INTRODUCTION

During plant–pathogen interactions, plants must sense and respond to many signals to balance growth and defence in an adverse environment (Jones and Dangl, 2006). The response to external stimuli through cellular signalling events can lead to resistance or susceptibility to pathogens, depending on the environment, pathogen virulence factors, and the genetics of the host plant.

Plants have evolved sophisticated strategies to suppress pathogen infection, possessing an immune system composed of two major layers: pathogen-associated molecular patterns (PAMPs)-triggered immunity (PTI), and effector-triggered immunity (ETI) (Spoel and Dong, 2012). These phenomena reflect the dynamic balance between the ability of the plant to activate defence responses against a pathogen, and the capability of the pathogen to suppress the plant’s immune system (Dodds and Rathjen, 2010). Plant-induced immunity studies the perception of pathogens and the activation of host defence regulatory mechanisms (Macho and Zipfel, 2014). The induced immunity of plants displaying increased resistance is often not attributed to direct activation of defences, but rather to a rapid, stronger activation of basal defence signalling on exposure to pathogens.

Plant immunity is often defined by what are considered the immunity hormones: salicylic acid (SA), jasmonic acid (JA), and ethylene (ET). These hormones are well known for differentially regulating defence responses against pathogens. In recent years, the involvement of other plant growth hormones such as auxin, gibberellic acid, abscisic acid, and cytokinins (CKs) in biotic stresses has been recognized. Previous reports have indicated that endogenous and exogenous CK treatment can result in pathogen resistance. We show here that CK induces systemic immunity in tomato (Solanum lycopersicum), modulating cellular trafficking of the pattern recognition receptor (PRR) LeEIX2, which mediates immune responses to Xyn11 family xylanases, and promoting resistance to Botrytis cinerea and Oidium neolycopersici in an SA- and ET-dependent mechanism. CK perception within the host underlies its protective effect. Our results support the notion that CK promotes pathogen resistance by inducing immunity in the host.

KEYWORDS
cytokinin, LeEIX2, plant immunity, PRR, tomato
defence responses against pathogens (Bari and Jones, 2009; Hatsugai et al., 2017). In recent years, the involvement of other more “classical” plant growth hormones, such as auxin, gibberellic acid (GA), abscisic acid (ABA), and cytokinins (CKs), in biotic stresses has been recognized (Chanclud and Morel, 2016; Shigenaga et al., 2017).

Cytokinin (CK) is an important developmental regulator, having activities in many aspects of plant life and its response to the environment. CKs are involved in diverse processes including stem-cell control, vascular differentiation, chloroplast biogenesis, seed development, growth and branching of root, shoot and inflorescence, leaf senescence, nutrient balance, and stress tolerance (Muller and Sheen, 2007). The roles of CK in plant growth and development have been reviewed extensively (Mok and Mok, 2001; Sakakibara, 2006; Werner and Schmülling, 2009; Keshishian and Rashotte, 2015).

In some cases, plant pathogens can secrete CKs, or induce CK production in the host plant. The hemibiotrophic actinomycete Rhodococcus fascians causes CKs. Recognition of R. fascians-derived CKs is essential for symptom development in Arabidopsis (Pertry et al., 2009). The spores of biotrophic rust and powdery mildew fungi contain CKs, which may be associated with green islands at the infection sites (Király et al., 1966, 1967). It has been suggested that to achieve pathogenesis in the host, CK-secreting biotrophs or hemibiotrophs manipulate CK signalling to regulate the host cell cycle and nutrient allocation (Jameson, 2000).

Works concerning the roles of CK in the plants’ interaction with microbes that do not produce CK are less abundant. High levels of CKs were found to increase the plants’ resistance to some viral pathogens and herbivores (Ballaré, 2011). Transgenic overexpression of CK-producing isopentenyl transferase (IPT) genes increased Arabidopsis resistance to Pseudomonas syringae (Choi et al., 2010), while overexpression of genes encoding CK oxidase (CKX), or mutating the endogenous CK Arabidopsis histidine kinase (AHK) receptors, enhanced Arabidopsis pathogen susceptibility (Choi et al., 2010, 2011; Argueso et al., 2012). In another study, CKs were found to mediate enhanced resistance to P. syringae in tobacco (Grosskinsky et al., 2011). Different mechanisms have been suggested for this enhanced resistance. In Arabidopsis, it was suggested that CK-mediated resistance functions through SA-dependent mechanisms, based on the finding that the Arabidopsis response regulator ARR2, a positive regulator of CK signalling, interacts with TGA3, a transcription factor involved in inducing SA-responsive genes, in the regulation of the disease marker gene pathogenesis-related 1 (PR1) against biotrophic infections in plants (Choi et al., 2010). An additional study suggested that CK signalling enhances the contribution of SA-mediated immunity in hormone disease networks (Naseem et al., 2012). CK was proposed to function in Arabidopsis immunity against the biotrophic Hyaloperonospora arabidopsis through repression of type-A response regulators (Argueso et al., 2012). In tobacco, an SA-independent phytoalexin-dependent mechanism was suggested (Grosskinsky et al., 2011).

In this work, we investigated the effects of CK on disease resistance and immunity in tomato, demonstrating that CK ameliorates disease outcomes of the tomato necrotrophic fungus Botrytis cinerea (Bc) and biotrophic fungus Oidium neolycopersici, that CK activates tomato immunity, and that CK signalling is activated in tomato in response to Bc. We show that high CK levels activate the plant defence machinery, and that CK response within the plant serves as a systemic immunity signal. CK promotes trafficking of the pattern recognition receptor (PRR) LeEIX2, and normal levels of PRRs were found to be required for CK-mediated Bc resistance. CK requires SA and ET, but not JA mechanisms, to exert its full effect in tomato defence.

2 | RESULTS

2.1 | Exogenous CK treatment ameliorates tomato disease

Works describing the role of CK in plant disease were obtained in only a few plant–pathogen experimental systems (Albrecht and Argueso, 2017). To investigate the role of CK in tomato fungal disease response, we examined the effect of CK on pathogenesis of a necrotrophic fungal pathogen, Bc, which is the causative agent of grey mould in over 1,000 different plant hosts, and a biotrophic fungal pathogen, O. neolycopersici, a causative agent of powdery mildew disease. The results are presented in Figure 1.

Wild-type (WT) Solanum lycopersicium “M82” tomato plants were treated with varying concentrations of the CK 6-benzyl-amino-purine (6-BAP) prior to pathogen infection, and the dose response of disease progression was measured as described in the methodology section. Disease was assessed 5–10 days after pathogen inoculation. CK pretreatment significantly decreased disease levels of the necrotrophic fungal pathogen Bc- (Figure 1a–f), and the biotrophic fungal pathogen O. neolycopersici (Figure 1h). Disease reduction increased with the application of higher CK concentrations (Figure 1f,h).

To test whether pretreatment with additional CK compounds has a similar effect, we examined Bc disease progression after pretreatment with kinetin, trans-zeatin, and thidiazuron (TDZ), as well as adenine, as a structurally similar control compound. Figure 1g demonstrates that all assayed CKs ameliorate Bc disease outcomes, with the exception of TDZ, a phenylurea-derived artificial CK that is structurally unrelated to the purine-type CKs, though it is known to bind strongly to the Arabidopsis CK receptors AHK3 and AHK4 (Romanov et al., 2006). Adenine, the control compound, has no significant effect on disease progression.

2.2 | Increased endogenous CK quantity or sensitivity improves tomato disease outcomes

To examine whether endogenous CK levels or response might have a similar effect, WT and CK tomato mutants were assessed for disease resistance or sensitivity in a similar manner. Figure 2 demonstrates that plants with elevated endogenous levels of CK [pBLS+IPT7 (Shani et al., 2010) or increased CK sensitivity (clausa)] (Bar et al., 2016) have significantly lower disease symptoms with Bc (Figure 2a–e) and O. neolycopersici (Figure 2g). pFIL»CKX4 (Shani et al., 2010), which
constantly breaks down its endogenous CK, had significantly increased disease levels in both cases (Figure 2e,g).

Modulating CK levels both exogenously (Figure 1) and endogenously (Figure 2a–e,g) improved tomato disease outcomes. To examine whether this is a systemic effect, CK was also applied by soil drench to the roots, with similar results in Bc disease resistance in leaves (Figure 2f), indicating that CK affects tomato disease resistance systemically.

