Treating seeds with activators of plant defence generates long lasting priming of resistance to pests and pathogens.

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Summary

- Priming of defence is a strategy employed by plants exposed to stress to enhance resistance against future stress episodes with minimal associated costs on growth. Here, we test the hypothesis that application of priming agents to seeds can result in plants with primed defences.
- We measured resistance to arthropod herbivores and disease in tomato (*Solanum lycopersicum*) plants grown from seed treated with jasmonic acid (JA) and/or β-aminobutryric acid (BABA).
- Plants grown from JA-treated seed showed increased resistance against herbivory by spider mites, caterpillars and aphids, and against the necrotrophic fungal pathogen, *Botrytis cinerea*. BABA seed treatment provided primed defence against powdery mildew disease caused by the biotrophic fungal pathogen, *Oidium neolycopersici*. Priming responses were long-lasting, with significant increases in resistance sustained in plants grown from treated seed for at least eight weeks, and were associated with enhanced defence gene expression during pathogen attack. There was no significant antagonism between different forms of defence in plants grown from seeds treated with a combination of JA and BABA.
- Long-term defence priming by seed treatments was not accompanied by reductions in growth, and may therefore be suitable for commercial exploitation.

Key Words: Herbivore; pathogen; defence; jasmonic acid; β-aminobutyric acid; seeds

Introduction

When under attack from pests and pathogens, plants are able to mount an array of inducible defence responses, ranging from the rapid synthesis of toxic metabolites and defensive proteins, to longer-term morphological changes, such as increases in trichome density. Such induced defences are generally recognised to impose a resource cost on the plant, manifest as reduced growth and reproductive fitness (Cipollini *et al*., 2003). Attempts to exploit such induced resistance responses via the application of synthetic chemicals that activate defence signalling pathways, such as benzothiadiazoles, have met with rather limited success to date, perhaps in part because these benefits are constrained by the inherent costs of defence (Heil *et al*., 2000). Besides direct competition for resources (allocation costs), other costs associated with direct activation of defences come in the form of trade-offs between different forms of defence (Walters & Heil, 2007). The different threats posed by different attackers, such as insect herbivores and biotrophic and necrotrophic pathogens, require different strategies to combat them, and these are regulated by different signalling pathways controlled by phytohormones such as jasmonic acid (JA), salicylic acid (SA) and ethylene. Antagonistic interactions between such defences are well documented, such as the reciprocal negative interactions between JA- and SA-dependent signalling (Kunkel & Brooks, 2002; Glazebrook, 2005). These costs and trade-offs mean that direct activation of defence provides maximum benefit only under specific circumstances, usually when there is a high threat from a single class of attacker.

As well as direct activation of defence, herbivore and pathogen attacks also result in long-term sensitisation of plant inducible defences to future biotic stress, a phenomenon commonly known as priming. Priming represents a state in which defences are not expressed, but in which the plant is able to respond more rapidly and/or more strongly to attack than

other plants which have not experienced prior stress (Conrath *et al*., 2006). Priming generally provides broad-spectrum enhanced resistance, but with minimal associated costs compared to direct activation of defence (van Hulten *et al*., 2006). Recent evidence suggests that priming can be an effective mechanism for crop protection in the field (Beckers & Conrath, 2007; Walters *et al*., 2008). The mechanisms underlying priming are poorly understood, but have been suggested to include increased expression of signalling proteins and transcription factors involved in inducible defence (Bruce *et al*, 2007; van der Ent *et al*., 2009), as well as epigenetic changes in defence genes which, following transient activation by an initial stress exposure, switch them into a primed state, poised for a rapid transcriptional response to subsequent attack (Bruce *et al*., 2007; van den Burg & Takken, 2009).

The establishment of fully activated and primed defence is often spatially and temporally separated in the plant. Infection of a single leaf with a microbial pathogen, for example, can lead to direct activation of defence in the infected leaf and primed defence in other parts of the plant, via the process known as systemic acquired resistance (SAR). Likewise, herbivory activates defences in both local and systemic leaves, and can also prime future direct and indirect defences (Kessler *et al*., 2006; Frost *et al*., 2007; Ton *et al*., 2007). The establishment of primed defence in systemic tissues implies long-range signalling. A number of hormonal and other chemical signals have been identified which can be transported either in the vascular system, or in the atmosphere, to elicit the primed state in receiving tissues (Heil & Ton, 2008; Pieterse *et al*., 2009). Exogenous application of such compounds, along with some synthetic chemicals, can also activate priming responses (Conrath *et al*., 2006). As well as influencing future responses in tissues distinct from the site of attack via priming, evidence is accumulating to suggest that the effects of biotic stress on future defence responses can extend to tissues not present at the time of stress perception, and even to subsequent

generations (Agrawal *et al*., 1999; Holeski, 2007; Boyko *et al*., 2010; Kathiria *et al*., 2010). Whilst the mechanisms underlying such responses are not yet understood, (Boyko & Kovalchuk, 2011), priming may be part of a phenomenon that can provide long-term adaptive benefits to plants and their offspring. The activation of primed resistance by chemical treatments may therefore provide a simple way of providing crop plants with long-term improvements in stress resistance with minimal impact on productivity. Here, we show that treatment of tomato seeds with jasmonic acid or beta-aminobutyric acid, (BABA), provides long-lasting increases in herbivore and pathogen resistance in plants grown from them.

Materials and Methods

Plants and seed treatments

Unless otherwise stated, tomato (*Solanum lycopersicum* L., cv. Carousel or Money Maker) were germinated and grown in a peat based compost mixture (Scotts M3) and cultivated in a heated, passively ventilated glasshouse (min 18 °C+/- 2 max 25 °C+/- 3) with supplementary lighting (Osram greenpower 600 W high pressure sodium lamps) to a minimum 250±25 µmol $m^{-2}s^{-1}$ PAR at the canopy. A minimum 16 hours photoperiod was maintained. Seeds were sown individually in 8 cm square form pots and watered daily from below to water holding capacity with excess water being removed from the flood trays. For mildew experiments plants were re-potted to 13 cm square form pots. For seed treatments, 20 to 40 seed were incubated in the dark at 4^{o}C in aqueous solutions of 3 mM JA (from a 1.2 M stock in ethanol) or 18 mM BABA. Control treatments included 0.25% ethanol where appropriate. Following incubation, seeds were washed twice for 10 minutes in distilled water before sowing.

