BABA when fruit were green; "BABA Red", plants treated with BABA when fruit were red and "Water", plants only treated with water. Fruit from the "BABA Green" treatment had smaller lesion diameters (**Figure 1b**) and were more likely to be classified in one of the lower disease necrosis classes (**Figure 1c**), than fruit from the other two treatments. However,

despite this, there were not significant differences between the three treatments (**Figure 1**). This illustrates that BABA-IR in fruits is not effective when plants are treated after the onset of fruit production.

Impact of BABA treatment on Fitness Parameters and fruit quality

Plants treated with BABA, particularly at high concentrations, can suffer costs to growth, development and fitness (van Hulten *et al.*, 2006; Wu *et al.*, 2010; Luna *et al.*, 2014b). Cost to yield or other fitness parameters were investigated following treatment with BABA at different developmental stages. At four weeks post "BABA seedling" treatment, there were significantly fewer fruit on average on BABA-treated plants. At five weeks, there was no longer a significant difference (**Figure 2a**). A similar delay was also observed for fruit ripening in the "BABA seedling" treatment plants. At eight weeks post BABA treatment, control plants began to form red fruit, whereas BABA-treated plants began to form red fruit a week later and in smaller numbers (**Figure 2b**). At week 10, the number of red fruit between treatments reached a similar amount. Although there were BABA-induced delays in fruit formation and ripening, by the time the fruit were harvested there was no difference in the yield of red tomatoes. In the second experiment, BABA was applied to plants once fruit had formed. As expected, there was no impact on fruit formation (**Figure S1a**). However, treatment with BABA when the fruit were green did delay fruit ripening. Consequently, at the time of harvesting there were significantly fewer red fruit on "BABA Green" plants (**Figure S1b**). This second experiment provides further evidence that BABA treatment can slow fruit development.

Post-harvesting of the tomatoes, size and water content of the fruit was assessed. No differences between treatments were found for either experiment (**Figure 2c, d; Figure S1c, d**), ruling out these parameters as being the cause of differences in resistance.

"BABA seedling" treatment induced changes in fruit metabolome – a resistance fingerprint

To gain further insights into the metabolic adjustments in response to BABA treatment, we conducted an untargeted metabolomics analysis by UPLC-qTOF-MS for the fruit of plants treated with BABA or water at the seedling stage (n = 4; **Figure 3**). Accurately detected *m/z* values (error = 0.4 ppm) were integrated using XCMS in R v. 3.1.3, providing 12,543 cations and 16,052 anions in ESI^+ and negative ESI^- ion mode, respectively. We performed a 3D principal component analysis (3D-PCA) from resulting ion intensities to obtain an overview of the metabolic profiles of fruit from water- and BABA-treated plants (**Figure 3a**). 3D-PCA displayed partial separation of water- and BABA-treated samples in ESI^- thus suggesting an impact of BABA on tomato metabolic profiles. This was confirmed with a hierarchical clustering from 289 significant markers (Student T-test $P < 0.01$) combined from ESI^- and ESI^+ analyses which indicated clear clustering of the water and BABA treatments (**Figure 3b**). In addition, quantitative differences were detected in an analysis aiming to investigate biologically-relevant differences between the two treatments using volcano plots (**Figure 3c**), which represented statistical significance (T-test, $P < 0.01$) against fold change (threshold of ± 2 fold). BABA treatment at the seedling stage led to 38 up-regulated (17 + 16) and 38 down-regulated (16 +22) metabolic markers considering both ion modes (**Figure 3c**).

Putative identifications were assigned to these 76 markers based on accurate mass measurements and online databases (**Table S1 and S2**). This putative identification revealed the largest single group to be lipids with 32% of the metabolites (**Figure 3d**). A third of these were glycerophospholipids, with a number of sterol lipids, fatty acids, fatty acyls and sphingolipids also being significantly up- or down-regulated (**Table S1 and S2**). Alkaloids, flavonoids, carbohydrates and terpenoids (lipids) collectively contribute another 30% of the 76 metabolites (**Figure 3d**). Overall, untargeted metabolomics indicate a long-lasting re-orchestration of plant metabolic profiles in tomato after chemical treatment by BABA. Interestingly, most of putatively identified metabolites fall into categories of compounds known to be involved in stress responses including plant-pathogen interactions (Bartwal *et al.*, 2013; Piasecka *et al.*, 2015).

