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TOR coordinates cytokinin and gibberellin signals mediating development and defense

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Abstract

Plants constantly perceive and process environmental signals and balance between the energetic demands of growth and defense. Growth arrest upon pathogen attack was previously suggested to result from a redirection of the plants' metabolic resources towards the activation of plant defense. The energy sensor Target of Rapamycin (TOR) kinase is a conserved master coordinator of growth and development in all eukaryotes. Although TOR is positioned at the interface between development and defense, little is known about the mechanisms by which TOR may potentially regulate the relationship between these two modalities. The plant hormones cytokinin (CK) and gibberellin (GA) execute various aspects of plant development and defense. The ratio between CK and GA was reported to determine the outcome of developmental programmes. Here, investigating the interplay between TOR-mediated development and TOR-mediated defense in tomato, we found that *TOR* silencing resulted in rescue of several different aberrant developmental phenotypes, demonstrating that TOR is required for the execution of developmental cues. In parallel, *TOR* inhibition enhanced immunity in genotypes with a low CK/GA ratio but not in genotypes with a high CK/GA ratio. TOR-inhibition mediated disease resistance was found to depend on developmental status, and was abolished in strongly morphogenetic leaves, while being strongest in mature, differentiated leaves. CK repressed TOR activity, suggesting that CK-mediated immunity may rely on TOR downregulation. At the same time, TOR activity was promoted by GA, and *TOR* silencing reduced GA sensitivity, indicating that GA signalling requires normal TOR activity. Our results demonstrate that TOR likely acts in concert with CK and GA signalling, executing signalling cues in both defense and development. Thus, differential regulation of TOR or TOR-mediated processes could regulate the required outcome of development-defense prioritisation.

KEYWORDS
cytokinin, gibberellin, immunity, tomato, TOR

1 | INTRODUCTION

Plants have developed sophisticated strategies and molecular mechanisms to protect themselves against attacks by pathogens (Jiang et al., 2020). When subjected to biotic stresses, plants activate an array of cellular and molecular processes which include the production of defense proteins and metabolites. However, this activation of defense responses is often energy-demanding, and can suppress plant growth by diverting energy and resources toward defense at the expense of growth, or by activation of conflicting pathways, or the sharing of components between immune and growth signalling (Eichmann & Schäfer, 2015). This is known as the 'growth-defense tradeoff', a phenomenon in which plants must constantly regulate and balance growth and defense to adapt to changes. It is now accepted that the tradeoff between growth and defense is carefully regulated by the plant, rather than a passive process in which energy diverted toward defense is simply not available for other needs (Karasov et al., 2017; Kliebenstein, 2016). Yet, our understanding of the mechanisms that enable plants to balance growth during biotic stress response is still limited.

In recent years, the conserved Target of Rapamycin (TOR) kinase has been established as a central eukaryotic regulatory hub, playing a role in the regulation of various cellular processes including metabolism, mRNA translation and transcription, cell division, rRNAs and ribosomal protein synthesis, and autophagy (Dobrenel et al., 2016; Zhang et al., 2016). The TOR signalling pathway fine-tunes growth and development by coordinating nutrient availability, energy status, and external cues. In plants, TOR signalling is particularly important for embryogenesis, meristem activation, leaf and root growth, senescence, and flowering (McCready et al., 2020). Under nutrient availability and when conditions are favourable for growth, the TOR signalling pathway is activated and developmental and anabolic processes are promoted while catabolic processes are repressed. When nutrients are limited or in the presence of environmental stresses, TOR is inactive and catabolic processes are promoted (Dobrenel et al., 2016; Saxton & Sabatini, 2017). Even though TOR signalling is involved in the regulation of multiple important signalling pathways, there is currently limited evidence of its cross-talk with the plant hormones gibberellin (GA) and cytokinin (CK), and its possible involvement in CK-mediated immunity.

Recent studies suggest that TOR acts as a negative regulator of plant immunity, as it has been demonstrated to antagonise the defense hormones jasmonic acid (JA) and salicylic acid (SA) in rice, suggesting that it may act as a switch between these modalities (De Vleesschauwer et al., 2018). Similarly, TOR was reported to negatively regulate JA biosynthesis and response in cotton (Song et al., 2017). Furthermore, mutants impaired in TOR complex and TOR-inhibited WT *Arabidopsis* plants were more resistant to *Fusarium* (Aznar et al., 2018), and in citrus spp., TOR inhibition was found to attenuate the growth of *Xanthomonas citri* (Soprano et al., 2018). In another study, *TOR* expression was downregulated upon NB-LRR activation. Suppression of *TOR* expression enhanced disease resistance, whereas *TOR* overexpression decreased it, suggesting that translational regulation executed by TOR plays an important role in the switch from growth to defense (Meteignier et al., 2018). TOR inhibition was also found to block growth and activate the SA signalling pathway in *Arabidopsis* (Dong et al., 2015; Moreau et al., 2012). In agreement with this, we previously showed that TOR inhibition or *TOR* silencing promote resistance against *Xanthomonas*, tobacco mosaic virus (TMV), *Alternaria alternata*, and *Botrytis cinerea* (*Bc*) in tomato and *N. benthamiana*, by SA-dependent activation of plant defense responses (Marash et al., 2022). Although the exact mechanism by which the inhibition of TOR primes resistance is not fully understood, it was suggested to selectively regulate translational control during plant immunity (Meteignier et al., 2018) and/or negatively regulate autophagy in plants, as was reported in yeast and mammals (Liu & Bassham, 2010).