Herbivore bioassays

Cultures of red spider mite (*Tetranychus urticae* Koch) were maintained on tomato plants prior to experiments. Adult mites were collected from stock plants and released onto leaves (10 mites per leaf) of tomato plants grown in glasshouses under natural light at Stockbridge Technology Centre. Mites were allowed to feed for 9 days. The extent of plant-mediated resistance to *T. urticae* was measured by counting using a binocular microscope, the number of living and dead mites on each plant and the number of mite eggs present. Eggs of tobacco hornworm caterpillars (*Manduca sexta* L.) were obtained from Chris Apark, University of Bath, UK. Eggs were hatched and caterpillars raised on an artificial diet under controlled environments. Two third instar larvae were placed on the fifth leaf of eight-week-old plants and allowed to feed for four days before grazed leaf area was measured. Stocks of green peach aphid (*Myzus persicae* (Sulzer)) were maintained on lettuce plants in a glasshouse. For experiments, five aphids were introduced onto a single leaf and allowed to feed on the plants for twelve days, with population counts conducted at regular intervals.

Powdery mildew bioassays

A stock culture of tomato plants (cv. Money Maker) heavily infected with *Oidium neolycopersici* was maintained in the glasshouse. For experimental work, spores were harvested from stock plants by washing 4-5 leaflets harbouring recently sporulated fungus into a collection tube with the inert solvent Fluorinert® (Pefluor compound FC-43, Apollo Scientific Ltd) at a final volume of approximately 5 mL. A suspension of $5x10^4$ spores per mL in Fluorinert® were sprayed onto the apical and first pair of leaflets on leaves four and five of 4-week-old tomato (var. Carousel) plants using a modified airbrush (Badger Co.). Six plants for each treatment were sprayed with 0.75 mL per plant. Inoculated plants were incubated in a glasshouse on a capillary mat flood bench to maintain high humidity around

the plant and encourage disease establishment. After 10 days, images of fungal growth were collected by high resolution CCD camera through Spot Basic imaging software (SPOT Imaging Solutions, USA). Additionally, the number of distinct disease colonies for each leaflet was counted by eye. Leaf area was derived using the area selection tool in Image-Pro PLUS (Media Cybernetics Inc).

Botrytis cinerea bioassays

Botrytis cinerea R16 (Faretra & Pollastro, 1991), kindly provided by Monica Höfte (Ghent University), was cultured on PDA plates in a plant growth chamber (Percival Scientific) set at 22±1°C with a 10 hour light cycle provided by Osram fluora lamps delivering 100±20 μmol $m^{-2}s^{-1}$. Conidia were isolated as described in Asselburgh *et al*., (2007) and re-suspended in sterile RO water before adding to the inoculation solution (50 mM glucose, 33.5 mM KH_2PO_4, pH 5) at a final concentration of 10^6/mL. Conidia were pre-germinated for 2.5 hours at room temperature. An excised leaf assay was performed (Audenaert *et al*., 2002) using leaf three or four from a 4-week-old tomato (var. Carousel) plant and two 5 μL droplets per leaflet. After incubation for 72 hours at 22°C in the dark, the infected leaves were imaged and recorded using SPOT Basic imaging software. Lesion diameter was measured using calibrated Screen Calipers (Iconico Inc. iconico.com). Measurements were converted to area by assuming lesions were circular.

Quantitative RT-PCR

Tomato plants (var. Carousel) were infected with *B. cinerea* as described above. Infected leaflets were frozen in liquid nitrogen after 0, 4, 8, 12, 16, and 24 hours following application of spores. Plant material was ground to a fine powder using a mortar and pestle and liquid nitrogen. RNA was extracted using a hot phenol method essentially as described by Verwoerd *et al*., (1989), scaled up accordingly. RNA was purified using Qiagen RNeasy spin columns. First strand cDNA

was synthesized from 4 μg total RNA using Reverse Transcriptase and primer OG1 (Table 1). PCR was performed in an ABI Prism 7000 cycler (Applied Biosystems) using EvaGreen qPCR master mix (qARTA•BIO Inc, Fremont, CA, USA), cDNA corresponding to 40 ng of total RNA and 0.2 μM of each primer in a 25 μL reaction at 95°C for 15 min, followed by 40 two-step cycles at 95°C for 15 sec and 60°C for 1 min. All gene-specific primers have been described previously (Table 1). Relative expression levels at each time point were calculated from cycle threshold (CT) values according to the ΔCT method (Applied Biosystems User Bulletin #2) using the tomato *Ubi3* gene as a reference.

Results

Seed Treatment with Jasmonic Acid Enhances Herbivore and Disease Resistance. To determine whether plants might respond to priming agents at the seed stage, seeds of tomato plants (*Solanum lycopersicum* cv. Carousel) were soaked in a 3 mM solution of jasmonic acid prior to germination. To examine the effect of the seed treatment on JA-dependent herbivore resistance responses, seven- to ten-week-old plants grown from control and JA-treated seed were challenged with different pest species, as detailed in 'Methods.' The red spider mite (*Tetranychus urticae*), is an important commercial pest of tomato. One week following introduction of spider mites, the visual damage caused by feeding activity was noticeably lower in JA seed treated plants than in controls, and both the populations (Fig. 1**a**) and reproductive rate (Fig. 1**b**) of mites measured nine days after infestation were significantly reduced by JA seed treatment. Feeding of tobacco hornworm (*Manduca sexta*) caterpillars was also reduced in JA seed-treated plants (Fig. 1**c**), and populations of the green peach aphid (*Myzus persicae*) were significantly lower on plants grown from treated seed than on controls (Fig. 1**d**). We also assessed the effect of the seed treatment on JA-dependent disease

resistance responses using a bioassay for infection by the necrotrophic fungal pathogen, *Botrytis cinerea*. Measurements of lesion areas following *Botrytis* inoculation (Fig. 1**e**), showed that plants grown from JA-treated seed are significantly more resistant to disease. The increase in resistance against herbivory and disease observed in these experiments could either be a consequence of constitutive activation of defence in plants grown from treated seed, or of priming of resistance responses following exposure of the embryo to JA.

JA seed treatment has minimal impact on growth and development. Direct activation of plant defence is commonly associated with reduced growth, an ecological cost which is minimised by priming of defences (van Hulten *et al.*, 2006). To determine the impact of the JA seed treatment on growth and development, a range of traits were measured. We observed a delay in germination of seed treated with 1 - 5 mM JA of approximately 1 day compared with control treated seeds (data not shown), but final germination percentage was not significantly altered (Fig 2**a**). At JA concentrations above 10 mM, however, inhibition of germination became significant (Fig. 2**a**). Furthermore, 3 mM JA seed treatment caused a reduction in growth of the primary root relative to controls in seedlings grown axenically on agar (Fig 2**b**). This is consistent with the known role of JA in root growth regulation (Wasternack, 2007). However, over the longer term, we observed no differences in growth and development between plants grown from control and JA-treated seed. Examples of characteristics determined include plant height (Fig. 2**c**), and fruit dry weight (Fig. 2**d**).