Fruit phytohormone content post "BABA seedling" treatment

Phytohormones including JA, SA and ABA are known to mediate plant defence responses (Conrath *et al.*, 2015). Importantly, SA and ABA have been demonstrated to play a crucial role in BABA-IR (Zimmerli *et al.*, 2000; Ton & Mauch-Mani, 2004). Furthermore, accumulation of the glycosylated form of these hormones has been proposed as a mechanism for priming of plant defence responses (Pastor *et al.*, 2013). Relative amounts of the main plant defence hormones were assessed in the fruits of plants treated with BABA or water at the seedling stage (**Figure 4**). The only hormone that differed significantly between treatments was ABA, with double the amount accumulated in the fruit of BABA-treated plants relative to that of the control treatment (**Figure 4**). SA, along with its glycosylated forms (glucosyl salicylate and salicylic acid glucosyl ester) did not differ between treatments. Neither did JA, the active form of JA jasmonic acid-isoleucine or methyl-jasmonate (**Figure**

4). Hence, the resistance profile against *B. cinerea* observed in tomato fruit after BABA treatment could be attributed to the accumulation of the defence hormone ABA.

Impact of post-harvest ABA treatment on the resistance phenotype

Following the observation that there is an accumulation of ABA in the fruit of "BABA seedling" plants, an additional experiment was established. Fruit of plants treated with water or BABA at the seedling stage were sprayed post-harvest with water or ABA. The following day, all tomatoes were infected with *B. cinerea*. As observed before, fruit from from "BABA seedling" plants were significantly more resistant to *B. cinerea* (**Figure 5**). Interestingly, ABA induced susceptibility in the fruit of "Water seedling" plants. However, this susceptibility phenotype was absent in the fruit of "BABA seedling" plants (**Figure 5**), therefore providing further evidence of the role of ABA in BABA-IR post-harvest.

Is BABA retained in the red fruit and present post-harvest?

As the plausible effect of BABA on human health and its movement into fruit has yet to be determined, we quantified the BABA content in harvested red fruit from the five treatments of experiments 1 and 2. BABA was not detected in the fruit of either water controls (**Figure 6**). It was however detected in tomatoes of the experiment one "BABA seedling" treatment (**Figure 6**). Furthermore, while BABA was not detected in the fruit of plants treated post ripening ("BABA Red" treatment), BABA contents were 8-fold greater in the fruit of the "BABA green" treatment than in the "BABA seedling" treatment (**Figure 6**). Hence, this suggests that not only is BABA translocated from vegetative tissue into fruit but also that BABA is metabolised very slowly.

Discussion

Here, we have described how treatment with BABA at the seedling stage can generate long-lasting protection, resulting in the fruit being more resistant to grey mould (*B. cinerea*) post-harvest (**Figure 1**). In addition, we have observed that BABA treatment induces a delay in fruit production and ripening however this was eliminated by the time harvest was reached (**Figure 2**). Thus, BABA-IR has the potential to reduce post-harvest losses in tomatoes without yield costs. While previous studies have demonstrated the ability of BABA-IR to protect tomato green tissue and be long lasting (Worrall *et al.*, 2012; Luna *et al.*, 2016), this is the first example of BABA-IR extending to protect fruit post-harvest.

The degree of necrosis in the fruit of the plants treated with BABA at the seedling stage was significantly less than in the controls, however, the fruit were not completely resistant. This is similar to what was observed in other publications that describe BABA-IR against *B. cinerea* (Luna *et al.*, 2016). Priming, the most likely explanation for the long-term induced resistance phenotype (Mauch- Mani *et al.*, 2017), enhances the basal defence response reducing damage but only in some occasions leads to full immunity (Luna *et al.*, 2014a). Therefore, BABA-IR against *B. cinerea* should be integrated with other control measures to provide an effective protection strategy (Conrath *et al.*, 2015; Luna, 2016).