Recent studies have shown that the TOR signalling pathway interacts with several plant hormones. TOR signalling interacts with the brassinosteroid (BR) signalling pathway during hypocotyl elongation through the BZR1 transcription factor (Zhang et al., 2016), and activates abscisic acid (ABA) receptors by phosphorylation (Wang et al., 2018). Additionally, TOR phosphorylates and stabilises the auxin (AUX) efflux facilitator PIN2, which affects the distribution gradient of PIN2 in *Arabidopsis* primary roots (Yuan et al., 2020). TOR monitors the level of sugar in meristematic regions and halts growth when the sugar level is low, blocking hormone signals that normally promote growth (Xiong et al., 2013). As plant growth rate is dictated by hormones, it seems that energy status and growth are integrated through the activity of TOR (Monson et al., 2022). Furthermore, TOR inhibition was shown to alter the expression of hundreds of genes, including genes that are linked to plant hormone signalling networks. When TOR is inhibited, the expression of genes involved in the signalling of growth hormones (AUX, GA, BR, and CK) is repressed, while the expression of stress/growth inhibiting hormones (ABA, JA, and SA) is upregulated (Dong et al., 2015). Although these findings demonstrated the existence of a relationship between TOR, GA, and CK signalling, the role of TOR in GA and CK-mediated immunity remains unclear.

CK is a plant hormone that regulates many aspects of plant growth and development including cell division, leaf senescence, apical dominance, vascular differentiation, chloroplast biogenesis, root development and stress responses (Zürcher & Müller, 2016). Previous studies have shown that CKs have a role in plant response to biotic stresses in tobacco (Großkinsky et al., 2011), rice (Jiang et al., 2013), and tomato (Gupta et al., 2020). Several studies have reported that CKs promote resistance through the SA signalling pathway (Choi et al., 2010; Naseem et al., 2012). In tomato, we have previously shown that CK-deficiency results in higher susceptibility to the fungi *Botrytis cinerea* (*Bc*) and *Oidium neolycopersici* (*On*), while high endogenous CK content, as well as external application of CK, confer increased resistance against these fungi, in a SA-defendant manner (Gupta et al., 2020). Moreover, we have shown that CKs directly inhibit the growth, development, and virulence of fungal pathogens (Gupta et al., 2021), and that CKs improve *Xanthomonas*

campestris pv. Vesicatoria (*Xcv*) and *Pseudomonas syringae* pv. *tomato Pst* disease outcomes in tomato (Gupta et al., 2021).

GAs are growth-promoting phytohormones that play critical roles throughout the plant's life cycle, including stem elongation, germination, leaf expansion, flowering, and fruit development (Davière & Achard, 2013). GAs regulate growth by de-stabilising DELLA, a class of nuclear growth-repressing proteins that act as key regulators of GA signalling and inhibit GA responses by interaction with multiple transcription factors (Locascio et al., 2013). Binding of GA to its receptor GA INSENSITIVE DWARF (GID1) results in degradation of DELLA and activation of responsive genes in the GA signalling pathway (Harberd et al., 2009; Hauvermale et al., 2012). GAs regulate plant growth in response to environmental changes as well as nutrient availability (Colebrook et al., 2014). Previous works demonstrated that GAs act as negative regulators of JA signalling (Campos et al., 2016; Major et al., 2020).

The opposing effects of GA and CK on many aspects of plant growth and development, such as shoot apical meristem formation, shoot and root elongation, and cell differentiation, often lead to their perception as antagonists (Ezura & Harberd, 1995; Jasinski et al., 2005). For instance, treatment with CK reduces GA activity by downregulation of GA biosynthesis genes and upregulation of two DELLA genes, *GAI* and *RGA* (Brenner et al., 2005). In addition, (Greenboim-Wainberg et al., 2005) have shown that GA inhibits CK responses in Arabidopsis. The balance between CK and GA is maintained by three main proteins: KNOX, SPY and SEC. KNOX proteins induce CK biosynthesis while inhibiting GA biosynthesis and promoting GA deactivation. On the other hand, SPY and SEC repress GA signals and promote CK signals (Weiss & Ori, 2007). CK and GA have development-reciprocal relations, in which CK inhibits GA biosynthesis and promotes its deactivation by DELLA, and GA inhibits CK response. Normal shoot apical meristem function requires high CK and low GA signals, whereas later developmental stages require the opposite: low CK and high GA signals (Weiss & Ori, 2007). Considering that TOR and CK were both implicated in SA-dependent plant responses to pathogens, it appears possible that TOR and CK may interact or share similar defense pathways.

Here, we assessed the involvement of TOR in the mediation of GA and CK signals in both immunity and development. By inhibiting or downregulating TOR, we observed partial rescue of abnormal development and defense phenotypes caused by imbalanced CK or GA levels. Our findings suggest that TOR plays a role in the mediation of developmental and defense signals originating from the balance between CK and GA.
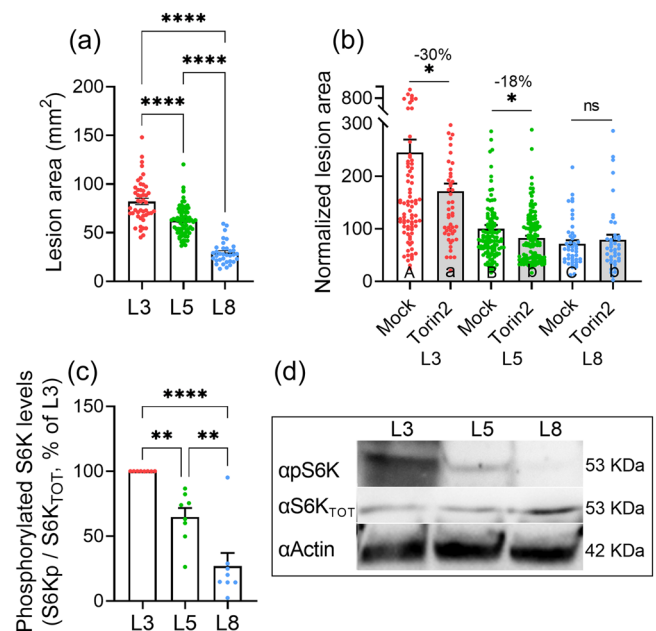
FIGURE 1 *TOR* activity and *TOR*-inhibition-mediated disease resistance depend on leaf developmental stage. (a) Leaves 3 (L3), 5 (L5), and 8 (L8) from *Solanum lycopersicum* cv. M82 5-week-old plants, were infected with *B. cinerea*. Asterisks denote statistical significance among indicated samples in Welch's analysis of variance (ANOVA) with Dunnett's post hoc test, $N > 35$, ****$p < 0.0001$. Bars represent mean ± SEM, all points shown. (b) Different leaves as indicated from *S. lycopersicum* cv. M82 5-week-old plants, were treated with Mock (1:5000 DMSO in DDW), or 2 μM Torin2. Plants were challenged with *Botrytis cinerea* (*Bc*) mycelia from a 72 h old culture, 24 h after treatment. Bars represent mean ± SEM, all points shown. Experiments were repeated three independent times. Asterisks indicate statistically significant decreases in *B. cinerea* infection upon Torin2 treatment as compared with Mock treatment, and letters indicate statistically significant differences among samples, upper case letters for Mock treated genotypes and lower case letters for samples treated with Torin2, in Welch's ANOVA with Dunnett's post hoc test, $N > 40$, *$p < 0.05$, ns, non-significant. Percentage of disease reduction is indicated above asterisks. (c and d) Total cellular proteins were prepared from the indicated leaves of *S. lycopersicum* cv. M82 5-week-old plants. TOR activation was expressed as the ratio between phosphorylated S6K and total S6K, detected using specific antibodies. Actin was detected as an additional control. Experiment was repeated four times with two biological repeats of two individual plants in each experiment, $N = 8$. Bars represent mean ± SEM, all points shown. Asterisks indicate statistically significant differences among indicated samples in one-way ANOVA with Tukey's post hoc test, **$p < 0.01$, ****$p < 0.0001$. (d) Representative blots. DMSO, dimethyl sulfoxide; TOR, Target of Rapamycin.