The priming agent β-aminobutyric acid influences plant pathogen responses when applied as a seed treatment. Whilst necrotrophic fungal pathogens such as *B. cinerea* are generally controlled by JA-dependent pathways, resistance against biotrophic pathogens is associated with salicylic acid (SA)-dependent pathways (Glazebrook, 2005). Various

compounds are known which can prime SA-dependent resistance. One, β-aminobutyric acid (BABA) is a non-protein amino acid which is well-known for its ability to prime a range of stress resistance responses in plants, including resistance against biotrophic pathogens (Zimmerli *et al*., 2000). We treated tomato seed with BABA to test whether it might also prime disease resistance when applied to the seed. Plants grown from treated seed were challenged with an important pathogen of tomato, powdery mildew (*Oidium neolycopersici*). Plants grown from treated seed suffered significantly lower levels of colonisation by powdery mildew (Fig. 3**a**), suggesting that like JA, BABA is able to prime defence responses in the growing plant when applied prior to germination.

Trade-offs between different resistance mechanisms are minimised by seed treatment-induced priming. Because JA- and SA-dependent signalling pathways can be antagonistic when directly activated (Kunkel & Brooks, 2002; Glazebrook, 2005), we were interested to determine whether there may be interactions between JA and BABA seed priming treatments in the context of resistance against necrotrophic and biotrophic pathogens. We found that a seed treatment with JA alone did not alter resistance to powdery mildew, consistent with the idea that JA-induced resistance responses are not effective against biotrophs (Fig. 3**a**). Importantly, however, the data also indicate that JA seed treatment does not increase susceptibility to powdery mildew via negative cross-talk. Furthermore, inclusion of JA in a combined treatment with BABA did not significantly impact on the ability of BABA to enhance resistance (Fig. 3**a**). We also performed the reciprocal experiment to test whether BABA priming might act antagonistically with JA-induced resistance against the necrotroph, *B. cinera*. In this case, we did detect a susceptibility to disease in BABA-treated plants, but found no interaction between treatments in two-way ANOVA, indicating that although in

isolation BABA treatment can be antagonistic to JA-dependent induced resistance, it did not interfere with the ability of JA to prime resistance against *Botrytis* (Fig. 3**b**).

JA-induced priming of *Botrytis* resistance depends on JA, ethylene and ABA signalling, and is associated with increased JA-dependent gene expression.

To determine the influence of different plant hormones on JA priming of pathogen resistance, we examined the effects of JA seed treatments on resistance to *Botrytis* infection in tomato mutants disrupted in JA, ethylene and ABA responses. In contrast to results from wild type plants, Fig. 4 shows that JA seed treatment was unable to increase *Botrytis* resistance in *JL5* (*def1*) and *Never ripe* plants, which are deficient in JA biosynthesis and ethylene perception respectively (Lanahan et al., 1994; Howe *et al*., 1996). Intriguingly, we found that in the ABA-deficient mutant, *flacca*, which is more resistant to *Botrytis* (Audenaert *et al*., 2002), JA seed treatment increased susceptibility to disease (Fig. 4).

A common feature of defence priming is that plants in the primed state exhibit more rapid and/or stronger transcriptional responses to stress. To investigate whether this might be a mechanism underlying the seed treatment-induced disease resistance, *Botrytis* inoculated leaves were sampled over a 24 hour time course for gene expression analysis. Quantitative real-time PCR (qPCR) was used to monitor expression of a number of well-known defence-related genes from tomato, including several regulated by JA. Although there was substantial inter-experiment variation in the exact timing of increases in expression, we found that *Botrytis* infection consistently resulted in early transcriptional activation of the JA biosynthetic gene *ALLENE OXIDE SYNTHASE 2* (*AOS2*), mid-phase activation of a JA-responsive defence gene *PROTEINASE INHIBITOR II* (*PinII*) and late activation of the pathogenesis-related gene *PR1b1*. For *AOS2* and *PR1b1*, we found no consistent difference

between the timing or peak expression levels between control and JA seed-treated plants, but in the case of the JA-dependent defence gene, *PinII*, we observed higher expression in JA seed-treated plants in all three replicate experiments performed. Representative data illustrating these responses are shown in Fig. 5.

Discussion

Entry into a primed state enhances plant resistance to future stress episodes with minimal costs to growth and development, and may therefore be a desirable trait to exploit commercially. Here, we show that seeds are receptive to agents that establish a primed state for pest and disease resistance, resulting in long-term increases in resistance to biotic attackers, including a range of arthropod herbivores and fungal pathogens. Seed treatment with JA reduced the performance of all three herbivores tested. Importantly, these species are representative of the three major herbivore feeding guilds. JA is well-known for its role in defence against chewing insects (Wasternack, 2007), including lepidopteran larvae, as exemplified here by *Manduca sexta* (Howe *et al*., 1996), and against cell content feeders such as spider mites (Li *et al*., 2002). Its role in defence against aphids and other phloem feeding herbivores is less clear, since these insects tend to activate SA-dependent responses in the plant. However, this appears to be a decoy strategy by which aphids repress plant responses regulated by JA that provide more effective resistance (Walling, 2008).

Consistent with its role in defence against necrotrophic pathogens, we also found that JA seed treatment enhances resistance against *Botrytis cinerea*. We used bioassays for *Botrytis* infection in a range of hormone mutants to begin to dissect the mechanism underlying the induced resistance afforded by JA seed treatment. As may be expected, we found that both

JA and ethylene signalling are required for elevated resistance in plants grown from JA treated seed. Our experiments do not distinguish between a requirement for these signals in perception of the seed treatment and subsequent expression of defence in infected leaves. Nevertheless, our data is consistent with a model in which the seed treatment acts via the typical JA and ethylene dependent pathways for defence against necrotrophic pathogens. ABA is known as a negative regulator of disease resistance (Ton *et al*., 2009), and the ABA-deficient tomato mutant, *sitiens*, is hyper-resistant to *Botrytis* (Audenaert *et al*., 2002). Surprisingly, we consistently found that JA seed treatment increased susceptibility to *Botrytis* in the ABA-deficient *flacca* mutant. Whilst the explanation for this response remains unclear, our data suggest a complex interaction between JA and ABA signalling during *Botrytis* infection.

BABA can prime resistance in many plant species against a range of stresses, including both necrotrophic and biotrophic pathogens (Zimmerli *et al*., 2000; Zimmerli *et al*., 2001; Ton & Mauch-Mani, 2004; Ton *et al*., 2005). When applied as a seed treatment in tomato, we found that it improved resistance against powdery mildew disease caused by the biotrophic fungal pathogen, *Oidium neolycopersici*, presumably via effects on SA-dependent resistance, as seen in other biotrophic and hemibiotrophic pathosystems (Zimmerli *et al*., 2000; Ton *et al*., 2005). However, we found that BABA seed treatments failed to promote *Botrytis* resistance. This contrasts with reports of BABA priming resistance to various necrotrophic pathogens, including *Botrytis cinerea*, in other plant species when applied as a soil drench (Zimmerli *et al*., 2001; Ton & Mauch-Mani, 2004).