### 2.3 CK disease amelioration is ET- and SA-dependent and JA-independent

It was previously reported that CK influences biotrophic disease resistance through regulation of SA in Arabidopsis (Choi et al., 2010; Naseem and Dandekar, 2012; Naseem et al., 2012). We found here that CK induces resistance to a necrotrophic pathogen in tomato. Because JA and ET are known to be involved in the response to necrotrophic pathogens (Thomma et al., 1998), we explored the involvement of SA, JA, and ET in the amelioration of disease outcomes by CK, conducting pathogenesis assays in SA, JA, and ET signalling/biosynthesis tomato mutants. Figure 3 shows that the SA-deficient NahG transgenic line (Brading et al., 2000) (Figure 3b,h,m), and the ET reduced sensitivity mutant Never-Ripe (Nr) (Lashbrook et al., 1998) (Figure 3d,i,m) have no significant Bc disease amelioration on CK treatment. However, the JA insensitive jai-1 mutant (Li et al., 2002) responds to CK with disease reduction (Figure 3f,i,m). Bc disease levels in these mutants without CK treatment matched those known in the literature when compared to their background genotypes: a moderate decrease in NahG (Mehari et al., 2015), similar levels in Nr...
2.4 | Altering CK response changes SA profiles on pathogen infection

Our results indicated that CK-induced pathogen resistance in tomato requires the SA pathway. To examine this further, we quantified SA in mock- and CK pretreated tissues, as well as in genotypes with altered CK levels/ response, in Bc-infected and uninfected tomato plants. External CK pretreatment caused an increase in internal SA content. However, the baseline levels of SA in genotypes with altered levels of CK or CK sensitivity resembled those of the background line (Figure 4). Bc inoculation results in a reduction in SA content after 48 hr. External CK pretreatment or increased endogenous CK sensitivity in the clausa mutant both maintain the SA reduction following Bc application (Figure 4).
Our results indicate that tomato pathogen resistance is modulated by both endogenous and exogenous CK, as was previously reported for *P. syringae* pv. *tomato* in *Arabidopsis* (Choi et al., 2010). Exogenous CK application primes *Arabidopsis* defence (Albrecht and Argueso, 2017). To examine whether the decrease in fungal disease in the presence of elevated CK levels is paired with increased plant defence in tomato, we tested known hallmarks of immune system activation: ET production, ion leakage (conductivity), and reactive oxygen species (ROS). Treating WT M82 plants with exogenous CK results in an increase in ET production and conductivity (Figure 5a,b). In addition to 6-BAP, we tested kinetin, trans-zeatin, and thidiazuron (TDZ). Adenine served as a negative control. Kinetin and trans-zeatin had similar activity to 6-BAP in the activation of plant defences. TDZ had no effect on ET production and a lower effect on conductivity than the other CKs (Figure 5c,d). Adenine has no significant effect on plant immune responses. We examined tomato genotypes with altered CK levels or response. Genotypes with elevated CK sensitivity or levels have elevated defence responses: the clausa mutant and the overexpressor of *IPT* both have elevated basal levels of ET when compared with M82 plants, and *IPT* also has elevated levels of conductivity (Figure 5e,f). The defence response mutant *jai-1* responded to CK with ET production and ion leakage at similar levels to those of its background cultivar M82 (Figure 5a,b); however, the SA-deficient *NahG* had reduced ET production in response to CK when compared with its background cultivar Moneymaker, and, unlike Moneymaker, did not respond to CK with an increase in ion leakage, while the reduced ET sensitivity mutant *Nr* did not respond significantly to CK at all when compared with its background cultivar Pearson (Figure 6a,b). Baseline defence responses without CK were not significantly different between the mutant genotypes and their background lines (Figure 6a,b). These results indicate that CK-induced immune responses are probably partially dependent on SA, fully dependent on ET, and JA-independent.

To examine if CK can augment defence responses elicited by a known elicitor of plant defence, we employed the Xyn11 family xylanase
ethylenic-ethylene inducing xylanase (EIX), which induces ETI in response to Cauxvars (Sharon et al., 1993; Ron et al., 2000; Elbaz et al., 2002; Bar and Avni, 2009; Leibman-Markus et al., 2017). The combination of CK and EIX induces immunity at greater levels than EIX or CK alone (see also Figure S1). We observed significant increases in ET production above 25 µM of 6-BAP added to EIX (Figure 7a). Ion leakage and ROS are also significantly increased with the addition of 6-BAP when compared with EIX or CK alone (Figures 7b and S2a,b). Interestingly, CK-regulated ROS homeostasis has been suggested as a possible mechanism underlying CK-activated defence (Albrecht and Argueso, 2017). Kinetin also has a similar enhancing effect on EIX-induced ET (Figures 7c and S1), while all tested CKs effect ion leakage (Figure 7d). Genotypes with elevated CK sensitivity or levels have elevated defence responses: the IPT overexpressor produced more ET in response to EIX, while both clausa and IPT had increased ion leakage and ROS production in response to EIX when compared to the background M82 cultivar (Figures 7e,f and S2c,d). The CKK overexpressor produced less ET in response to EIX.

To analyse the alterations to tomato gene expression in CK-induced immunity, we examined the expression of several known defence genes in response to CK treatment, with and without subsequent pathogen inoculation. CK induces the expression of Pto-interacting 5 (Pti5, Solyc02g077370), pathogenesis-related proteins (PR1a, Solyc01g106620), and PR-1b (Solyc00g174340), and pathogen-induced 1 (Pi-1, Solyc01g097270), and reduces the expression of proteinase inhibitor 2 (PI-2, Solyc03g020000) (Figure 8a). The addition of CK prior to Bc inoculation causes a decrease in defence gene expression, correlating with reduced disease (Figure 8b). Positive correlations between Bc disease levels and defence gene expression were reported previously (Harel et al., 2014; Mehari et al., 2015). The chosen genes are all hallmarks of Bc response. Pi-2 and Pi-1 are JA-responsive and considered induced systemic resistance (ISR) markers (Ament et al., 2004; Martínez-Medina et al., 2013; Iberkleid et al., 2014; Cui et al., 2019). Pti5 is ethylene-responsive, though it was found not to require ET, JA, or SA for its defensive up-regulation (Thara et al., 1999). PR1a is SA-responsive and considered a systemic acquired resistance (SAR) marker (López-Ráez et al., 2010; Martínez-Medina et al., 2013). PR1b is up-regulated by both SAR and ISR activation (Harel et al., 2014; Li et al., 2017). Distinctions between ISR and SAR are not clear-cut in tomato, and they can overlap (Liu et al., 2016; Betsuyaku et al., 2018).

Examining the expression of defence genes in CK altered genotypes revealed that CK affects defence gene expression endogenously as well. Pti5, PR1b, and PI1 being increased with CK treatment and on IPT overexpression, while PI2 and PR1a are increased on CKX overexpression (Figure 9a–e). On Bc inoculation, PI2, known to correlate with Bc disease levels (Harel et al., 2014), is reduced in IPT and clausa (Figure 9f), while PR1b is increased (Figure 9j). With the exception of PR1a (Figure 9h), clausa behaves like IPT following Bc inoculation, similarly to its disease resistant phenotype (Figure 2).

2.6 | Pathogenic processes activate the CK pathway in tomato

We demonstrated that pretreatment with CK and increased endogenous/signalling CK genotypes results in disease resistance in tomato (Figures 1 and 2). Is this an endogenously employed mechanism in
Do tomato plants activate their CK machinery on pathogen attack? To answer this question, we examined endogenous modulations to the CK pathway during pathogenesis.

During Bc infection, the expression of CK-responsive type-A tomato response regulators (TRRs) increases and the expression of CKX genes is also significantly altered (Figure 10a). The amount of active CKs decreases, significantly in the case of trans-zeatin and iso-pentenyl-riboside, 48 hr after Bc inoculation (Figure 10b). Using the CK activity response synthetic promoter TCS (two-component signalling sensor) fused to the VENUS fluorescent protein as a reporter (Zürcher et al., 2013; Bar et al., 2016), we determined that the CK pathway is activated on Bc infection in mature leaf tissue. Quantification of TCS-driven VENUS fluorescence in transgenic M82 tomato plants stably expressing pTCS::3×VENUS showed that the TCS-driven VENUS signal is significantly higher than the mock-treated plants 24-96 hr after inoculation (Figure 10c,d). Bc droplet inoculation causes a specific response in the leaf tissue in contact with the fungus; following the spread of infection and necrosis of the tissues, the CK-responsive VENUS halo spreads out from the site of pathogen inoculation (Figure 10d). Mock-treated leaves (droplet “inoculation” with infection medium) produced little to no TCS-driven VENUS signal (Figure 10c,d).
2.7 | CK-regulated PRR presence on endomembrane compartments mediates CK-induced disease resistance