2 | RESULTS

2.1 | TOR-inhibition mediated immunity depends on leaf developmental stage

Age has been previously linked to alterations in disease susceptibility (Goss & Bergelson, 2006). To examine *Botrytis cinerea* (*Bc*) susceptibility in the context of "leaf developmental age", we first compared *Bc* sensitivity across different aged leaves on the same plants. In tomato, leaves follow a well described developmental programme, and differentiate as they mature (Shleizer-Burko et al., 2011). We found that as leaves differentiate, they become more susceptible to *Bc* (Figure 1a).

In our previous work, we found that downregulation or specific inhibition of TOR promotes immunity through the SA pathway (Marash et al., 2022). Since TOR has been implicated in development-defense tradeoffs, and we observed that disease susceptibility depends on leaf developmental stage (Figure 1a), we tested whether TOR-inhibition mediated immunity could be dependent on leaf developmental stage. We used Torin2, a potent ATP-competitive inhibitor of TOR activity in plants (Shi et al., 2018; Xiong et al., 2013; Ye et al., 2022) to inhibit TOR. We previously demonstrated that disease reduction observed with Torin2 is due to plant *TOR* inhibition, with nonspecific effects on additional plant kinases or on *B. cinerea* *BcTOR* being minimal (Marash et al., 2022). While Torin2 reduced *Bc*-induced disease in leaves 3 and 5 (L3 and L5), disease levels in leaf 8 (L8) treated with Torin2 were similar to those observed in untreated leaves (Figure 1b). This finding demonstrates that the extent of TOR-mediated immunity increases with leaf developmental age. This could be dependent on TOR activity, therefore, we examined TOR-kinase activity throughout leaf maturation, by analysing the phosphorylation status of S6 kinase 1 (S6K1), a conserved TOR substrate that has been previously used as an indication of TOR activity in plants (Cao et al., 2019; Li et al., 2017; Liu et al., 2021; Song et al., 2022; Xiong & Sheen, 2012; Ye et al., 2022). The antibodies were tested and found to detect total and phosphorylated S6K tomato proteins (Supporting Information: Figure S1). TOR activity was lowest in L8, the youngest, most developmentally morphogenetic leaf, and gradually increased with age, with the highest activity observed in the mature, no longer morphogenetic L3 (Figure 1c,d). This is in agreement with an earlier report demonstrating that TOR's activity level increases with leaf age in Arabidopsis (Brunkard et al., 2020). Thus, when TOR activity is high, as in L3, TOR inhibition results in disease resistance, and when TOR activity is low, as in L8, TOR inhibition does not promote disease resistance (Figure 1).

Our previous work found that CK can confer disease resistance (Gupta et al., 2020). Since young leaves undergoing morphogenesis are usually high in CK and low in GA, while differentiated organs have an opposite CK/GA balance (Israeli et al., 2021; Shwartz et al., 2016), we hypothesised that the reduction in *Bc* disease susceptibility in younger leaves could relate to the CK/GA ratio. To test this hypothesis and to better understand the mechanisms that may confer resistance to plants with high CK levels, we investigated *Bc* susceptibility in leaves of different developmental stages in genotypes with altered CK/GA ratios. We used the following genotypes, all in the M82 background: *pBLS»IPT7*, which contains elevated endogenous levels of CK (Shani et al., 2010) referred to hereinafter as "*pBLS»IPT*" or "*IPT*"; *clausa*, which has increased CK sensitivity coupled with decreased CK content (Bar et al., 2016), referred to hereinafter as "*clausa*" or "*clau*"; *pFIL»CKX3*, which has reduced CK levels (Shani et al., 2010; Shwartz et al., 2016), referred to hereinafter as "*pFIL»CKX*" or "*CKX*"; *pFIL»GFP-PROΔ17*, which has low GA signalling, referred to hereinafter as "*pFIL» PROΔ17*" or "*PROΔ17*" (Nir et al., 2017); *ga20ox3*, which is predicted to have reduced GA levels; and *procera^AGRAS*, referred to hereinafter as "*procera*" or "*pro*", which has increased GA signalling (Livne et al., 2015). All the genotypes used in this study are detailed and justified in Table 1 in the materials section. As shown in

Supporting Information: Figure S2A, when compared with the background line M82, genotypes with high CK/GA ratio (*IPT*, *clausa*, *ga20ox*, and *PROΔ17*) exhibit significantly higher resistance to *Bc*, whereas genotypes with low CK/GA ratio (*CKX*, *pro*) are significantly more sensitive to *Bc*. This finding is in agreement with our and other previous studies (Bari & Jones, 2009; De Bruyne et al., 2014; Gupta et al., 2020; Wang et al., 2013). To examine the connection between the CK/GA ratio and leaf developmental age-related resistance, we examined the age-related *Bc* sensitivity of genotypes with altered CK/GA ratios. We found that leaf developmental age-related resistance was preserved when the CK/GA ratio was low (*CKX*, *pro*), but abolished when the CK/GA ratio was high (*IPT*, *clausa*, and *PROΔ17*) (Supporting Information: Figure S2B), indicating that CK-mediated resistance supersedes and/or is the same as leaf developmental age-related resistance.