Importantly, we found that biotic stress resistance afforded by seed treatments was long-lasting, with significant effects on herbivore resistance evident in plants at least 8-9 weeks

old. Although other examples of the use of seed treatments to improve biotic stress resistance exist in the literature, those we are aware of measured short-term responses occurring in seedlings a few days old rather than mature plants (Jensen *et al*., 1998; Latunde-Dada & Lucas, 2001; Shailasree *et al*., 2001; Buzi *et al*., 2004). A long-lasting effect on stress resistance as seen here is suggestive of a priming response rather than constitutive activation of defences. Apart from some early effects of JA seed treatments on germination and seedling root growth, which are consistent with known effects of JA on these processes (Wasternack, 2007), we were unable to detect long-term effects on vegetative and reproductive growth at the concentration used here. Constitutive activation of plant defence is commonly associated with reductions in growth and reproductive fitness (Heil *et al*., 2000; Cipollini *et al*., 2003; Walters & Heil, 2007). Priming of defences on the other hand, minimises these costs whilst improving future resistance to attack (van Hulten *et al*., 2006), consistent with the effects of the seed treatments employed here. Measurements of defence gene expression also support the idea that JA seed treatment primes future JA-dependent defences. Expression of the genes assayed was similar in control and treated plants prior to biotic stress, which argues against constitutive activation of defence, but at least in the case of *PinII*, induced levels of transcripts in response to *Botrytis* infection were significantly elevated in JA seed-treated plants. Perhaps significantly, proteinase inhibitors, including PinII, have recently been demonstrated to be essential for resistance against *Botrytis* (El Oirdi *et al*., 2011).

The antagonism between the JA- and SA-dependent pathogen resistances responses assayed here via their effects on *Botrytis* and powdery mildew respectively, is well-studied (Kunkel & Brooks, 2002; Glazebrook, 2005). Although the exact mechanism by which this antagonism arises is still to be fully elucidated, it is clear that interactions occur at the signalling level when endogenous JA and SA levels are elevated. It might, therefore, be predicted that

priming, rather than constitutive activation of JA- and SA-dependent resistances would have minimal impact on the other response. This prediction was found to be broadly correct in our experiments, since neither treatment prevented the ability of the other to increase resistance against the corresponding pathogen in combined JA and BABA seed treatments. However, BABA seed treatment alone tended to increase susceptibility to *Botrytis* in the absence of a JA seed treatment. One possible explanation for this observation is that BABA priming of SA-dependent defence can interfere with the endogenous JA-dependent resistance against necrotrophic pathogens. However, BABA primes resistance against *Botrytis* and other necrotrophs via SA-independent mechanisms in other systems (Zimmerli *et al*., 2001; Ton & Mauch-Mani, 2004). In Arabidopsis, one of these SA-independent mechanisms is via priming of ABA-dependent responses (Ton & Mauch-Mani, 2004). The suppression of resistance to *Botrytis* by ABA in tomato (Audenaert *et al*., 2002), may provide an alternative explanation for the negative effect of BABA seed treatment on *Botrytis* resistance. In either case, our data indicate that increased susceptibility to *Botrytis* following BABA seed treatment is overcome when plants are also primed for JA responses.

Long-term effects of the environment on plant genomes have recently attracted growing attention, particularly with regard to epigenetic mechanisms for the regulation of gene expression. A number of studies of mutants affected in DNA methylation and histone modification, which function as epigenetic regulators of gene expression, show that genome-wide changes in chromatin status have pleiotropic consequences, ranging from development to stress tolerance (Reinders *et al*., 2009; Kim *et al*., 2010; Luo *et al*., 2011), including JA-dependent responses (Zhou *et al*., 2005; Wu *et al*., 2008; Berr *et al*., 2010). Since the level of transcriptional activity of stress-related genes is maintained by epigenetic marks, it follows that changes in chromatin modifications as a consequence of stress may be one mechanism

by which priming may operate. For example, van den Burg & Takken (2009) recently put forward a model for priming during SAR based on histone replacement at defence-related gene promoters following pathogen recognition, and Jaskiewicz *et al*. (2011) showed that changes in histone acetylation and methylation were correlated with priming during SAR. It is possible, therefore, that the long-term priming effects we observe here as a consequence of JA and BABA seed treatments, may be mediated via epigenetic modifications of JA- and BABA-responsive genes in embryonic tissues during imbibition. In this way, these genes could become more responsive to JA- and SA-dependent signalling pathways during biotic attack on the growing plant. Interestingly, there is also mounting evidence for trans-generational changes in the sensitivity of plant resistance responses mediated by stress-induced epigenetic changes (Boyko *et al*., 2010; Kathiria *et al*., 2010; Scoville *et al*., 2011).

The control of pests and pathogens in crop plants by synthetic pesticides with direct toxic activity is becoming increasingly less desirable, and the use of more environmentally-friendly approaches are required for a more sustainable future. The use of elicitors of plant defences, or 'plant activators' as they have been termed, has been proposed as an alternative approach to crop protection (Vallad & Goodman, 2004; Bruce, 2010). However, commercial success in this area is currently limited. Priming of natural plant defences, as well as minimising yield penalties, should be compatible with other facets of integrated pest management strategies, such as the use of biological control. Since seed treatments are economically more attractive than chemical application to plants in the field and require no action by growers, the approaches we have described here may have useful applications in agriculture and horticulture.

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References

Agrawal AA, Laforsch C, Tollrian R. **1999**. Transgenerational induction of defences in animals and plants. *Nature* **401:** 60-63.

Asselbergh B, Curvers K, Franca SC, Audenaert K, Vuylsteke M, Van Breusegem F, Höfte M. **2007**. Resistance to *Botrytis cinerea* in *sitiens*, an abscisic acid-deficient tomato mutant, involves timely production of hydrogen peroxide and cell wall modifications in the epidermis. *Plant Physiology* **144:** 1863-1877.

Audenaert K, De Meyer GB, Höfte MM. **2002**. Abscisic acid determines basal susceptibility of tomato to *Botrytis cinerea* and suppresses salicylic acid-dependent signaling mechanisms. *Plant Physiology* **128:** 491-501.

Beckers GJ & Conrath U. **2007**. Priming for stress resistance: from the lab to the field. *Current Opinion in Plant Biology* **10:** 425-431.