How does CK affect immune signalling and disease resistance? Other than the evidence provided here (Figure 3) and by others (Choi et al., 2010; Argueso et al., 2012; Naseem et al., 2012) that SA is required for CK-induced immunity, partially through binding of the CK-responsive ARR2 to the SA pathway activator TGA3 (Choi et al., 2010), and that phytoalexins can play a part (Grosskinsky et al., 2011), no additional cellular mechanisms have been reported. PRRs are the first line of defence and immune-activation in plant cells. Based on the evidence in the literature that CK modulates endocytic trafficking of PIN1 in its regulation of auxin (Marhavý et al., 2011), we were prompted to investigate whether CK may also affect trafficking of immune receptors as a possible mechanism for promoting immune responses.
and disease resistance. Because we determined that CK enhances EIX-induced immune responses (Figures 7, S1, and S2), we examined whether it affects trafficking of the PRR LeEIX2, the receptor for the family 11 xylanase EIX (Ron and Avni, 2004; Bar and Avni, 2009). We have previously demonstrated that LeEIX2 colocalizes with several early endosome/TGN markers, and is internalized on VHA1/Rab11e endosomes (e.g., Bar and Avni, 2009; Bar et al., 2009, 2010; Sharfman et al., 2011, 2013; Leibman-Markus et al., 2018; Pizarro et al., 2019). Ligand-dependent internalization of LeEIX2 is required for signal propagation, which occurs, in part, directly from endosomes (Ron and Avni, 2004; Bar and Avni, 2009; Sharfman et al., 2011). As can be seen in Figure 11, CK enhances both the endosomal presence (Figure 11a,k) and vesicular size (Figure 11b,k) of LeEIX2 endosomes, without affecting the total cellular content of the protein (Figures 11c

**FIGURE 7** Cytokinin (CK) and ethylene-inducing xylanase (EIX) augment each other in inducing immunity. (a) and (b) *Solanum lycopersicum* “M82” leaves were treated with indicated concentrations of 6-benzylaminopurine dissolved in 1 µM NaOH and 1 µg/ml EIX. (c) and (d) *S. lycopersicum* “M82” leaves were treated with 100 µM of indicated CK compounds or the control adenine and 1 µg/ml EIX. (e) and (f) Leaves of the increased CK transgene pBLS> IPT7, the increased CK-sensitivity mutant clausa, and the reduced CK content transgene pFIL>CKX4, all in a *S. lycopersicum* “M82” background, were treated with 1 µg/ml EIX. (a), (c), and (e) Ethylene production was measured using gaschromatography. Presented values are normalized to M82 mock average. Average ± SEM of five independent experiments is presented, n ≥ 10. Asterisks represent statistical significance in a two-tailed t test (*p < .05, **p < .01, ***p < .001, ****p < .0001). (b), (d), and (f) Conductivity levels of samples immersed in water for 40 hr was measured. Average ± SEM of four independent experiments is presented, n ≥ 8. Asterisks represent statistical significance in a two-tailed t test (*p < .05, **p < .01, ***p < .001)
and S3). On EIX treatment, which enhances the endosomal presence (Figure 11d,h) and size (Figure 11e,h) of LeEIX2 in the mock-treated samples, as previously reported (Pizarro et al., 2018), there is no further increase with the addition of CK (Figure 11d,e), though the level of the receptor in the cell appears to increase slightly with the combination of both treatments (Figure 11f). CK enhances cellular immunity (Figures 5 and 8), and LeEIX2 presence on endosomes (Figure 11a–l). LeEIX2 endosomal presence is required for EIX-induced immunity (Ron and Avni, 2004; Sharfman et al., 2011; Bar and Avni, 2012), which CK enhances (Figure 7). Does this CK-mediated enhancement of PRR endosomal presence act as a mechanism for increased disease resistance? To examine this, we used a SlPRA1A overexpressing line, which was previously shown to have a decreased presence of receptor-like protein (RLP)-type PRRs in the cell plasma membrane, due to receptor degradation, along with a reduction in LeEIX2-mediated immune responses (Pizarro et al., 2018). Wild-type (WT), 35S-driven green fluorescent protein (GFP)-overexpressing, and 35S-driven SlPRA1A-GFP overexpressing, and behaves like an overexpression of IPT transcription factor mutant clausa, which has increased CK sensitivity and behaves like an overexpression of IPT developmentally (Bar et al., 2016), possesses decreased amounts of endogenous CK. Despite decreased endogenous CK, clausa retains the CK protective effect and exhibits increased defence responses (Figure 5) and pathogen resistance (Figure 2), indicating that CK-induced immunity is dependent on host signalling pathways. Although variable in steady state, on Bc infection, the defence gene expression pattern in clausa resembles that of the IPT-overexpressing line, in agreement with increased pathogen resistance observed in both genotypes. This demonstrates that defence gene expression in the steady state can be uncoupled from subsequent pathogen resistance in genetically primed plants in certain cases, and testifies to the flexibility of host responses.

Previous results coupled with our work suggest that CK can increase basal immunity levels, acting to potentiate defence responses (Choi et al., 2010; Argueso et al., 2012). Interestingly, comparing CK

![FIGURE 8](image)

**FIGURE 8** Cytokinin (CK) induces defence gene expression as a stand-alone treatment and reduces defence gene expression following *Botrytis cinerea* (Bc) infection. (a) Gene expression analysis of defence genes in M82 mock and CK (100 μM 6-benzylaminopurine)-treated plants was measured by quantitative reverse transcription PCR (RT-qPCR). Relative expression normalized to mock. Plants treated with 1 μM NaOH were used as mock. The expression of *RPL8* was used as an internal control. Average ± SEM of four independent experiments is shown, n ≥ 9. (b) Gene expression analysis of defence genes in mock and CK-treated Bc-infected plants was measured by RT-qPCR in samples harvested 24 hr after Bc inoculation. Plants treated with 1 μM NaOH were used as mock. The expression of *RPL8* was used as an internal control. Relative expression normalized to untreated mock. Average ± SEM of four independent experiments is shown, n ≥ 10. Results for (a) and (b) were analysed for statistical significance in one-way analysis of variance with a Dunnett post hoc test, p < .0001 in both cases. Asterisks represent statistical significance in a two-tailed t test comparing each gene (*p < .05; **p < .01; ***p < .001)

3 | DISCUSSION

Several previous reports have indicated that endogenous and exogenous CK treatment can result in resistance to pathogens (Albrecht and Argueso, 2017). Our work supports the notion that the main mode of action for CK-induced pathogen resistance is through induced immunity (Figures 2 and 5). In *Arabidopsis*, CK-treated plants demonstrated up-regulation of defence gene expression and callose deposition coupled with decreased pathogen growth (Choi et al., 2010; Argueso et al., 2012). CK treatment alone was also previously shown to induce ET biosynthesis (Coenen and Lomax, 1998) and PR1a expression (Choi et al., 2010).

Here, we show that CK acts systemically to induce immunity to foliar fungal pathogens when applied by soil drench to tomato roots (Figure 2f). CK induces ET biosynthesis and ion leakage, both exogenously, using different CK compounds, and endogenously, using CK mutants. CK also induces defence gene expression in tomato (Figures 8 and 9), as was previously reported for *Arabidopsis* (Choi et al., 2010; Argueso et al., 2012) and rice (Jiang et al., 2013). Interestingly, the MYB transcription factor mutant clausa, which has increased CK sensitivity and behaves like an overexpression of IPT developmentally (Bar et al., 2016), possesses decreased amounts of endogenous CK. Despite decreased endogenous CK, clausa retains the CK protective effect and exhibits increased defence responses (Figure 5) and pathogen resistance (Figure 2), indicating that CK-induced immunity is dependent on host signalling pathways. Although variable in steady state, on Bc infection, the defence gene expression pattern in clausa resembles that of the IPT-overexpressing line, in agreement with increased pathogen resistance observed in both genotypes. This demonstrates that defence gene expression in the steady state can be uncoupled from subsequent pathogen resistance in genetically primed plants in certain cases, and testifies to the flexibility of host responses.

Previous results coupled with our work suggest that CK can increase basal immunity levels, acting to potentiate defence responses (Choi et al., 2010; Argueso et al., 2012). Interestingly, comparing CK
FIGURE 9  Defence gene expression in altered cytokinin (CK) genotypes in steady-state and following Botrytis cinerea (Bc) infection. Gene expression analysis of defence genes in the increased CK transgenic line pBLS-IPT7, the increased CK-sensitivity mutant clausa, and the reduced CK content transgenic line pFIL-CKX4, as well as in M82 treated with 100 µM 6-benzylaminopurine, in steady state (a)–(e) and following Bc infection in samples harvested 24 hr after pathogen inoculation (f)–(j), was measured by quantitative reverse transcription PCR. (a) and (f) proteinase inhibitor 2 (PI2); (b) and (g) Pto-interacting 5 (Pti5); (c,h) pathogenesis-related protein (PR1a); (d) and (i) PR1b; (e) and (j) pathogen-induced 1 (PI1) genes. All samples normalized to M82 levels in steady-state. Average ± SEM of three to five independent experiments is shown, n ≥ 6. The expression of RPL8 was used as an internal control. Different letters represent statistical significance in a two-tailed t test comparing each gene among all genotypes (p ≤ .04)
with EIX, a well-known elicitor of plant defence responses, a priming agent, demonstrates that ≥25 µM CK induces ET biosynthesis to similar levels as EIX, confirming that CK elicits plant responses similar to those elicited by priming.