2.2 | TOR inhibition mediates disease resistance and immunity effected by the CK/GA ratio

CK-mediated resistance and downregulation of TOR both promoted immunity in tomato through the SA pathway (Marash et al., 2022). As it emerged from our results that the CK/GA ratio affects not only development but also disease resistance, we next turned to examine the connection between TOR and the CK/GA ratio in the context of immunity. We found that inhibiting TOR reduced *Bc* disease symptoms in the M82 background and in genotypes with low CK/GA ratio (*CKX*, *pro*). However, we did not observe any additional decrease in *Bc* disease level when TOR was inhibited in genotypes with high CK/GA ratios (*IPT*, *clau*, *ga20ox*, and *PROΔ17*) (Figure 2a). We confirmed these results using another TOR inhibitor, WYE132 (Supporting Information: Figure S3). We also silenced the expression of the tomato *SlTOR* gene by virus-induced gene silencing (VIGS), which reduces *SlTOR* transcription level by 50% in *TRV2:SlTOR* leaves (Marash et al., 2022), and observed similar results (Supporting Information: Figure S4). This suggests that TOR might be required to transmit the output of the GA/CK ratio in defense.

As differences in *Bc*-sensitivity could be due to changes in cellular immunity, we examined the effect of TOR inhibition on defense responses in genotypes with an altered CK/GA ratio. In mock-treated samples, consistent with our previous report characterising CK-mediated immunity (Gupta et al., 2020), flg-22 elicited ROS levels (as measured in relative luminescent units [RLU]) were higher in *IPT*, and lower in *CKX* plants (Figure 2c and Supporting Information: Figure S5). Flg-22 elicited ROS production was enhanced in the mock samples of the high CK/GA ratio genotype *PROΔ17*, and interestingly, in the low CK/GA ratio genotype *pro* (Figure 2c and Supporting Information: Figure S5). Torin2 treatment led to increased ROS production in M82 plants as previously reported (Marash et al., 2022) and in the low CK/GA ratio genotype *CKX* (Figure 2c and Supporting Information: Figure S5). However, Torin2 did not affect any of the high CK/GA ratio genotypes *IPT*, *ga20ox3*, or *PROΔ17* (Figure 2c and Supporting Information: Figure S5). We did not observe any significant change in the low
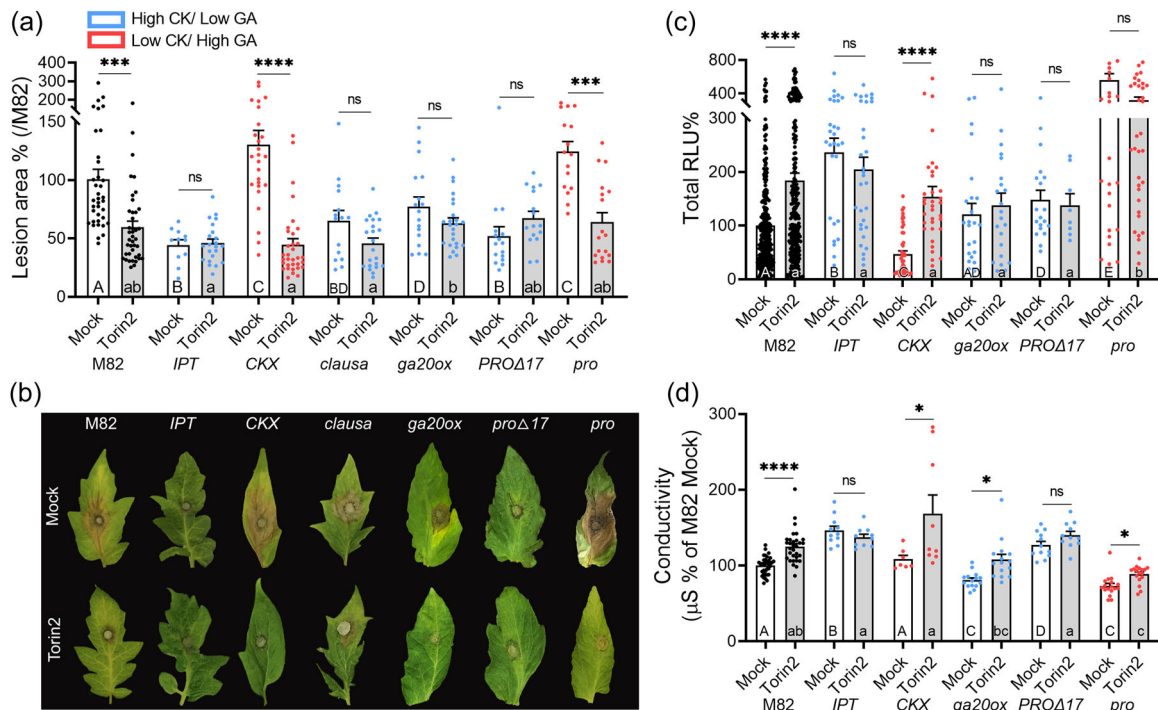
FIGURE 2 TOR-inhibition-mediated disease resistance depends on the CK/GA balance. *Solanum lycopersicum* plants of altered CK/GA genotypes: increased CK content *pBLS»IPT7* ("*IPT*"), decreased CK content *pFIL»CKX3* ("*CKX*"), increased CK sensitivity and decreased GA sensitivity *clausa* mutant ("*clausa*"), decreased GA content mutant ("*ga20ox*"), decreased GA signalling *pFIL»proΔ17* ("*proΔ17*"), increased GA signalling *procera* ("*pro*") and their WT background M82, were treated with Mock (1:5000 DMSO in DDW), or 2 μM Torin2. Plants were challenged with *Botrytis cinerea* (*Bc*) mycelia from a 72 h old-culture 24 h after treatment. (a) *Bc* necrotic lesion size. Asterisks indicate statistically significant disease reduction upon Torin2 treatment when compared with Mock treatment. Different letters indicate statistically significant differences among samples, upper case letters for Mock treated genotypes and lower case letters for samples treated with Torin2, in a one-way ANOVA with a Tukey post hoc test, $N > 12$, $p < 0.044$ (***$p < 0.001$, ****$p < 0.0001$, ns, non-significant). Experiments were repeated six independent times. (b) Representative *Bc* infected leaf images. (c) Plants were challenged with the immunity elicitor flg-22 (1 μM) 24 h after Torin2 treatment. ROS production was measured immediately after flg-22 application every 3 min, using the HRP-luminol method, and expressed as Relative Luminescent Units (RLU). Average total RLU per treatment, expressed as % of M82 control, is plotted. (d) Conductivity as a result of wounding was measured 24 h after Torin2 treatment. Bars represent mean ± SEM, all points shown. Experiments were repeated three independent times. (c and d) Asterisks indicate statistically significant increases in ROS production or conductivity upon Torin2 treatment when compared with Mock treatment. Different letters indicate statistically significant differences among samples, upper case letters for Mock treated genotypes and lower case letters for samples treated with Torin2 in Kruskal–Wallis ANOVA with Dunn's post hoc test, $N > 20$, $p < 0.0001$. ANOVA, analysis of variance; CK, cytokinin; DMSO, dimethyl sulfoxide; GA, gibberellin; ROS, reactive oxygen species; TOR, Target of Rapamycin.