The fruit from plants treated with BABA after the formation of fruit were not more resistant to *B. cinerea* (**Figure 1**). In the case of the "BABA red" treatment, the explanation for this is likely the lack of BABA accumulating in the fruit (**Figure 6**). Ripened fruit are no longer sinks for metabolites and therefore BABA was not transported into those fruits. For the "BABA green" treatment the explanation must be different, as BABA did accumulate in the red fruit (**Figure 6**). A possibility is that the BABA treatment led to direct induction of SA-dependent defences in the tomatoes therefore triggering an extensive downregulation of JA-dependent defences through hormonal crosstalk (Koornneef & Pieterse, 2008).

The benefits of BABA-IR would be minimized if there were costs to yield or fruit quality associated with BABA treatment. Interestingly, for the potential of using BABA commercially, only transient alterations to development were observed. Treatment with BABA at the seedling stage delayed fruit formation (**Figure 2a**), while treatment with BABA at both seedling and fruiting developmental stages delayed ripening (**Figure 2b**; **Figure S1b**). Alterations in development, as a result of the application of a priming stimulus, have previously been observed. Redman *et al.* (2001) demonstrated that application of the phytohormone and priming cue JA to tomato plants, results in reduced fruit number and delayed fruit ripening.

In addition, Luna *et al.* (2014*b*) detailed how *Arabidopsis* plants treated with BABA showed a transient growth reduction, with a lower fresh weight than control plants at six but not 28 days post treatment. Therefore, treatments with priming-inducing chemicals can slow growth and/or alter development, with these effects being transient or permanent throughout the life of the plants. Additional fitness parameter assessed in this study included tomato diameter and percentage water content of fruit. For both, no differences were observed between the BABA treatment and water controls (**Figure 2; Figure S1**). This allowed us to confirm firstly that BABA treatment did not reduce the quality of tomatoes but also that differences in

499 resistance were not an artefact of BABA induced changes in fruit diameter and water content.
500 In summary, BABA treatment represents a potential strategy to reduce post-harvest losses
501 with a minimal penalty in developmental parameters.

502 Treatment with BABA at the seedling stage induced changes in the metabolic profiles
503 of red fruit (**Figure 3; Figure 4)**. Overall these were fairly minor, which is similar to findings
504 of previous studies looking at the metabolic alterations in the green tissue of *Arabidopsis*
505 following BABA treatment (Pastor *et al.*, 2014) and tomato following hexanoic acid
506 application (Camañes *et al.*, 2015). However, those differences that were observed could
507 have participated in the post-harvest resistance phenotype.

508 Lipids were identified to substantially contribute to the significantly up-regulated
509 metabolites in the tomatoes of BABA-treated plants (**Figure 3d**). Signalling and regulation of
510 plant defence responses is known to involve lipids, including sphingolipids and lipid-derived
511 metabolites such as the major regulator of plant defence responses against necrotrophic
512 pathogens – JA (Shah, 2005). Furthermore an accumulation of signalling molecules, allowing
513 basal defences to be activated faster upon a challenge, is a well described hypothesis for the
514 mechanism behind priming (Beckers *et al.*, 2009; Pastor *et al.*, 2013; Conrath *et al.*, 2015).
515 Thus, the accumulation of lipids could act to prime defence mechanisms and in turn explain
516 the induced resistance phenotype observed upon challenge with *B. cinerea*.