Though mechanisms of action were reported to differ in different host plants (Choi et al., 2010; Grosskinsky et al., 2011; Albrecht and Argueso, 2017), it is generally accepted that, in Arabidopsis, CK induces immunity to biotrophic pathogens through SA-dependent pathways. In agreement, our results demonstrate that a functioning SA pathway is required to achieve CK-induced pathogen resistance (Figure 3b,h,m). CK was able to induce ET biosynthesis, to a lesser level than in the WT, in an SA-deficient background (Figure 6a), demonstrating that NahG plants have a low level of CK-induced immunity that is significant but insufficient to promote disease resistance. Interestingly, though we have shown that CK-induced pathogen resistance requires the SA pathway (Figure 3), and external CK pretreatment results in an increase in SA levels (Figure 4), absolute SA levels or Bc-mediated SA content reduction do not directly correspond with pathogen resistance (Figure 4), suggesting that CK-mediated pathogen resistance may require additional signalling mechanisms.

Previous reports have disclosed an increase in SA levels following Bc inoculation in some cases (El-Oirdi et al., 2011), correlating with disease progression, where hypervirulence resulted in increased SA levels within hours of Bc inoculation. The level of Bc disease achieved and the resultant increase in SA levels depend on both the host and the pathogen isolate. In our experimental system, the combination of tomato host and Bc strain results in a progression of pathogenesis that is not hyperaggressive. While lower SA levels may be correlated with increased Bc resistance in certain cases in tomato (Angulo et al., 2015; Martínez-Hidalgo et al., 2015; Mehari et al., 2015), our results demonstrate that failure to reduce SA levels after Bc infection is not a defining feature in tomato CK-mediated disease resistance.

The role of SA signalling in plant Bc resistance is a subject of debate and depends greatly on the host plant and experimental conditions.
system investigated. Decreasing endogenous SA level in tomato NahG plants, used also in our study, has reportedly resulted in all possible Bc disease phenotypes, including increased susceptibility (Audenaert et al., 2002a; Achuo et al., 2004), unchanged disease levels (Audenaert et al., 2002b), and, as we observed, increased resistance (Angulo et al., 2015; Martínez-Hidalgo et al., 2015; Mehari et al., 2015). The situation is equally complex in other plant species. Most works indicate that SA may negatively regulate defence responses to Bc (see also AbuQamar et al., 2017), as we observed here; however, its role appears highly complex and is not yet elucidated. We also show here that normal ET sensitivity is required for CK-induced immunity and disease resistance in tomato (Figures 3 and 6). Defence responses induced by CK (Figure 6) were compromised in an ET sensitivity deficient background. Finally, we found that
JA sensitivity is not required for CK-induced pathogen resistance (Figures 3 and 6).

Distinctions between SAR and ISR are not always clear-cut. Occurrences of overlaps and/or coactivation between these pathways have been previously reported (Liu et al., 2016; Betsuyaku et al., 2018). Our work suggests that in tomato, CK activates systemic resistance that requires SA signalling (Ryals et al., 1996). This is supported by the fact that CK-induced immunity requires a functioning SA pathway. Further support comes from the fact that CK and EIX, an elicitor protein derived from the JA pathway ISR elicitor *Trichoderma* (Shoresh et al., 2005), augment the levels of defence elicited by each alone (Figure S1), indicating that they potentiate plant immunity primarily through different pathways. However, evidence of overlap between SAR and ISR also exists in the context of CK, with CK activating classical ISR genes (Figure 8a), and requiring ET, though not JA, to induce pathogen resistance (Figure 3). This is perhaps not surprising, given that CK signalling was reported to be upstream of ET in several cases (Robert-Seilaniantz et al., 2011; Zdarska et al., 2015).

Our work demonstrates that CK affects internalization of the PRR LeEIX2, which mediates immune responses to the xylanase EIX (Ron and Avni, 2004). LeEIX2 was shown to be internalized following ligand application, and to require this internalization for a full mounting of defence responses (Ron and Avni, 2004; Bar and Avni, 2009). Interestingly, CK increased both LeEIX2 internalization and EIX-mediated defence responses (Figures 7, 11, S1, and S2), suggesting that increased internalization could be at least one of the mechanisms underlying the increase in immune responses. Supporting this hypothesis, we found that in the SIPRA1A overexpressing line, in which expression and plasma membrane presence of RLP-type PRRs, including LeEIX2, are greatly reduced (Pizarro et al., 2018), CK is no longer able to mediate resistance to Bc, suggesting that it requires normal levels of PRRs to do so.

CK-based direct regulation of receptor endocytosis has been shown for PIN1 (Marhavý et al., 2011), where the authors determined that this endocytic regulation is a specific mechanism to rapidly modulate the auxin distribution in CK-mediated developmental processes, through a branch of the CK signalling pathway that does not involve transcriptional regulation. Therefore, in addition to regulating immunity and disease resistance through the SA pathway, CK may regulate endocytic trafficking independent of transcriptional regulation, accounting for the rapid plant response.

To the best of our knowledge, our work is the first one reporting that CK induces resistance to Bc and *O. neolycopersici* in tomato. This also points to an overlap between SAR and ISR in the case of CK-induced immunity, as the host plant requires JA signalling to resist necrotrophic pathogens such as Bc (Thomma et al., 1998; Durrant and Dong, 2004; Liu et al., 2016) (see also Figure 3f,m), yet, CK is able to prime this resistance. It would seem that in the case of CK-mediated immunity, a functioning SA pathway is required to achieve immunity upstream of the plants’ primary pathogen response, exerting similar effects on a biotrophic and a necrotrophic fungus (Figure 1). The relationship between CK and SA appears to be required for full immunity induction even in the absence of a pathogen (Figure 6). Our work contradicts results achieved in tobacco (Grosskinsky et al., 2011), a solanaceous host, in two respects: first, SA signalling was found not to be required for CK-induced immunity to *P. syringae* pv. *tomato* and, second, CK was found not to induce resistance to Bc. In that work, the authors found significant roles for the phytoalexins, scopoletin, and capsidiol in CK-induced immunity to *P. syringae* pv. tomato in tobacco; the lack of SA pathway requirement in that particular system could be attributed to the time-course of infection and phytoalexin production (Albrecht and Argueso, 2017). The lack of protectant effect for CK against Bc in tobacco may stem from different host biology, as responses to Bc were reported to differ between tomato and tobacco, in particular in relation to SA-induced immunity (Achuo et al., 2004). Differences in experimental design could also account for varying results.