CK/GA ratio genotype *pro*, again, possibly due to extremely high initial ROS levels. Similar results were obtained when quantifying ion leakage in response to Torin2, with increases in the background M82 line and in the low CK/GA ratio genotypes *CKX* and *pro*, but not in the high CK/GA genotypes, apart from *ga20ox*3 (Figure 2d).

We continued to assess the effect of TOR downregulation on disease resistance in lines with an altered CK/GA ratio by examining susceptibility to the hemibiotrophic bacterial pathogen *Xanthomonas campestris* pv. *Vesicatoria* (*Xcv*), the causal agent of bacterial spot disease (Moss et al., 2007), and the obligate biotrophic fungus *Oidium neolycopersici* (*On*), the causal agent of powdery mildew in tomato (Jacob et al., 2008), upon silencing of *TOR*. We compared disease symptoms in L5 of *TOR*-silenced and non-silenced plants after inoculated with *Xcv* or *On*. Leaves of *TOR*-silenced M82 plants showed lower *Xcv* disease symptoms and exhibited a significant decrease in *On* disease severity in

comparison to non-silenced plants. With the exception of *CKX*, none of the genotypes displayed a significant reduction in *Xcv* disease symptoms upon silencing (Supporting Information: Figure S6A). In the case of *On* disease symptoms, *CKX* and *ga20ox* both showed a reduction in disease symptoms (Supporting Information: Figure S6B).
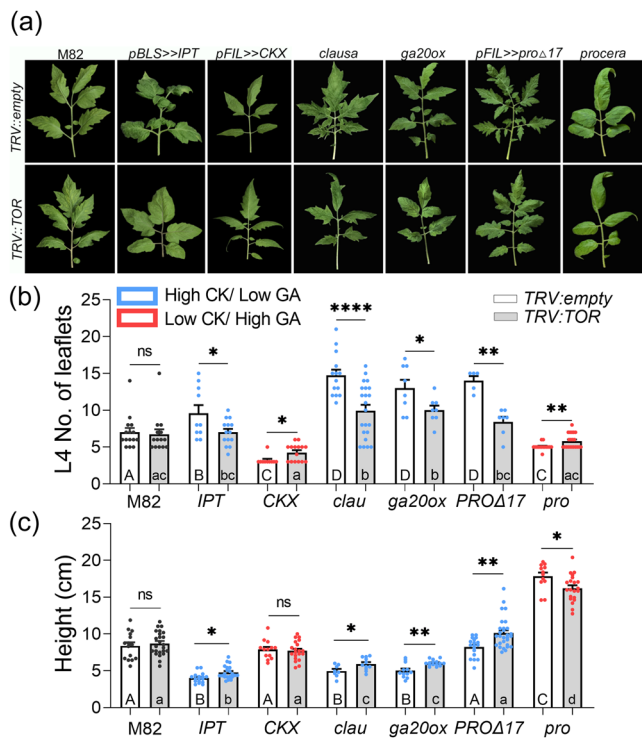
2.3 | TOR mediates CK-driven developmental cues

Transgenic tomato lines with altered leaf CK content have altered developmental programmes, resulting in quantifiable phenotypic changes in leaf development. *pBLS»IPT7* has significantly more complex leaves, while *pFIL»CKX3* has significantly simpler leaves, when compared with their M82 background (Shani et al., 2010). Likewise, *pFIL»PROΔ17* and *clausa* have more complex leaves (Israeli et al., 2021), while *pro*^ΔGRAS has

simpler leaves in comparison to the M82 background (Livne et al., 2015). To investigate whether TOR plays a role in GA and CK-mediated leaf development, we compared the leaf complexity of lines with different GA and CK levels upon *TOR* silencing. Interestingly, while the leaves of the WT M82 plants did not show any significant developmental changes in response to *TOR* silencing, we observed a reduction in leaf complexity in the highly complex *IPT*, *clausa*, *ga20ox3*, and *PROΔ17* plants, and an increase in leaf complexity in the simple-leaved *CKX* and *pro* plants (Figure 3a,b), suggesting that TOR is required to execute the developmental cues generated by CK and GA. In addition, *TOR* silencing significantly promoted shoot length (plant height) in *IPT*, *clau*, *ga20ox3* and

(a)

(b)

(c)