517 Secondary metabolites including alkaloids, terpenoids, and flavonoids were
518 significantly up- and down-regulated in the fruit of BABA-treated plants (**Figure 3d**). All
519 have previously been reported to play roles in plant defence responses (Bartwal *et al.*, 2013;
520 Piasecka *et al.*, 2015) and therefore likely play a role in the post-harvest induced resistance.
521 For instance, all the groups are known to contain phytoalexins, anti-microbial/herbivory
522 compounds which are synthesised and accumulated in response to challenge. Thus, many of

523 the metabolites featured in the resistance fingerprint could play a role in the enhanced
524 resistance of fruit of BABA-treated plants against *B. cinerea.*

525 In addition to the global metabolic analysis, a targeted study of phytohormones was
526 carried out. SA and JA are the two phytohormones most readily associated with plant defence
527 (Bari & Jones, 2009). However, neither varied significantly between treatments in this study,
528 nor did other SA and JA conjugates that have previously been shown to accumulate during
529 the priming phase (Camañes *et al.*, 2012). Remarkably, we identified differences between
530 treatments for the plant hormone ABA, which was significantly accumulated in the fruit of
531 BABA-treated plants (**Figure 4**). During the ripening of tomatoes, ABA is known to
532 accumulate and reach a peak just as the fruit begins to redden (Zhang *et al.*, 2009). In an
533 antagonistic interplay with ethylene, ABA steadily declines as fruit mature and redden (Sun
534 *et al.*, 2012; Leng *et al.*, 2014). The fruit of BABA-treated plants were delayed in ripening
535 and therefore, despite having turned red by the time of harvest, they could potentially still be
536 at an earlier developmental stage (**Figure 2b**). Thus, delayed development could explain the
537 elevated ABA levels in the fruit of BABA-treated plants.

538 ABA has been associated with the defence response of tomato plants against *B.*
539 *cinerea* (Asselbergh & Höfte, 2007). It is therefore plausible that the increased resistance to
540 *B. cinerea* in the fruit of BABA-treated plants may be the consequence of the delayed
541 development and in turn elevated ABA. However, the role of ABA in plant defence is highly
542 controversial (Asselbergh *et al.*, 2008; Ton *et al.*, 2009). For instance, Ton and Mauch-Mani
543 (2004) concluded that BABA-induced callose deposition in *Arabidopsis*, which helped
544 provide resistance against two necrotrophic pathogens, required an intact ABA-dependent
545 signalling pathway. Furthermore, Asselbergh and Höfte (2007) concluded that ABA is
546 required for callose deposition and therefore basal resistance against *B. cinerea* in tomato.
547 However, the tomato ABA mutant *sitens*, which is impaired in ABA biosynthesis, has been

548 shown to be more resistant to *B. cinerea* than wild-type plants (Audenaert *et al.*, 2002). In
549 order to clarify the role of ABA in BABA-IR phenotype post-harvest, we exogenously
550 applied ABA to harvested fruit one day prior to inoculation with *B. cinerea*. ABA treatment
551 induced susceptibility in the fruit from water pre-treated plants (**Figure 5**), yet, surprisingly,
552 this phenotype was abolished in fruit from BABA pre-treated plants. These results indicate
553 that ABA has a BABA-dependent role in induced resistance.

554 The BABA-dependent role of ABA in induced resistance could arise from BABA's
555 ability to prime multiple defence processes that are regulated by complex interacting
556 signalling pathways. For instance, in *Arabidopsis*, BABA independently primes SA-
557 dependent defences (Zimmerli *et al.*, 2000) and the cell wall defence callose deposition (Ton
558 & Mauch-Mani, 2004). Both mechanisms have been shown to play a role in tomatoes
559 resistance to *B. cinerea* (Audenaert *et al.*, 2002; Asselbergh & Höfte, 2007), yet they are
560 seemingly contradictorily regulated by ABA. Via negative crosstalk, ABA represses SA-
561 dependent defences (Audenaert *et al.*, 2002), whereas, priming of callose deposition needs
562 intact ABA signalling (Ton & Mauch-Mani, 2004; Asselbergh & Höfte, 2007). Moreover, the
563 role of exogenously applied ABA has been further linked to environmental conditions and
564 the threshold of reactive oxygen species (ROS) in the cell (Luna *et al.*, 2011). In this study, it
565 is possible that elevated ABA in fruit suppressed SA-dependent defences. Yet, the fruit of
566 BABA-treated plants did not suffer from ABA induced susceptibility as they are primed for
567 callose deposition. Future work is required to dissect the exact role of ABA in BABA-IR in
568 tomato fruit.