**FIGURE 11** Cytokinin (CK) enhances cellular trafficking of the pattern recognition receptor LeEIX2. *Nicotiana benthamiana* epidermal cells transiently expressing LeEIX2-GFP were treated with CK or Mock as indicated (a)–(i), and subsequently treated with 1 μg/ml EIX (d)–(f), h, j, and l) for 5 min, followed by live cell imaging. (a)–(f) Graphs depicting the analysis of confocal microscope images acquired using a Zeiss LSM780 confocal microscope system with a C-Apochromat 40×/1.2 W Corr M27 objective, using a 488 nm excitation laser (3% power, 493–535 nm emission range), (g)–(l) Representative images taken from the membrane/endosomal plane, bar = 10 μm. (a)–(c) and (g)–(l) Effect of CK (6-benzylaminopurine, 6-BAP, concentrations as indicated) on endosomal presence (a) and (g)–(k) vesicular size (b) and (g)–(k), and total green fluorescent protein (GFP) cellular content of LeEIX2 (c, d)–(f) and (h)–(l) Effect of the combination of CK and EIX (1 μg/ml) on endosomal presence (d) and (h)–(l), vesicular size (e) and (h)–(l) and total GFP cellular content of LeEIX2 (f). Image analysis (21–38 images per treatment) was conducted with ImageJ using the raw images and the 3D object counter tool for quantifying endosome numbers and size, and the measurement analysis tool for quantifying pixel intensity. Graphs represent average ± SEM, n ≥ 20 per treatment. Statistical significance was determined in a one-way analysis of variance with a Dunnett post hoc test. p = .0005 (a), p < .0001 (b, d), and (e), p = .26 (c), p = .056 (f). Asterisks (a)–(c) represent statistically significant differences from the mock treatment (**p < .01, ***p < .0001). Letters (d)–(f) represent statistically significant differences between samples (p ≤ .038 where different letters are indicated). (m) *Solanum lycopersicum* cv “M82” plants, wild type (WT) or expressing GFP or SlPRA1A, both driven by the 35S promoter, were spray-treated with 100 μM 6-BAP dissolved in 1 μM NaOH, and inoculated with 10 μl of *Botrytis cinerea* spore solution (10⁶ spores/ml) 24 hr later. The lesion area was measured 7 days after *B. cinerea* inoculation using ImageJ. Graph represents the results of four independent experiments ± SEM, n ≥ 20 for each genotype × treatment combination. Results were analysed for statistical significance using one-way analysis of variance with a Bonferroni post hoc test, p < .0001. Asterisks indicate statistically significant differences between mock and CK treatment, letters indicate statistically significant differences in a two-tailed t test conducted among mock samples only.
Why does CK induce immunity in plants? Are classical developmental hormones also “defence” hormones, or is CK-induced immunity attributable to hormonal crosstalk? The swiftness of CK-induced immune processes seems to indicate that CK action is relatively "direct" (see Figures 5 and 7), and supports the idea of regulation of PRR trafficking as one of the underlying mechanisms. In developmental contexts, CK can be viewed as a “juvenility” factor, promoting meristem maintenance and morphogenetic processes, and delaying differentiation and senescence (Kurakawa et al., 2007; Gordon et al., 2009). Simplistically, could senescence-like processes activated by pathogen-derived tissue destruction in both biotrophic and necrotrophic infections make it evolutionarily economical for the plant to adapt those pathways for use in the war against pathogens, by recognizing levels of self-CK as a signal to activate immunity? Certainly, delayed senescence/enhanced juvenility can correlate with increased pathogen resistance (Pogány et al., 2004; Grosskinsky et al., 2011). It is worth nothing that, although CK signalling it is not normally active in mature, differentiated tissue such as a mature tomato leaf (Shani et al., 2010; Farber et al., 2016), on pathogen attack, the CK system is activated (Figure 10), suggesting that CK defensive function could be spatiotemporally regulated. CK signalling outside of a defined developmental window in specific tissues could facilitate the translation of CK into a defence signal. Thus, it would seem that the previously accepted paradigm, that pathogenesis processes cause a shunting of available plant resources towards immunity, shutting off growth programmes to divert all available resources towards defence (Berens et al., 2017; Karasov et al., 2017), can be updated to reflect that some developmental programmes are not shut off but rather modified and appropriated for defence purposes.

4 | MATERIALS AND METHODS

4.1 | Plant materials and growth conditions

Seeds of the S. lycopersicum "M82" were used throughout the study. Tomato mutant and transgenic lines employed in the assays were as follows: seeds of the increased CK: pBLSIPT7, decreased CK: pFIL»CKX4, increased CK sensitivity: clausa (allele e2522 from an EMS population (Menda et al., 2004)), and JA-insensitive jai-1, all in an M82 background, were obtained from Professor Naomi Ori, the Hebrew University of Jerusalem (Shani et al., 2010; Bar et al., 2016). Seeds of the decreased SA: NahG and its parental WT Moneymaker, decreased ET sensitivity: Never-Ripe (Nn) and its parental WT Pearsonwere obtained from Professor Yigal Elad (Mehari et al., 2015). Plants were grown from seeds in soil (Green Mix; Even-Ari) in a growth chamber, under long-day conditions (16 hr:8 hr, light:dark) at 24 °C.

4.2 | CK treatments

CK (6-benzylaminopurine, 6-BAP; Sigma-Aldrich) was sprayed onto 4-5-week-old plants, or soil drenched onto the roots (100 ml, 15 cm-diameter pot). 6-BAP solutions were prepared from a stock in 1 µM NaOH, and diluted into an aqueous solution to the desired 6-BAP concentration, with the addition of Tween 20 (100 µL/L). Mock plants were sprayed or soil-drenched with the aforementioned solution of NaOH with Tween 20. The CK analogs kinetin (6-furfurylaminopurine riboside), trans-zeatin (6-[4-hydroxy-3-methyl but-2-enylamino] purine), and thidiazuron (TDZ), as well as the control adenine (all from Sigma-Aldrich) were prepared in 1 µM NaOH (kinetin and zeatin), 1 M HCl (adenine), and 100% dimethyl sulphoxide (TDZ). Pathogen inoculations were carried out 24 hr after spray treatments and 3 days after soil drench, as detailed above.

4.3 | Pathogen infection and disease monitoring

Pathogenesis assays were conducted both on whole plants (Figures 1, 2g, 4, 8, 9, and 10) and on detached leaves (Figures 1g, 2, 3, and 11). For disease assays conducted on whole plants, inoculation was conducted as described below, and, as indicated, leaflets were photographed on the plant 5–10 days postinoculation (Figure 1f–h and 2f), or removed and immediately photographed (Figures 1a–e and 10c–d). For gene expression analyses (Figures 8, 9, and 10a), leaf tissue (1 cm diameter around the inoculation site) was removed 24 hr after inoculation and immediately processed for RNA/cDNA preparation and RT-qPCR analyses (see below). For metabolomic analyses (Figures 4 and 10b), leaf tissue (entire spray-inoculated leaflets) was removed 48 hr after inoculation and immediately processed for hormone extraction (see below).

B. cinerea isolate Bc16 cultures were maintained on potato dextrose agar (PDA; Difco) plates and incubated at 22°C for 5–7 days. Bc spores were harvested in 1 mg/ml glucose and 1 mg/ml K2HPO4 and filtered through cheesecloth. Spore concentration was adjusted to 106 spores/ml using a haemocytometer. Each tomato leaflet was either spray inoculated with the spore suspension or inoculated with two droplets of 10 µl spore suspension as indicated.

O. neolycopersici was isolated from young leaves of tomato plants grown in a commercial greenhouse. Conidia of O. neolycopersici were collected by rinsing infected leaves with sterile water. Concentrations of these conidial suspensions were determined under a light microscope using a haemocytometer. The conidial suspensions were adjusted to 105/ml and then sprayed onto plants at 5 ml per plant. All suspensions were sprayed within 10–15 min of the initial conidial collection. Suspensions were applied with a hand-held spray bottle and plants were left to dry in an open greenhouse for up to 30 min.

Inoculated plants were kept in a temperature-controlled growth chamber at 22 °C and inoculated excised leaves were kept in a humid growth chamber at 22 °C under long-day conditions (16 hr:8 hr, light:dark).

Controls consisted of plants or leaves treated with water/buffer without pathogen inoculation. The area of the necrotic lesions or percentage of infected leaf tissue was measured 5–10 days postinoculation using ImageJ.
4.4 | Plant immunity assays

Immunity assays (Figures 5–7, S1, and S2) were conducted on leaf discs from indicated genotypes.

4.4.1 | ET measurement

ET production was measured as previously described (Leibman-Markus et al., 2017). Leaf discs 0.9 cm in diameter were harvested from indicated genotypes, and average weight was measured for each plant. Discs were washed in water for 1–2 hr for EIX and steady-state assays or incubated for 3–4 hr in different concentrations of CK. For each sample, six discs were sealed in a 10-ml flask containing 1 ml of assay medium (with or without 1 µg/ml EIX or with or without CK) for 4 hr (for EIX) or overnight (for CK) at room temperature. ET production was measured by gas chromatography (Varian 3350).

4.4.2 | Ion leakage (conductivity) measurement

Leaf discs 0.9 cm in diameter were harvested from indicated genotypes. Discs were washed in a 50-ml water tube for 3 hr. For each sample, five discs were floated in a 12-well plate containing 1 ml of water, with or without 1 µg/ml EIX or with or without CK, adaxial surface down, at room temperature with agitation. Conductivity was measured in the water solution after 40 hr of incubation using a conductivity meter (EUTECH instrument con510).

4.4.3 | Measurement of ROS generation

ROS was measured as previously described (Leibman-Markus et al., 2017). Leaf discs 0.5 cm in diameter were harvested from indicated genotypes. Discs were floated in a white 96-well plate (SPL Life Sciences) containing 250 µl distilled water for 4–6 hr at room temperature. After incubation, water with and without different concentrations of CK and its analogs was removed and a ROS measurement reaction containing either 1 µg/ml EIX or water (mock) was added. Light emission was measured immediately and over indicated time using a luminometer (Turner BioSystems Veritas).