FIGURE 3 The CK/GA balance governs effects of *TOR* silencing on leaf development. *Solanum lycopersicum* plants of altered CK/GA genotypes: increased CK content *pBLS»IPT7* ("*IPT*"), decreased CK content *pFIL»CKX3* ("*CKX*"), increased CK sensitivity and decreased GA sensitivity *clausa* mutant ("*clau*"), decreased GA content mutant ("*ga20ox*"), decreased GA signalling *pFIL»proΔ17* ("*PROΔ17*"), increased GA signalling *procera* ("*pro*") and their WT background M82, were *TOR*-silenced using VIGS. 4 weeks after silencing, leaf complexity was quantified by counting the leaflets on leaf No. 4 (a and b), and height was measured (c). Experiment was conducted three times. Bars represent mean ± SEM, all points shown. Asterisks indicate statistically significant changes in leaf complexity (b) or plant height (c) upon *TOR* silencing, and different letters indicate statistically significant differences among samples, upper case for control-silenced and lower-case for TOR-silenced, in Welch's ANOVA with Dunnett's post hoc test, or in student's *t*-test with Welch's correction. (b) N > 5 individual plants, (c) N > 8 individual plants. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$, ns, non-significant. ANOVA, analysis of variance; CK, cytokinin; GA, gibberellin; ROS, reactive oxygen species; SEM, standard error of mean; TOR, Target of Rapamycin.

PROΔ17, while it reduced the height of *pro*. No significant difference in plant height was observed in *CKX*, which was similar in height to M82.

To further examine the importance of TOR in mediating hormonal signals leading to abnormal tomato leaf phenotypes, we down-regulated *TOR* in several classical tomato mutants, and examined the effect on leaf phenotypes. A dominant mutation in the TCP transcription factor *LA* (*LANCEOLATE*), known as *La2*, results in highly simple leaves, and overexpression of the *miR* that regulates *LA* expression, *miR319* (as in *pBLS»JAW*) causes highly complex leaves (Ori et al., 2007). *LA* was demonstrated to be involved in the regulation of the balance between CK and GA during leaf development (Israeli et al., 2021), and its activity is mediated in part by positive regulation of GA (Yanai et al., 2011) and negative regulation of CK (Efroni et al., 2013; Israeli et al., 2021). *CLAUSA* (Figure 3) and *LA* jointly regulate leaf development through the CK-GA balance (Israeli et al., 2021). The classical mutants *BIPPINATE* (*bip*) and *DOUBLE-DISSECTED LEAF* (*ddl*) were both previously found to be related to the KNOX-BELL machinery (Kimura et al., 2008; Nakayama et al., 2021). BELL proteins negatively regulate KNOX genes, and as such, can affect CK and GA levels (Bolduc & Hake, 2009; Jasinski et al., 2005; Sakamoto et al., 2001; Yanai et al., 2005). *POTATO-LEAF* (*c*) is a MYB transcription factor mutant shown to be involved in branching and boundary formation (Busch et al., 2011). *TOR* inhibition partially rescued the phenotypes observed in all these mutants (Supporting Information: Figure S7), demonstrating TORs involvement in the mediation of hormonal signals and execution of development, in accordance with previous studies (McCready et al., 2020).

2.4 | GA-response is mediated by TOR

Exogenous GA can affect developmental programmes and phenotypes (Jasinski et al., 2008; Sun, 2010). Given our results that TOR mediates hormonal signals, we hypothesised that *TOR* silencing would reduce GA sensitivity. Therefore, we next characterised the effect of treatments with different concentrations of GA on growth and immunity phenotypes of *TOR*-silenced plants. GA-treated plants showed a concentration-dependent increase in *B. cinerea* disease susceptibility (Figure 4a) and plant height (Figure 4b), and a reduction in leaf complexity (Figure 4c,d) in comparison with untreated plants, as previously described (Fleishon et al., 2011). In response to GA treatment, *TOR*-silenced plants showed a milder increase in disease susceptibility and plant height, and a milder reduction in leaf complexity, as compared with non-silenced plants (Figure 4a–d). *TOR*-silenced plants were more resistant to *B. cinerea* infection in the mock treatment, as previously described (Marash et al., 2022). Apart from plant height, which remained significantly differential among control and TOR-silenced plants, GA treatment at the highest concentration of 100 μM had similar effects on *TOR*-silenced and non-silenced plants (Figure 4). The reduced GA-sensitivity upon *TOR* silencing suggests that GA-response is mediated, at least in part, by TOR.
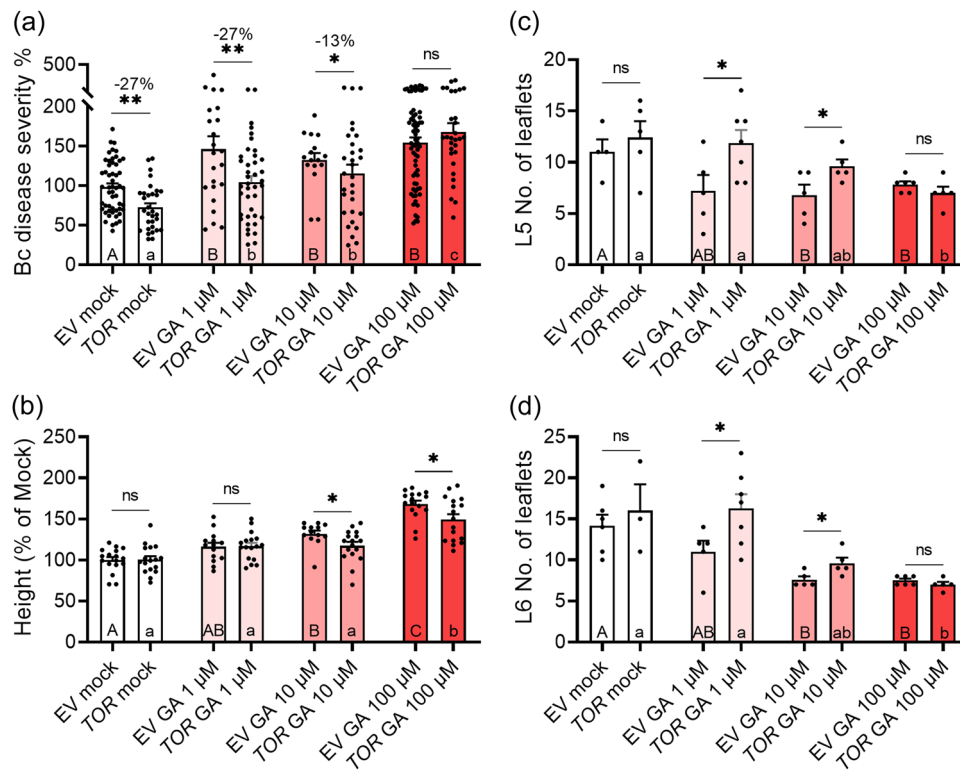
FIGURE 4 *TOR* silencing affects GA response. *Solanum lycopersicum* cv M82 plants were *TOR*-silenced using VIGS. One week after silencing, mock and *TOR*-silenced plants were treated with indicated concentration of GA3, three times a week, for 2 weeks, by spraying. 4 weeks after silencing, plants were challenged with *B. cinerea* (*Bc*) mycelia from a 72 h old-culture (a), height was measured (b), and leaf complexity was quantified by counting the leaflets on leaves 5 (c) and 6 (d). Experiment was conducted three times. Bars represent mean ± SEM, all points shown. Asterisks indicate statistically significant changes in *B. cinerea* disease (a), plant height (b), or leaf complexity (c and d) upon *TOR* silencing, and different letters indicate statistically significant differences among samples, upper case for control-silenced and lower-case for TOR-silenced, in Welch's ANOVA with Dunnett's post hoc test (a), one-way ANOVA with Bonferroni's post hoc test (b), or in student's *t*-tests with Welch's correction (c and d). (a) $N = 30$, (b) $N = 17$, (c) $N = 5$, (d) $N > 3$. *$p < 0.05$, **$p < 0.01$, ns, non-significant. ANOVA, analysis of variance; CK, cytokinin; GA, gibberellin; SEM, standard error of mean; TOR, Target of Rapamycin.