569 Chemical residues in fruit products are highly scrutinized by health authorities and
570 legislation (The European Parliament and the Council of the European Union, 2009). Our
571 analysis surprisingly detected traces of BABA in the fruit of plants treated at the seedling
572 stage (**Figure 6**). Importantly, until very recently, BABA was thought to be a xenobiotic

573 compound. However, it has now been shown to occur naturally in multiple different plant
574 species (Thevenet *et al.*, 2017). Moreover, BABA has been shown to accumulate in plants
575 after biological stresses, such as fungal pathogen infection (Thevenet *et al.*, 2017).
576 Nevertheless, as our work was based on artificial treatments with BABA, future work is
577 required to evaluate the plausible implications on human health. Previous studies, carried out
578 days after treatments with ^{14}C-labbelled BABA, have suggested that BABA accumulates in
579 above-ground tissue of *Arabidopsis* and tomato plants post root treatment (Cohen & Gisi,
580 1994; Jakab *et al.*, 2001). Our study has confirmed that traces of BABA accumulate in fruit,
581 therefore suggesting that artificial BABA is not rapidly metabolized and accumulates in plant
582 tissue. Toxicity tests of BABA should be done in the context that BABA blocks its receptor
583 protein in *Arabidopsis,* an aspartyl-tRNA synthetase (Luna *et al.*, 2014a) which is highly
584 conserved among different organisms including humans. Moreover, BABA has been shown
585 to be a partial agonist of the major mammalian inhibitory neurotransmitter glycine
586 (Schmieden & Betz, 1995). However, preliminary studies have shown BABA to have no
587 effect on the behaviour or survival of mice treated with high concentrations (Cohen *et al.*,
588 2016).

589 In summary, BABA offers extraordinary opportunities due to its outstanding
590 performance. Firstly, BABA induces resistance in numerous plant species against a range of
591 biotic (Ton & Mauch-Mani, 2004; Ton *et al.*, 2005; Luna *et al.*, 2016) and abiotic stresses
592 (Jakab *et al.*, 2005). Secondly, BABA-IR is long-lasting as described here and in other
593 publications (Slaughter *et al.*, 2012; Worrall *et al.*, 2012; Luna *et al.*, 2014b, 2016). Thirdly,
594 BABA is a priming-inducing agent that provides a robust and consistent resistance response.
595 Thus, BABA is an excellent tool to study the genetic and molecular mechanisms to fully
596 exploit the priming phenomenon. BABA-induced priming should play a leading role in the

development of new strategies that exploit the plant immune system to ultimately produce sufficient food for the world's ever growing population.

Supplementary Data

Table S1. Putative identification of candidate metabolic markers up-regulated in the "BABA seedling" treatment compared to the water treatment.

Table S2. Putative identification of candidate metabolic markers down-regulated in the "BABA seedling" treatment compared to the water treatment.

Figure S1. Fitness parameters of plants from experiment 2.

Acknowledgements

The work by SWW and SP was supported by two Undergraduate Vacation Bursaries provided by the British Society of Plant Pathology (BSPP). PP work is supported by the Plant Production and Protection (P3) centre of the University of Sheffield. VP work is supported by Plan de Promoción de la Investigación Jaume I P1.1B2015-33. Also we thank the SCIC of

the Universitat Jaume I for the technical support. The project was funded from a Sheffield Undergraduate Research Experience (SURE) grant and the BBSRC Future Leader Fellowship BB/P00556X/1 to EL. We thank Dr. Flors for his comments on earlier versions of the manuscript.

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769 **Figure 1.** Post-harvest disease resistance of tomatoes. In experiment one, two week old
770 seedlings were either soil drenched with 0.5 mM BABA or water. In experiment two, mature
771 plants were either treated with water or 1 mM BABA when the fruit were green or when the
772 fruit were red. (a) Representative pictures of diseases lesions in tomatoes from the five
773 treatments. (b) The mean lesion diameters, ± standard error of the mean, of tomatoes at three
774 days post inoculation (dpi). Asterisks indicate statistically significant differences (Student T-
775 test; $p<0.05$; n=8). (c) The percentage of tomatoes from each treatment classified into each of
776 four classes based on external necrosis at four dpi. Class one (white) - no external mycelium
777 or signs of necrosis, healthy tomatoes; class two (pink) – external mycelium + necrosis
778 diameter < 10 mm; class three (light red) – external mycelium + necrosis diameter > 10 mm;
779 class four (dark red) – tissue collapse, whole tomato necrotic, lesion diameter = tomato
780 diameter. Asterisk indicates statistically significant differences (Pearson's Chi-Squared test;
781 $p<0.05$, n=32).