4.5 | RNA extraction and RT-qPCR

Plant total RNA was extracted from tomato plants 24 hr after B. cinerea inoculation using TRI-reagent (Sigma-Aldrich) according to the manufacturer’s instructions. RNA was isolated from plants infected as indicated (whole-plant assays). RNA (3 µg) was converted to first-strand cDNA synthesis using reverse transcriptase (Promega) and oligo d(T)15. RT-qPCR was performed according to the Power SYBR Green Master Mix protocol (Life Technologies) using a Rotor-Gene Q machine (Qiagen). Table S1 lists the specific primers used in this work. Relative expression quantification was calculated using copy number method for gene expression experiments (D’haene et al., 2010). The housekeeping gene coding for ribosomal protein RPL8 (accession number Solyc10g006580) was used for the normalization of gene expression in all analyses.

4.6 | Phytohormone analysis

Hormone extraction was performed according to Shaya et al. (2019). Plants were inoculated with Bc as described above. At 48 hr after inoculation, entire leaflets were harvested from the inoculated plants. Phytohormones were quantified in the harvested tissue. Briefly, frozen tissue was ground to a fine powder using a mortar and pestle. A total of 200–450 mg of powder was transferred to a 2-ml tube containing 1 ml of extraction solvent (ES) mixture (79% IPA:20% methanol:1% acetic acid) supplemented with 20 ng of each deuterium-labelled internal standard (IS). The tubes were incubated for 60 min at 4 °C with rapid shaking and centrifuged at 14,000 × g for 15 min at 4 °C. The supernatant was collected and transferred to 2-ml tubes. ES (0.5 ml) was added to the pellet and the extraction steps were repeated twice. The combined extracts were evaporated using speed-vac at room temperature. Dried samples were dissolved in 200 µl of 50% methanol and filtered with a 0.22-µm cellulose syringe filter. Five to ten microlitres were injected for each analysis. LC-MS-MS analyses were conducted using a UPLC-Triple Quadrupole MS (WatersXevo TQMS). Separation was performed on a Waters Acuity UPLC BEH C18 1.7 µm 2.1 × 100 mm column with a VanGuard precolumn (BEH C18 1.7 µm 2.1 × 5 mm). The mobile phase consisted of water (phase A) and acetonitrile (phase B), both containing 0.1% formic acid in the gradient elution mode. The flow rate was 0.3 ml/ min and the column temperature was kept at 35 °C. Acquisition of LC-MS data was performed using MassLynx v. 4.1 software (Waters). Quantification was done using isotope-labelled ISs. Solvent gradients and MS-MS parameters are detailed in Table S2.

4.7 | Imaging of CK-response synthetic promoter pTCS::3 × VENUS

Stable transgenic M82 tomato pTCS::3 × VENUS plants (T1) that express VENUS driven by the synthetic two-component signalling sensor pTCS (Zürcher et al., 2013; Bar et al., 2016) were spot-inoculated with Bc and kept at 22 °C under long-day conditions (16 hr light/8 hr dark) for up to 4 days. VENUS expression was analysed using a Nikon SMZ-25 stereomicroscope equipped with a Nikon-D2 camera and NIS Elements v. 5.11 software. ImageJ software was used for analysis and quantification of captured images.

4.8 | Trafficking imaging and analysis

As previously reported (Leibman-Markus et al., 2018), LeEIX2 cDNA (Solyc07g008630) C-terminally tagged with GFP was cloned into
the S0l site of pBINPLUS (van Engelen et al., 1995), between the CaMV 35S promoter containing the translation enhancer signal and the NOS terminator, using the following primers: LeEIX2 forward primer 5′-ATCTCGACATGGCAGGAAAAAAGTAAATC-3′ and LeEIX2 reverse primer 5′-ATGCAGCTTCCTTAGC TTCCCTTCAGTGC-3′. Nicotiana benthamiana epidermal cells transiently expressing LeEIX2-GFP were infiltrated with 100 nM, 100 µM 6-BAP or mock (distilled water with 0.2% Tween). Leaf discs of 1 cm diameter from the different conditions (6 hr after infiltration) were treated with EIX (1 µg/ml) for 5 min and live cell imaging was conducted. A total of eight plants per treatment were studied in three separate experiments. Confocal microscopy images were acquired using a Zeiss LSM780 confocal microscope system with a C-Apochromat 40x/1.2 W Corr M27 objective. GFP images were acquired using a 488 nm excitation laser (3% power), with the emission collected in the range of 493–535 nm. Images of 8 bits and 1,024 × 1,024 pixels were acquired using a pixel dwell time of 1.27, pixel averaging of 8, and pinhole of 1 airy unit. Image analysis (21–38 images per treatment) was conducted with Fiji-ImageJ using the raw images and the 3D object counter tool for quantifying endosome numbers, and the measurement analysis tool for quantifying pixel intensity (Schindelin et al., 2012).

4.9 Western blot

Western blot was performed on N. benthamiana leaves transiently expressing LeEIX2-GFP. One hundred milligrams of plant tissue were ground to a fine powder with liquid nitrogen, and three volumes of extraction buffer (50 mM Tris-HCl, pH 7.5, 2 mM MgCl2, 150 mM NaCl. 140 mM β-mercaptoethanol, 2 mM phenylmethylsulphonyl fluoride [PMSF], and one Complete Protease Inhibitor tablet, without EDTA [Roche] per 50 ml) were added. Samples were centrifuged, and supernatant cytosolic fraction was discarded. Pellets were ground using two volumes of extraction buffer with 1% Triton X-100 and incubated in a rotating wheel at 4 °C for 20 min before centrifugation. Supernatant samples (TSM) were collected and boiled after adding sample buffer (8% sodium dodecyl sulphate, 40% glycerol, 200 mM Tris-Cl, pH 6.8, 388 mM dithiothreitol, and 0.1 mg/ml bromophenol blue). Samples were separated by electrophoresis on sodium dodecyl sulphate-polyacrylamide gels, blotted onto nitrocellulose membranes, and incubated with rat anti-GFP (Chromotek), followed by appropriate secondary antibodies and horseradish peroxidase-based detection.

4.10 Statistical analysis

All data are presented as mean ± SEM. Differences between two groups were analysed for statistical significance using a two-tailed t test. Differences among three groups or more were analysed for statistical significance with a one-way analysis of variance (ANOVA). Regular ANOVA was used for groups with equal variances and Welch’s ANOVA for groups with unequal variances. When a significant result for a group in an ANOVA was returned, significance in differences between the means of different samples in the group were assessed using a post hoc test. Tukey’s test was employed for samples with equal variances when the mean of each sample was compared to the mean of every other sample. Bonferroni was employed for samples with equal variances when the mean of each sample was compared to the mean of a control sample. Dunnett’s test was employed for samples with unequal variances. All statistical analyses were conducted using Prism 8.

ACKNOWLEDGMENTS

The authors would like to thank Yigal Elad for the B. cinerea strain, and seeds of NahG and Nr mutants; Naomi Ori for seeds of clausa, pFIL>>CKX, pTCS::3 x VENUS, and jai-1; Adi Avni for EIX and seeds of p35S::SIPRA1A-GFP; Tzahi Arazi for seeds of p35S::GFP; and Mira Weissberg-Carmeli and Felix Shaya for the metabolomic analyses. The research was supported by the Israeli Ministry of Agriculture. R.G. is supported by the Indo-China ARO Postdoctoral Fellowship Program. M.B. thanks members of the Bar group for continuous discussion and support.

AUTHOR CONTRIBUTIONS

M.B. and R.G. conceived and designed the study, R.G., L.P., M.L.-M., and I.M. formulated the methodology and carried out the experiments. R.G., L.P., M.L.-M., and M.B. analysed the data. All authors contributed to the writing of the manuscript.

DATA AVAILABILITY STATEMENT

The authors declare that the data supporting the findings of this study are available within the paper and its supplementary information files. Raw data are available from the corresponding author upon reasonable request.

ORCID

Maya Bar https://orcid.org/0000-0002-7823-9121

REFERENCES

AbuQamar, S., Chai, M.F., Luo, H., Song, F. and Mengiste, T. (2008) Tomato protein kinase 1b mediates signaling of plant responses to necrotrophic fungi and insect herbivory. The Plant Cell, 20, 1964–1983.
AbuQamar, S., Moustafa, K. and Tran, L.S.P. (2017) Mechanisms and strategies of plant defense against Botrytis cinerea. Critical Reviews in Biotechnology, 37, 262–274.
Achou, E.A., Audenaert, K., Meziane, H. and Höfte, M. (2004) The salicylic acid-dependent defence pathway is effective against different pathogens in tomato and tobacco. Plant Pathology, 53, 65–72.
Albrecht, T. and Argueso, C.T. (2017) Should I fight or should I grow now? The role of cytokinins in plant growth and immunity and in the growth-defence trade-off. Annals of Botany, 119, 725–735.
Ament, K., Kant, M.R., Sabelis, M.W., Haring, M.A. and Schuurink, R.C. (2004) Jasmonic acid is a key regulator of spider mite-induced volatile terpenoid and methyl salicylate emission in tomato. Plant Physiology, 135, 2025–2037.
Angulo, C., de la Leyva, M.O., Finiti, I., López-Cruz, J., Fernández-Crespo, E., García-Agustín, P. et al. (2015) Role of dioxygenase α-DOX2 and SA in basal response and in hexanoic acid-induced resistance of
tomato (Solanum lycopersicum) plants against Botrytis cinerea. Journal of Plant Physiology, 175, 163–173.