Berr A, McCallum EJ, Alioua A, Heintz D, Heitz T, Shen WH. **2010**. Arabidopsis histone methyltransferase *SET DOMAIN GROUP8* mediates induction of the jasmonate/ethylene pathway genes in plant defense response to necrotrophic fungi. *Plant Physiology* **154:** 1403-1414.

Boyko A, Blevins T, Yao Y, Golubov A, Bilichak A, Ilnytskyy Y, Hollander J, Meins F, Kovalchuk I. **2010**. Transgenerational adaptation of *Arabidopsis* to stress requires DNA methylation and the function of Dicer-like proteins. *PLoS ONE* **5:** e9514-

Boyko A & Kovalchuk I. **2011**. Genome instability and epigenetic modification - heritable responses to environmental stress? *Current Opinion in Plant Biology* **14:** 260-266.

Bruce TJA, Matthes MC, Napier JA, Pickett JA. **2007**. Stressful "memories" of plants: Evidence and possible mechanisms. *Plant Science* **173:** 603-608.

Bruce TJA. **2010**. Tackling the threat to food security caused by crop pests in the new millennium. *Food Security* **2:** 133-141.

Buzi A, Chilosi G, De Sillo D, Magro P. **2004**. Induction of resistance in melon to *Didymella bryoniae* and *Sclerotinia sclerotiorum* by seed treatments with acibenzolar-S-methyl and methyl jasmonate but not with salicylic acid. *Journal of Phytopathology* **152:** 34-42.

Cipollini D, Purrington CB, Bergelson J. **2003**. Costs of induced responses in plants. *Basic and Applied Ecology* **4:** 79-89.

Conrath U, Beckers GJM, Flors V, García-Agustín P, Jakab G, Mauch F, Newman MA, Pieterse CMJ, Poinssot B, Pozo MJ *et al*. **2006**. Priming: Getting Ready for Battle. *Molecular Plant-Microbe Interactions* **19:** 1062-1071.

El Oirdi M, Abd El Rahman T, Rigano L, El Hadrami A, Cecilia Rodriguez M, Daayf F, Vojnov A, Bouarab K. **2011**. *Botrytis cinerea* manipulates the antagonistic effects between immune pathways to promote disease development in tomato. *Plant Cell* **23:** 2405-2421.

Faretra F & Pollastro S. **1991** . Genetic basis of resistance to benzimidazole and dicarboimide fungicides in *Botryotinia fuckeliana* (*Botrytis cinerea*). *Mycological Research* **95:** 943-951.

Flors V, Leyva Mdl, Vicedo B, Finiti I, Real MD, García-Agustín P, Bennett AB, González-Bosch C. **2007**. Absence of the endo-β-1,4-glucanases Cel1 and Cel2 reduces susceptibility to *Botrytis cinerea* in tomato. *Plant Journal* **52:** 1027-1040.

Fowler JH, Narváez-Vásquez J, Aromdee DN, Pautot V, Holzer FM, Walling LL. **2009**. Leucine aminopeptidase regulates defense and wound signaling in tomato downstream of jasmonic acid. *Plant Cell* **21:** 1239-1251.

Frost CJ, Appel M, Carlson JE, De Moraes CM, Mescher MC, Schultz JC. **2007**. Within-plant signalling via volatiles overcomes vascular constraints on systemic signalling and primes responses against herbivores. *Ecology Letters* **10:** 490-498.

Glazebrook J. **2005**. Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. *Annual Review of Phytopathology* **43:** 205-227.

Heil M, Hilpert A, Kaiser W, Linsenmair KE. **2000**. Reduced growth and seed set following chemical induction of pathogen defence: does systemic acquired resistance (SAR) incur allocation costs? *Journal of Ecology* **88:** 645-654.

Heil M & Ton J. **2008**. Long-distance signalling in plant defence. *Trends in Plant Science* **13:** 264-272.

Holeski LM. **2007**. Within and between generation phenotypic plasticity in trichome density of *Mimulus guttatus*. *Journal of Evolutionary Biology* **20:** 2092-2100.

Howe GA, Lightner J, Browse J, Ryan CA. **1996**. An octadecanoid pathway mutant (JL5) of tomato is compromised in signaling for defense against insect attack. *Plant Cell* **8:** 2067-2077.

Jaskiewicz M, Conrath U, Peterhaensel C. 2011. Chromatin modification acts as a memory for systemic acquired resistance in the plant stress response. *EMBO Reports* **12**: 50-55.

Jensen BD, Latunde-Dada AO, Hudson D, Lucas JA. **1998**. Protection of *Brassica* seedlings against downy mildew and damping-off by seed treatment with CGA 245704, an activator of systemic acquired resistance. *Pesticide Science* **52:** 63-69.

Kathiria P, Sidler C, Golubov A, Kalischuk M, Kawchuk LM, Kovalchuk I. **2010**. Tobacco mosaic virus infection results in an increase in recombination frequency and resistance to viral, bacterial, and fungal pathogens in the progeny of infected tobacco plants. *Plant Physiology* **153:** 1859-1870.

Kessler A, Halitschke R, Diezel C, Baldwin IT. **2006**. Priming of plant defense responses in nature by airborne signaling between *Artemisia tridentata* and *Nicotiana attenuata*. *Oecologia* **148:** 280-292.

Kim JM, To TK, Nishioka T, Seki M. **2010**. Chromatin regulation functions in plant abiotic stress responses. *Plant Cell and Environment* **33:** 604-611.

Kunkel BN & Brooks DM. **2002**. Cross talk between signaling pathways in pathogen defense. *Current Opinion in Plant Biology* **5:** 325-331.

Lanahan MB, Yen HC, Giovannoni JJ, Klee HJ. **1994**. The Never ripe mutation blocks ethylene perception in tomato. *Plant Cell* **6:** 521-530.

Latunde-Dada AO & Lucas JA. **2001**. The plant defence activator acibenzolar-S-methyl primes cowpea [*Vigna unguiculata* (L.) Walp.] seedlings for rapid induction of resistance. *Physiological and Molecular Plant Pathology* **58:** 199-208.

Li CY, Williams MM, Loh YT, Lee GI, Howe GA. **2002**. Resistance of cultivated tomato to cell content-feeding herbivores is regulated by the octadecanoid-signaling pathway. *Plant Physiology* **130:** 494-503.

Luo, M., Liu, X., Singh, P., Cui, Y., Zimmerli, L. and Wu, K. (2011) Chromatin modifications and remodeling in plant abiotic stress responses. *Biochimica et Biophysica Acta*. doi:10.1016/j.bbagrm.2011.06.008

Peiffer M, Tooker JF, Luthe DS, Felton GW. **2009**. Plants on early alert: glandular trichomes as sensors for insect herbivores. *New Phytologist* **184:** 644-656.