2.5 | TOR-inhibition-mediated immunity and CK-mediated immunity do not augment each other

To further examine the relationship between TOR inhibition and CK in plant defense, we assessed *Bc* disease sensitivity of M82 plants upon Torin2 and 6-BAP treatment. Plants were treated either with Torin2, 6-BAP, or both. As we previously reported (Gupta et al., 2020; Marash et al., 2022), both 6-BAP and Torin2 treatments promoted disease resistance, as lesion size was reduced by about 40% with either Torin2 or CK (Supporting Information: Figure S8A). Treatment with both Torin2 and 6-BAP, however, had no additive effect on disease resistance. To assess the effect of 6-BAP and Torin2 on plant defense responses, we analysed ROS accumulation and ion leakage with or without Torin2 and 6-BAP treatment (Supporting Information: Figure S8B,C). ROS accumulation and ion leakage were increased by 6-BAP and Torin2 treatments. However, no additive effect on the induction of defense responses was observed upon combined treatment with both Torin2 and 6-BAP, suggesting that TOR inhibition and CK likely promote disease resistance through overlapping pathways.

2.6 | TOR inhibition alters CK and GA pathway genes and reduces CK response

Since *TOR* inhibition reduced the sensitivity to signalling cues mediated by CK and GA, we analysed the effect of Torin2 on the expression of CK and GA metabolic and signalling genes. CK is synthesised by IPT enzymes (Kakimoto, 2001; Sakamoto et al., 2006; Takei et al., 2001), activated by LOG enzymes (Kurakawa et al., 2007; Kuroha et al., 2009), and perceived by a response regulator array (Argyros et al., 2008; Ishida et al., 2008). Type-A response regulators (*TRRs* in tomato) are known to be CK-responsive (Fleishon et al., 2011). The deactivation of CKs can happen either through conjugation or irreversible degradation by *Cytokinin oxidase/dehydrogenases* (*CKXs*) (Mok and Mok, 2001; Werner et al., 2006). The expression of the CK-responsive response regulator genes, *TRR3/4* and *TRR16B* was downregulated by Torin2 treatment in developing leaves and unaffected in mature leaves, while the expression of the CK inactivating genes *CKX2* and *CKX5* was upregulated in developing leaves and downregulated in mature leaves (Figure 5a,b). Expression levels of *TRR5/6/7* and *CKX6* were unaffected in both tissues.
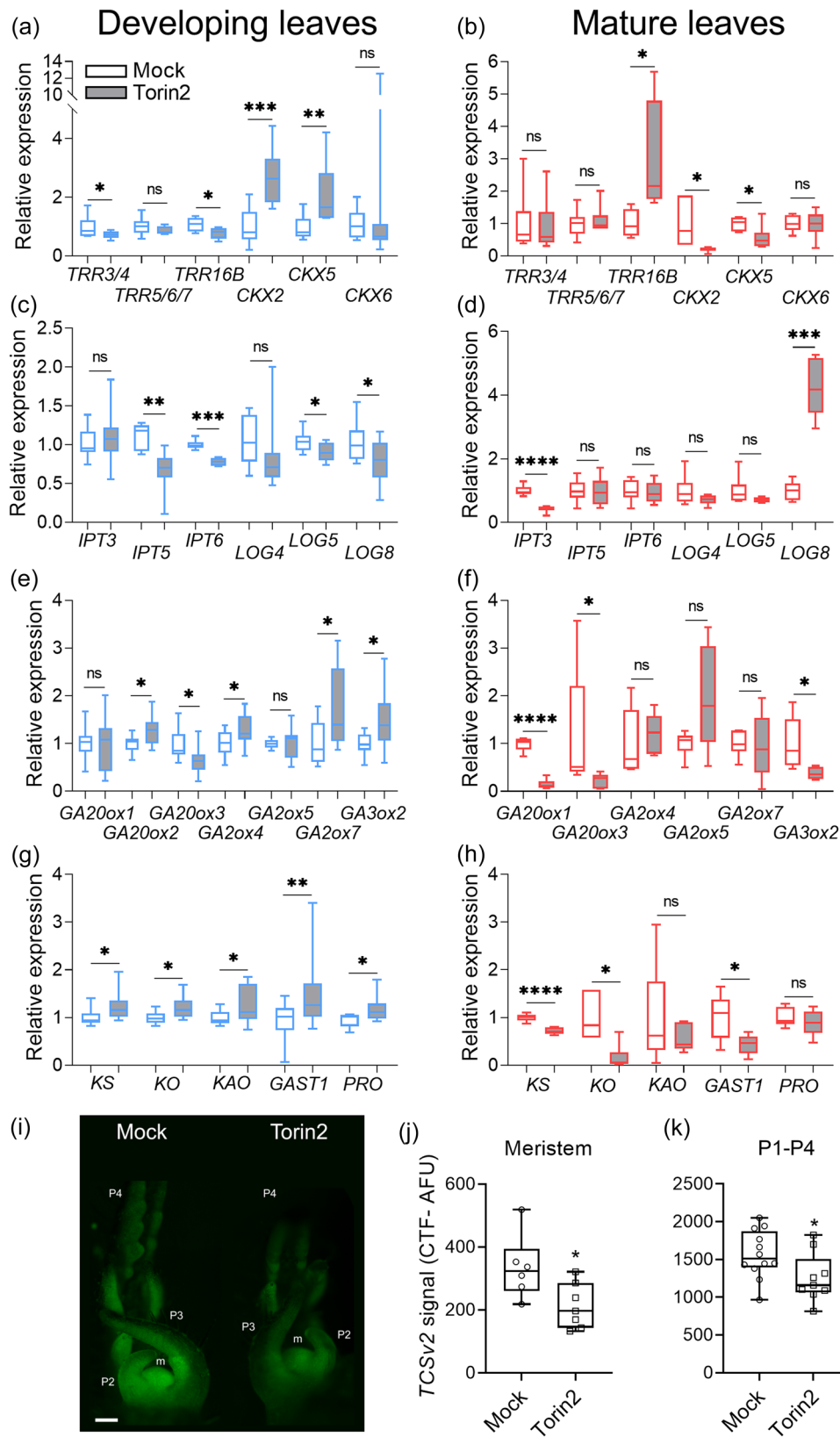
FIGURE 5 (See caption on next page).