Argueso, C.T., Ferreira, F.J., Epplle, P., To, J.P.C., Hutchison, C.E., Schaller, G.E. et al. (2012) Two-component elements mediate interactions between cytokinin and salicylic acid in plant immunity. PLoS Genetics, 8, e1002448.

Audenaert, K., De Meyer, G.B. and Höfte, M.M. (2002a) Abscisic acid determines basal susceptibility of tomato to Botrytis cinerea and suppresses salicylic acid-dependent signaling mechanisms. Plant Physiology, 128, 491–501.

Audenaert, K., Pattery, T., Cornelis, P. and Höfte, M. (2002b) Induction of systemic resistance to Botrytis cinerea in tomato by Pseudomonas aeruginosa 7NSK2: Role of salicylic acid, pyochelin, and pyocyanin. Molecular Plant-Microbe Interactions, 15, 1147–1156.

Ballaré, C.L. (2011) Jasmonate-induced defenses: a tale of intelligence, collaborators and rascals. Trends in Plant Science, 16, 249–257.

Bar, M. and Avni, A. (2009) EHD2 inhibits ligand-induced endocytosis and signaling of the leucine-rich repeat receptor-like protein LeEix2. The Plant Journal, 59, 600–611.

Bar, M. and Avni, A. (2012). Endocytosis of LeEix and EHD proteins during plant defense signalling. In: Šamaj, J. (Ed.) Endocytosis in Plants. Berlin, Heidelberg: Springer, pp. 297–311.

Bar, M., Sharfman, M., Ron, M. and Avni, A. (2010) BAK1 is required for the attenuation of ethylene-inducing xylanase (Eix)-induced defense responses by the decoy receptor LeEix1. The Plant Journal, 63, 791–800.

Bar, M., Sharfman, M., Schuster, S. and Avni, A. (2009) The coiled-coil domain of EHD2 mediates inhibition of LeEix2 endocytosis and signaling. PLoS ONE, 4, e7973.

Bar, M., Israeli, A., Levy, M., Ben Gera, H., Jiménez-Gómez, J.M., Kouril, S. et al. (2016) CLAUSA is a MYB transcription factor that promotes leaf differentiation by attenuating cytokinin signaling. The Plant Cell, 28, 1602–1615.

Bari, R. and Jones, J.D.G. (2009) Role of plant hormones in plant defence responses. Plant Molecular Biology, 69, 473–488.

Berens, M.L., Berry, H.M., Mine, A., Argueso, C.T. and Tsuda, K. (2017) Evolution of hormone signaling networks in plant defense. Annual Review of Phytopathology, 55, 401–425.

Betsuyaku, S., Katou, S., Takebayashi, Y., Sakakibara, H., Nomura, N. and Fukuda, H. (2018) Salicylic acid and jasmonic acid pathways are activated in spatially different domains around the infection site during effector-triggered immunity in Arabidopsis thaliana. Plant and Cell Physiology, 59, 8–16.

Brading, P.A., Hammond-Kosack, K.E., Parr, A. and Jones, J.D.G. (2000) Salicylic acid is not required for Cf-2 and Cf-9-dependent resistance of tomato to Cladosporium fulvum. The Plant Journal, 23, 305–318.

Chanclud, E. and Morel, J.-B. (2016) Plant hormones: a fungal point of view. Molecular Plant Pathology, 17, 1289–1297.

Choi, J., Huh, S.U., Kojima, M., Sakakibara, H., Paek, K.-H. and Hwang, I. (2010) The cytokinin-activated transcription factor ARR2 promotes plant immunity via TGA3/NPR1-dependent salicylic acid signaling in Arabidopsis. Developmental Cell, 19, 284–295.

Choi, J., Choi, D., Lee, S., Ryu, C.-M. and Hwang, I. (2011) Cytokinins and plant immunity: old foes or new friends? Trends in Plant Science, 16, 388–394.

Coenen, C. and Lomax, T.L. (1998) The diaeotropic gene differentially affects auxin and cytokinin responses throughout development in tomato. Plant Physiology, 117, 63–72.

Cui, H., Sun, Y., Zhao, Z. and Zhang, Y. (2019) The combined effect of elevated O₃ levels and TYLCV infection increases the fitness of Bemisia tabaci Mediterranean on tomato plants. Environmental Entomology, 48, 1425–1433.

D’haene, B., Vandesompele, J. and Hellemans, J. (2010) Accurate and objective copy number profiling using real-time quantitative PCR. Methods, 50, 262–270.

Dodds, P.N. and Rathjen, J.P. (2010) Plant immunity: towards an integrated view of plant-pathogen interactions. Nature Reviews Genetics, 11, 539–548.

Durrant, W.E. and Dong, X. (2004) Systemic acquired resistance. Annual Review of Phytopathology, 42, 185–209.

El-Oirdi, M., El-Rahman, T.A., Rigano, L., El-Hadrami, A., Rodríguez, M.C., Daiyf, F. et al. (2011) Botrytis cinerea manipulates the antagonistic effects between immune pathways to promote disease development in tomato. The Plant Cell, 23, 2405–2421.

Elbaz, M., Avni, A. and Weil, M. (2002) Constitutive caspase-like machinery executes programmed cell death in plant cells. Cell Death and Differentiation, 9, 726–733.

van Engelen, F.A., Moltzoff, J.W., Conner, A.J., Nap, J.-P., Pereira, A., Stiekema, W.J. (1995) pBINPLUS: An improved plant transformation vector based on pBIN19. Transgenic Research, 4, 288–290.

Farber, M., Attila, Z. and Weiss, D. (2016) Cytokinin activity increases stoma density and transpiration rate in tomato. Journal of Experimental Botany, 67, 6351–6362.

Gordon, S.P., Chickarmane, V.S., Ohno, C. and Meyerowitz, E.M. (2009) Multiple feedback loops through cytokinin signaling control stem cell number within the Arabidopsis shoot meristem. Proceedings of the National Academy of Sciences of the United States of America, 106, 16529–16534.

Grosskinsky, D.K., Naseem, M., Abdelmohsen, U.R., Plickert, N., Engelke, T., Griebel, T. et al. (2011) Cytokinins mediate resistance against Pseudomonas syringae in tobacco through increased antimicrobial phytoalexin synthesis independent of salicylic acid signaling. Plant Physiology, 157, 815–830.

Harel, Y.M., Mehari, Z.H., Rav-David, D. and Elad, Y. (2014) Systemic resistance to gray mold induced in tomato by benzo(thiazoleazole and Trichoderma harzianum T39. Physopathology, 104, 150–157.

Hatsugai, N., Igarashi, D., Mase, K., Lu, Y., Tsuda, Y., Chakravarty, S. et al. (2017) A plant effector-triggered immunity signaling sector is inhibited by pattern-triggered immunity. The EMBO Journal, 36, 2758–2769.

Ikerkleid, I., Ozalvo, R., Feldman, L., Elbaz, M., Patricia, B. and Horowitz, S.B. (2014) Responses of tomato genotypes to avirulent and Mi -virulent Meloidogyne javanica isolates occurring in Israel. Phytopathology, 104, 484–496.

Jameson, P.E. (2000) Cytokinins and auxins in plant-pathogen interactions – An overview. Plant Growth Regulation, 32, 369–380.

Jiang, C.-J.-J., Shimono, M., Sugano, S., Kojima, M., Liu, X., Inoue, H. et al. (2013) Cytokinins act synergistically with salicylic acid to activate defense gene expression in rice. Molecular Plant-Microbe Interactions, 26, 287–296.

Jones, J.D.G. and Dangl, J.L. (2006) The plant immune system. Nature, 444, 323–329.

Karasov, T.L., Chae, E., Herman, J.J. and Bergelson, J. (2017) Mechanisms to mitigate the trade-off between growth and defense. The Plant Cell, 29, 666–680.

Keshishian, E.A. and Rashotte, A.M. (2015) Plant cytokinin signalling. Essays in Biochemistry, 58, 13–27.

Király, Z., Pozsar, B. and Hammady, M.E. (1966) Cytokinin activity in rust infected plants: juvenility and senescence in diseased leaf tissues. Acta Phytopathologica Academiae Scientiarum Hungaricae, 1, 29–37.