Pieterse CMJ, Leon-Reyes A, van der Ent S, van Wees SCM. **2009**. Networking by small-molecule hormones in plant immunity. *Nature Chemical Biology* **5:** 308-316.

Reinders J, Wulff BBH, Mirouze M, Mari-Ordonez A, Dapp M, Rozhon W, Bucher E, Theiler G, Paszkowski J. **2009**. Compromised stability of DNA methylation and transposon immobilization in mosaic Arabidopsis epigenomes. *Genes & Development* **23:** 939-950.

Scoville AG, Barnett LL, Bodbyl-Roels S, Kelly JK, Hileman LC. **2011**. Differential regulation of a MYB transcription factor is correlated with transgenerational epigenetic inheritance of trichome density in Mimulus guttatus. *New Phytologist* **191:** 251-263.

Shailasree S, Sarosh BR, Vasanthi NS, Shetty HS. **2001**. Seed treatment with β-aminobutyric acid protects Pennisetum glaucum systemically from Sclerospora graminicola. *Pest Management Science* **57:** 721-728.

Ton J & Mauch-Mani B. **2004**. β-amino-butyric acid-induced resistance against necrotrophic pathogens is based on ABA-dependent priming for callose. *Plant Journal* **38:** 119-130.

Ton J, Jakab G, Toquin V, Flors V, Iavicoli A, Maeder MN, Metraux JP, Mauch-Mani B. **2005**. Dissecting the beta-aminobutyric acid-induced priming phenomenon in arabidopsis. *Plant Cell* **17:** 987-999.

Ton J, D'Alessandro M, Jourdie V, Jakab G, Karlen D, Held M, Mauch-Mani B, Turlings TCJ. **2007**. Priming by airborne signals boosts direct and indirect resistance in maize. *Plant Journal* **49:** 16-26.

Ton J, Flors V, Mauch-Mani B. **2009**. The multifaceted role of ABA in disease resistance. *Trends in Plant Science* **14:** 310-317.

Vallad GE & Goodman RM. **2004**. Systemic acquired resistance and induced systemic resistance in conventional agriculture. *Crop Science* **44:** 1920-1934.

van den Burg HA & Takken FLW. **2009**. Does chromatin remodeling mark systemic acquired resistance? *Trends in Plant Science* **14:** 286-294.

van der Ent S, van Hulten M, Pozo MJ, Czechowski T, Udvardi MK, Pieterse CMJ, Ton J. **2009**. Priming of plant innate immunity by rhizobacteria and β-aminobutyric acid: differences and similarities in regulation. *New Phytologist* **183:** 419-431.

van Hulten M, Pelser M, van Loon LC, Pieterse CMJ, Ton J. **2006**. Costs and benefits of priming for defense in Arabidopsis. *Proceedings of the National Academy of Sciences of the United States of America* **103:** 5602-5607.

Verwoerd TC, Dekker BMM, Hoekema A. **1989**. A small-scale procedure for the rapid isolation of plant RNAs. *Nucleic Acids Research* **17:** 2362-2362.

Walling LL. **2008**. Avoiding effective defenses: Strategies employed by phloem-feeding insects. *Plant Physiology* **146:** 859-866.

Walters D & Heil M. **2007**. Costs and trade-offs associated with induced resistance. *Physiological and Molecular Plant Pathology* **71:** 3-17.

Walters DR, Paterson L, Walsh DJ, Havis ND. **2008**. Priming for plant defense in barley provides benefits only under high disease pressure. *Physiological and Molecular Plant Pathology* **73:** 95-100.

Wasternack C. **2007**. Jasmonates: An update on biosynthesis, signal transduction and action in plant stress response, growth and development. *Annals of Botany* **100:** 681-697.

Worrall D, Elias L, Ashford D, Smallwood M, Sidebottom C, Lillford P, Telford J, Holt C, Bowles D. **1998**. A carrot leucine-rich-repeat protein that inhibits ice recrystallization. *Science* **282:** 115-117.

Wu K, Zhang L, Zhou C, Yu CW, Chaikam V. **2008**. HDA6 is required for jasmonate response, senescence and flowering in *Arabidopsis*. *Journal of Experimental Botany* **59:** 225-234.

Zhou CH, Zhang L, Duan J, Miki B, Wu KQ. **2005**. *HISTONE DEACETYLASE19* is involved in jasmonic acid and ethylene signaling of pathogen response in Arabidopsis. *Plant Cell* **17:** 1196-1204.

Zimmerli L, Jakab C, Métraux JP, Mauch-Mani B. **2000**. Potentiation of pathogen-specific defense mechanisms in Arabidopsis by β-aminobutyric acid. *Proceedings of the National Academy of Sciences of the United States of America* **97:** 12920-12925.

Zimmerli L, Métraux JP, Mauch-Mani B. **2001**. β-aminobutyric acid-induced protection of Arabidopsis against the necrotrophic fungus *Botrytis cinerea*. *Plant Physiology* **126:** 517-523.

Table 1. Oligonucleotide primers used for cDNA synthesis and qPCR.

	Forward primer	Reverse primer	Reference
OG1		$GAGAGAGGATCCTCGAGT_{(15)}$	Worrall *et al*., 1998
AOS2	TCTCTTCCTCTTCCTTCTCTTCACC	CGCCGGGTATAGTCCTGGTAGATA	Fowler *et al*., 2009
Ubi3	ACTCTTGCCGACTACAACATCC	CTCCTTACGAAGCCTCTGAACC	Fowler *et al*., 2009
PR1b1	CCGTGCAATTGTGGGTGTC	GAGTTGCGCCAGACTACTTGAGT	Flors *et al*., 2007
Pin II	GGATTTAGCGGACTTCCTTCTG	ATGCCAAGGCTTGTACTAGAGAATG	Peiffer *et al*., 2009

Figure Legends

Figure 1. Seed treatment with jasmonic acid enhances plant resistance to herbivory and disease. (**a, b**) Tomato plants grown from control or 3 mM JA-treated seed were grown in a glasshouse and 10 adult female red spider mites (*T. urticae*) introduced onto a single leaflet of each plant when they were 7 weeks old. Mite performance was measured by counting live mites (**a**) and eggs laid (**b**) 9 days later. Data show means plus SEM (n = 10). Differences were significant by a student's t-test (P=0.0012 for survival and P<0.0001 for egg laying). (**c**) Leaf area measured after *M. sexta* caterpillar grazing. Data show means plus SEM (n = 10). Differences were significant by a student's t-test (P=0.011). (**d**) Aphid (*M. persicae*) population on leaflets of tomato plants grown from JA-treated seed as a percentage of the population on control plants. (**e**) Areas of disease lesions on leaves of 4-week-old tomato plants three days following inoculation with *B. cinerea*. Data show means plus SEM (n = 8 leaves with 10 inoculations per leaf). Populations are significantly different by a Mann-Whitney U test (P<0.0001).