782

783 **Figure 2.** Fitness parameters after seedling treatments with water or BABA. (a) Number of
784 fruit produced at four, five and six weeks post treatment. Asterisks indicates $p<0.01$ (Mann-

785 Whitney U test). (b) Number of red fruit per plant at eight, nine and ten weeks post treatment.
786 (c) Diameters of tomatoes harvested for infection at 10 weeks after treatment. (d) Percentage
787 water content of tomatoes. Bars represent means ± standard error of the mean. Asterisks
788 indicate $p<0.05$ (Mann-Whitney U test).
789
790 **Figure 3.** Untargeted metabolomic analysis of red tomatoes 10 weeks after treatments of
791 seedlings with water or BABA. (a) Principal component analysis in positive and negative
792 electrospray ionisation modes. (b) Pearson's hierarchical clustering of significantly up or
793 down regulated metabolites ($p<0.01$; Student T-test). (c) Volcano plot analysis of up or down
794 regulated putative metabolites. Pink balls represent significant putative metabolites (Student
795 T-test; $p<0.01$; 2-fold difference between treatments). (d) Classification of the 76 putatively
796 identified metabolites that were significantly up or down regulated. Pie charts indicate the
797 total number of up (38) and down (38) regulated putative compounds. Miscellaneous
798 metabolites are those where a putative identity has been found but no class was assigned.
799 Unknown metabolites are those which could not be assigned a putative identity.
800
801 **Figure 4.** Effect of BABA treatment on relative phytohormone content in harvested red fruit.
802 bars represent mean (± standard error of the mean) content of salicylic acid (SA),
803 glycosylated SA (SAG/SGE), abscisic acid (ABA), jasmonic acid (JA), jasmonic acid-
804 isoleucine (JA-Ile) and methyl jasmonate (MeJA) in the tomatoes of BABA-treated plants
805 relative to the amount found in the fruit of water-treated plants. Asterisk indicates $p<0.05$
806 (Student T-test).
807
808 **Figure 5.** Effect of post-harvest exogeneous ABA application on disease resistance.
809 Harvested fruit of plants soil-drenched with either water or 0.5 mM BABA at the seedling

stage, were treated with water (-ABA) or ABA (+ABA) one day prior to infection with *B. cinerea*. (a) The mean lesion diameters, ± standard error of the mean, of tomatoes at five days post inoculation (dpi). A two-way ANOVA was used to analyse the per plant mean lesion diameter distributions. There was a significant effect of the seedling treatment ($F = 17.84$, d.f. = 1,44, $p < 0.001$) and interaction between seedling and fruit treatments ($F = 6.04$, d.f. = 1,44, $p < 0.05$). (b) The percentage of tomatoes from each treatment classified into each of four classes based on external necrosis at five dpi. Class one (white) - no external mycelium or signs of necrosis, healthy tomatoes; class two (pink) – external mycelium + necrosis diameter < 10 mm; class three (light red) – external mycelium + necrosis diameter > 10 mm; class four (dark red) – tissue collapse, whole tomato necrotic, lesion diameter = tomato diameter. Asterisk indicate statistically significant differences in class distributions compared to the -ABA Water seedling fruit (Pearson's Chi-square test; $p < 0.05$, $n = 48$).

Figure 6. Accumulation of BABA in harvested red fruit. Relative intensity of BABA was quantified for each of the five treatments- BABA Seedling, Water Seedling, Water, BABA Green and BABA Red - in comparison to the water treatments. Bars represent means ± standard error of the mean (n=4). n.d. indicates not detected.