Király, Z., El Hammady, M., Pozsár, B., Király, Z., Hammady, M.E. and Pozsar, B. (1967) Increased cytokinin activity of rust-infected bean and broad bean leaves. Phytopathology, 57, 93–94.

Kurakawa, T., Ueda, N., Maekawa, M., Kobayashi, K., Kojima, M., Nagato, Y. et al. (2007) Direct control of shoot meristem activity by a cytokinin-activating enzyme. Nature, 445, 652–655.

Lashbrook, C.C., Tieman, D.M. and Klee, H.J. (1998) Differential regulation of the tomato ETR gene family throughout plant development. The Plant Journal, 15, 243–252.
Leibman-Markus, M., Schuster, S. and Avni, A. (2017) LeEIX2 Interactors’ analysis and EIX-mediated responses measurement. In: Shan, L. and He, P. (Eds.) Plant Pattern Recognition Receptors: Methods and Protocols. New York, NY: Springer New York, pp. 167–172.

Leibman-Markus, M., Pizarro, L., Schuster, S., Lin, Z.J.D.D., Gershony, O., Bar, M. et al. (2018) The intracellular nucleotide-binding leucine-rich repeat receptor (SINRCA4) enhances immune signalling elicited by extracellular perception. Plant, Cell & Environment, 41, 2313–2327.

Li, L., Li, C., Lee, G.J. and Howe, G.A. (2002) Distinct roles for jasmonate synthesis and action in the systemic wound response of tomato. Proceedings of the National Academy of Sciences of the United States of America, 99, 6416–6421.

Li, Y., Qin, L., Zhao, J., Muhammad, T., Cao, H., Li, H. et al. (2017) SIMAPK3 enhances tolerance to tomato yellow leaf curl virus (TYLCV) by regulating salicylic acid and jasmonic acid signaling in tomato (Solanum lycopersicum). PLoS ONE, 12, e0172466.

Liu, L., Sonbol, F.M., Huot, B., Gu, Y., Withers, J., Mwimba, M. et al. (2016) Salicylic acid receptors activate jasmonic acid signalling through a non-canonical pathway to promote effector-triggered immunity. Nature Communications, 7, 13099.

López-Ráez, J.A., Verhage, A., Fernández, I., García, J.M., Azcón-Aguilar, C., Flors, V. et al. (2010) Hormonal and transcriptional profiles highlight common and differential host responses to arbuscular mycorrhizal fungal and the regulation of the oxylipin pathway. Journal of Experimental Botany, 61, 2589–2601.

Macho, A.P. and Zipfel, C. (2014) Plant PRRs and the activation of innate immune signaling. Molecular Cell, 54, 263–272.

Marhavý, P., Bielach, A., Abas, L., Abuzeinéh, A., Duclercq, J., Tanaka, H. et al. (2011) Cytokinin modulates endocytic trafficking of PIN1 auxin efflux carrier to control plant organogenesis. Developmental Cell, 21, 796–804.

Martínez-Hidalgo, P., García, J.M. and Pozo, M.J. (2015) Induced systemic resistance against Botrytis cinerea by Micromonospora strains isolated from root nodules. Frontiers in Microbiology, 6, 922.

Martínez-Medina, A., Fernández, I., Sánchez-Guzmán, M.J., Jung, S.C., Pascual, J.A. and Pozo, M.J. (2013) Deciphering the hormonal signaling network behind the systemic resistance induced by Trichoderma harzianum in tomato. Frontiers in Plant Science, 4, 206.

Mehari, Z.H., Elad, Y., Rav-David, D., Graber, E.R. and Meller Harel, Y. (2017) SlMAPK3 analyzing salicylic acid and jasmonic acid signaling in tomato (Solanum lycopersicum) from a saturated mutation library. Annual Review of Phytopathology, 65, 39–47.

Melo, E., Romanov, G.A., Lomin, S.N. and Schmülling, T. (2006) Biochemical characteristics and ligand-binding properties of Arabidopsis cytokinin receptor AHK3 compared to CRE1/AHK4 as revealed by a direct binding assay. Journal of Experimental Botany, 57, 4051–4058.

Ron, M. and Avni, A. (2004) The receptor for the fungal elicitor ethylene-inducing xylanase is a member of a resistance-like gene family in tomato. The Plant Cell, 16, 1604–1615.

Ron, M., Kantety, R., Martin, G.B., Avidan, N., Eshed, Y., Zamir, D. et al. (2000) High-resolution linkage analysis and physical characterization of the EIX-responder locus in tomato. Theoretical and Applied Genetics, 100, 184–189.

Ryalss, J.A., Neuenschwander, U.H., Willits, M.G., Molina, A., Steiner, H.Y. and Hunt, M.D. (1996) Systemic acquired resistance. The Plant Cell, 8, 1809–1819.

Sakakibara, H. (2006) Cytokinins: activity, biosynthesis, and translocation. Annual Review of Plant Biology, 57, 431–449.

Shendeh, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T. et al. (2012) Fiji: an open-source platform for biological-image analysis. Nature Methods, 9, 676–682.

Shani, E., Ben-Gera, H., Shliezer-Burko, S., Burko, Y., Weiss, D. and Ori, N. (2010) Cytokinin regulates compound leaf development in tomato. The Plant Cell, 22, 3206–3217.

Sharman, M., Bar, M., Ehrlich, M., Schuster, S., Melech-Bonfil, S., Ezer, R. et al. (2011) Endosomal signaling of the tomato leucine-rich repeat receptor-like protein LeEix2. The Plant Journal, 68, 413–423.

Sharman, M., Bar, M., Schuster, S., Leibman, M. and Avni, A. (2013) Sterol-dependent induction of plant defense responses by a microbe-associated molecular pattern from Trichoderma viride. Plant Physiology, 164, 819–827.

Sharon, A., Fuchs, Y. and Anderson, J.D. (1993) The elicitation of ethylene biosynthesis by a Trichoderma xylanase is not related to the cell wall degradation activity of the enzyme. Plant Physiology, 102, 1325–1329.

Shaya, F., David, I., Yitzhak, Y. and Izhaki, A. (2019) Hormonal interactions during early physiological partenocarpic fruitlet abscission in persimmon (Diospyros Kaki Thunb.) “Triumph” and “Shinshu” cultivars. Scientia Horticulturae, 243, 575–582.

Shigenaga, A.M., Berens, M.L., Tsuda, K. and Argueso, C.T. (2017) Towards engineering of hormonal crosstalk in plant immunity. Current Opinion in Plant Biology, 38, 164–172.

Shoresh, M., Yedidia, I. and Chet, I. (2005) Involvement of jasmonic acid/ethylene signaling pathway in the systemic resistance induced in cucumber by Trichoderma asperellum T203. Phytopathology, 95, 76–84.

Spoel, S.H. and Dong, X. (2012) How do plants achieve immunity? Defence without specialized immune cells. Nature Reviews Immunology, 12, 89–100.

Thara, V.K., Tang, X., Gu, Y.Q., Martin, G.B. and Zhou, J.-M. (1999) Pseudomonas syringae pv. tomato induces the expression of tomato EREBP-like genes PtI4 and PtI5 independent of ethylene, salicylate and jasmonate. The Plant Journal, 20, 475–483.

Thomma, B.P., Eggemont, K., Penninckx, I.A., Mauch-Mani, B., Vogelsang, R., Cammue, B.P. et al. (1998) Separate jasmonate-dependent and
salicylate-dependent defense-response pathways in Arabidopsis are essential for resistance to distinct microbial pathogens. Proceedings of the National Academy of Sciences of the United States of America, 95, 15107–15111.

Werner, T. and Schmülling, T. (2009) Cytokinin action in plant development. Current Opinion in Plant Biology, 12, 527–538.

Zdarska, M., Dobisová, T., Gelová, Z., Pernisová, M., Dabravolski, S. and Hejátko, J. (2015) Illuminating light, cytokinin, and ethylene signalling crosstalk in plant development. Journal of Experimental Botany, 66, 4913–4931.

Zürcher, E., Tavor-Deslex, D., Lituiev, D., Enkerli, K., Tarr, P.T. and Müller, B. (2013) A robust and sensitive synthetic sensor to monitor the transcriptional output of the cytokinin signaling network in planta. Plant Physiology, 161, 1066–1075.

SUPPORTING INFORMATION
Additional supporting information may be found online in the Supporting Information section.

How to cite this article: Gupta R, Pizarro L, Leibman-Markus M, Marash I, Bar M. Cytokinin response induces immunity and fungal pathogen resistance, and modulates trafficking of the PRR LeEIX2 in tomato. Molecular Plant Pathology. 2020;21:1287–1306. https://doi.org/10.1111/mpp.12978