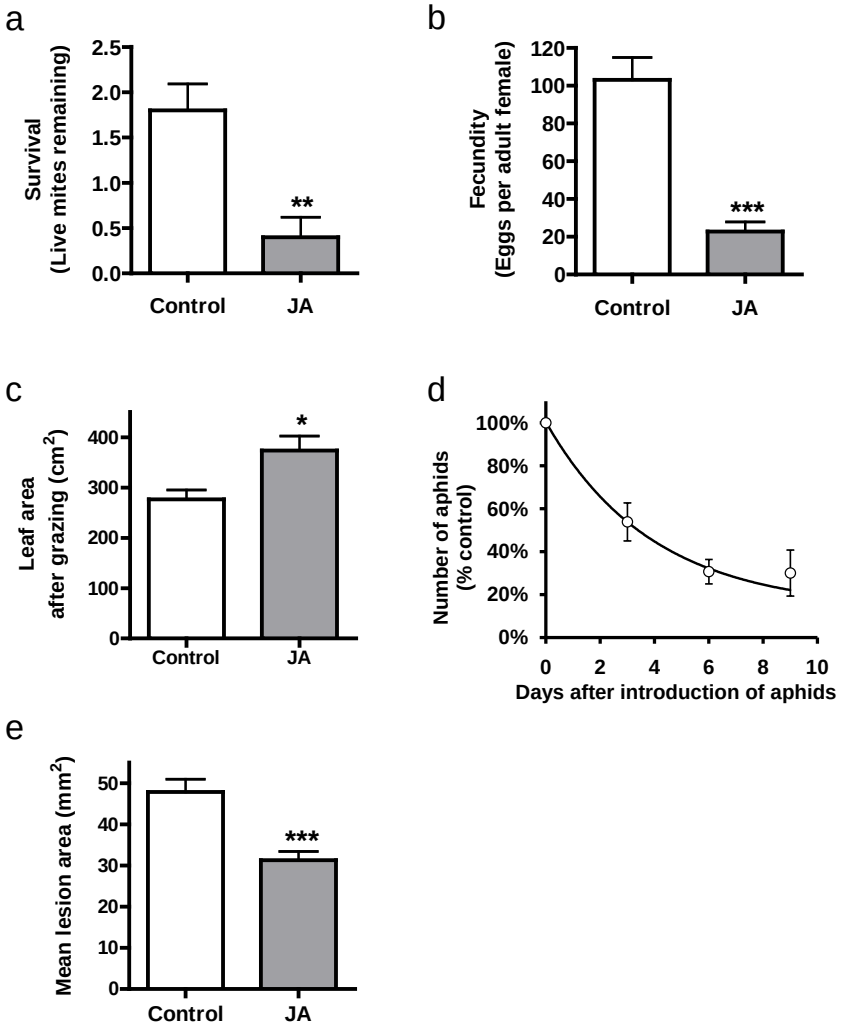

Figure 2. JA seed treatment has minimal impact on growth and development.
(**a**) Germination of seeds of tomato following treatment with JA over a range of concentrations. The illustrated data are means of 10 replicate populations +/- SEM, and the line is a fitted dose response. The dashed lines represent the 95% confidence interval. (**b**) JA inhibition of primary root growth of seedlings grown for 10 days on MS (Murashige and Skoog) agar. Data show mean root length +/- SEM (n=20). Means are significantly different by student's *t*-test ($P<0.0001$). There were no significant effects on the vegetative or reproductive growth of tomato grown from seed treated with 3 mM JA. Plots show (**c**) plant height and (**d**) fruit dry weight measurements taken at successive weekly harvests. Data are means of 8 replicate plants +/- SEM, and the lines are fitted growth response curves. Open circles; control, filled circles; JA. Neither the fitted growth response nor 2-way analysis of variance (treatment x harvest) showed any significant difference between control plants and those grown from JA-treated seed.

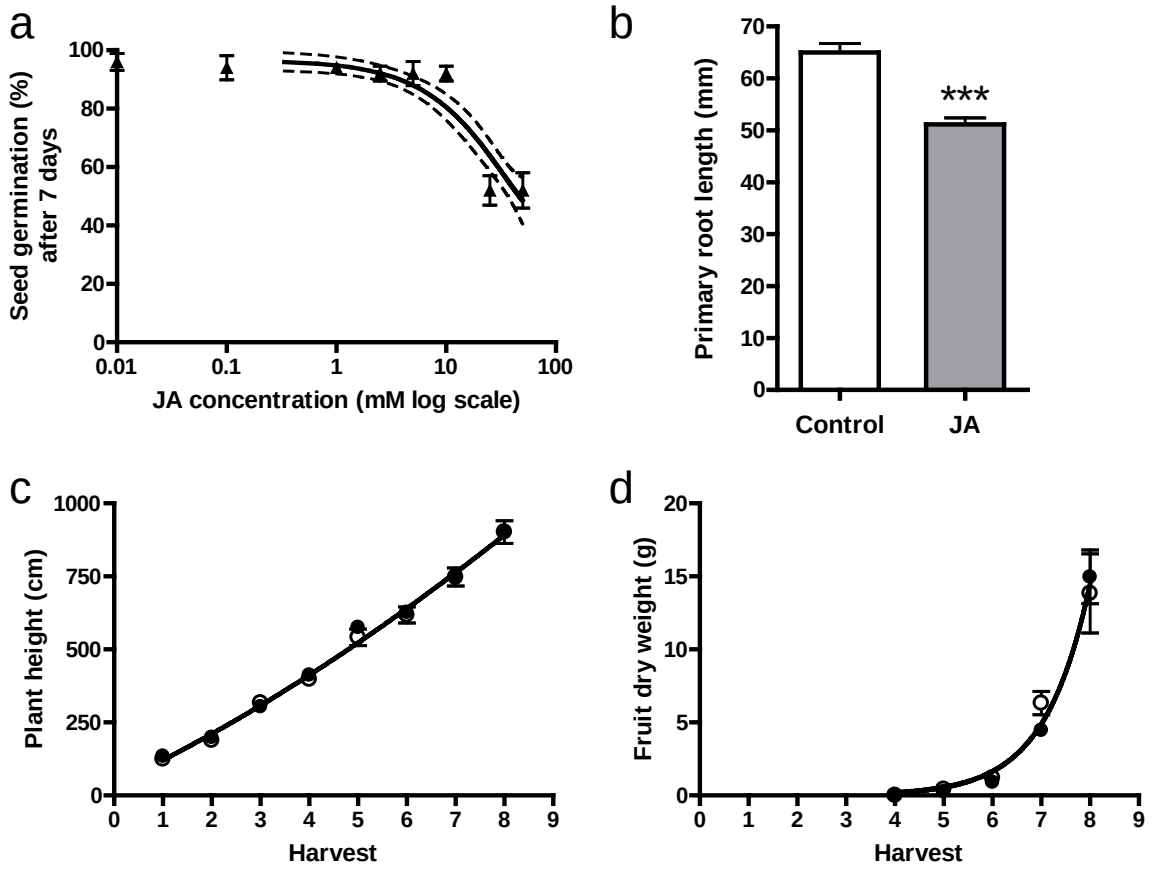

Figure 3. Effects of BABA and JA seed treatments on disease.

Tomato plants were grown in a glasshouse from seeds treated with either a control solution, 3 mM JA, 18 mM BABA, or 3 mM JA + 18 mM BABA, and challenged with either *O. neolycopersici* (**a**) or *B. cinerea* (**b**). (**a**) Plants were inoculated with spores of *O. neolycopersici* when they were 4 weeks old. Bars show mean colony number per cm^2 leaf area plus SEM (n = 6 leaves x 3 leaflets), 2 weeks after inoculation. From 2-way ANOVA, the effect of JA is not significant (P = 0.49), whereas the effect of BABA treatment is highly significant (P = 0.0008). The JA x BABA interaction was also not significant (P = 0.188). (**b**) *B. cinerea* lesion areas were measured as in Fig. 1. Bars show mean lesion area plus SEM (n = 8 leaves with 10 inoculations per leaf). From 2-way ANOVA, the effect of JA is significant at $P < 0.0001$, BABA at P = 0.0002, but the JA x BABA interaction was not significant (P = 0.121). Letters above bars indicate statistically significantly different means as determined by a Tukey post-hoc test.

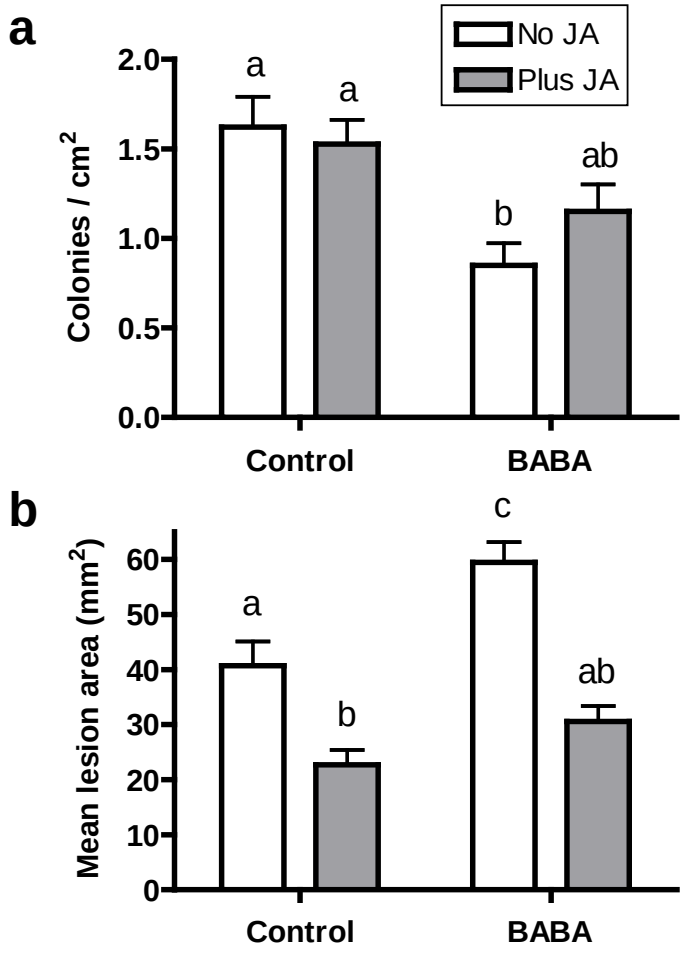

Figure 4. JA seed treatment-induced Botrytis resistance depends on JA, ethylene and ABA signalling. Mean lesion areas in leaves from treated plants relative to control untreated plants from WT (Carousel) and the *JL5/def1*, *Never ripe* and *flacca* mutants. Data show means plus SE from several independent experiments (Carousel, n=9; *JL5/def1*, n= 6; *Never Ripe*, n=4; *flacca*, n = 3), in which relative lesion areas were determined from 60-80 replicate measurements per genotype.

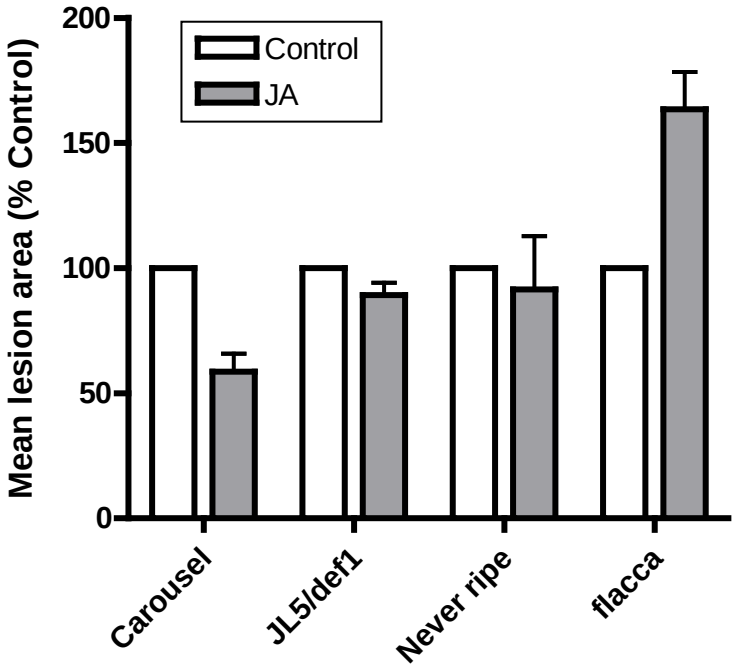

Figure 5. Transcriptional responses of defence genes to *Botrytis* infection are altered by JA seed treatment.

Relative steady state mRNA levels of the genes *AOS2*, *PinII* and *PR1b1* measured by real time PCR during a time course following inoculation of tomato leaves with *Botrytis cinerea*. Clear bars represent data from control, untreated plants, and filled bars, data from JA seed-treated plants. Error bars represent standard deviations determined from 3 technical replicate assays. Data shown are representative of 3 independent time course experiments.