Expression of the CK-biosynthesis genes *IPT5* and *IPT6* was down-regulated in developing leaves upon Torin treatment, and unaffected in mature leaves. *IPT3* was unaffected in developing leaves and downregulated in mature leaves (Figure 5c,d). Expression of the CK activating LOG enzymes *LOG5* and *LOG8* was reduced by Torin in developing leaves (Figure 5c,d). Torin also induced *LOG8* expression in mature leaves. *LOG4* was unaffected in both tissues. Overall, TOR inhibition causes CK biosynthesis, CK activation, and CK signalling, to be inhibited in developing leaves, and somewhat promoted (apart from *IPT3*) in mature leaves.

The production of GA from Geranylgeranyl diphosphate (GGDP) requires multiple enzymes, including *ent*-kaurene synthase (*KS*), *ent*-kaurene oxidase (*KO*), *ent*-kaurenoic acid oxidase (*KAO*), GA20 oxidase (*GA20ox*), and GA3 oxidase (*GA3ox*). The GA metabolic genes *GA20ox* and *GA3ox* convert precursors of GA into its active form through a series of oxidation reactions, while the *GA2ox* genes are essential for inactivating GA (Hedden, 2020). We found that the expression of key genes involved in the biosynthesis of active forms of GA: *GA20ox2 and GA3ox2*, was upregulated by Torin2 in developing leaves (Figure 5e). *GA3ox2* was downregulated by Torin2 in mature leaves, and *GA20ox1* was unaffected in developing leaves and downregulated in mature leaves, while *GA20ox3* was down-regulated in both developing and mature leaves (Figure 5e,f). The expression of the GA-inactivating enzymes *GA2ox4* and *GA2ox7* was upregulated in developing leaves and unaffected in mature leaves (Figure 5e,f). The expression levels of genes involved in early GA biosynthesis, *KS*, *KO*, and *KAO*, were elevated by Torin2 in developing leaves, and reduced in mature leaves (except for *KAO* which was unaffected in mature leaves) (Figure 5g,h). Likewise, the expression of the GA response and signal transduction genes *PROCERA* and *GAST1* (Shi & Olszewski, 1998) was upregulated in developing leaves, while *GAST1* was downregulated in mature leaves (Figure 5g,h). Overall, TOR inhibition causes GA biosynthesis, GA activation, and GA signalling, to be promoted in developing leaves, and inhibited in mature leaves. These results suggest the existence of a feedback regulatory mechanism between GA, CK and TOR that may help plants to fine-tune the level of these hormones upon pathogen attack.

To further examine the effect of TOR inhibition on CK signalling in young developing shoots, we used the CK-response transgenic reporter line *pTCSv2::3xVENUS*, which expresses *VENUS* under the control of the CK-responsive synthetic promoter TCSv2 (Steiner et al., 2020, 2016; Zürcher et al., 2013). We found that in the presence of Torin2, there is a reduction in CK signalling. Torin treated *pTCSv2::3xVENUS* shoots showed a significant reduction in VENUS signal relative to mock-treated shoots, in the meristem (Figure 5i,k) and four youngest leaf primordia (Figure 5i,l). Similar results were achieved in TCS-expressing plants in which *SlTOR* was silenced by VIGS (Supporting Information: Figure S9). This aligns with the reduction in *TRR3/4*, *TRR16B*, *IPT5*, and *IPT6*, and the increase in *CKX2* and *CKX5* expression in developing leaves upon Torin2 treatment (Figure 5a,c).

2.7 | CK and GA can affect TOR activity

To further investigate a possible feedback regulatory mechanism between GA, CK, and TOR, we examined whether CK and GA modulate TOR activity. We treated plants with 100 µM GA and CK, separately or simultaneously, and tested the phosphorylation status of S6K1 after 4 h, in both developing and mature leaves. As shown in Figure 6, exogenous CK treatment reduced the phosphorylation of S6K1 by TOR in both developing and mature leaves, suggesting that CK may negatively regulate TOR activity. By contrast, GA treatment, as well as GA and CK co-treatment, significantly increased the level of TOR-phosphorylated S6K in developing and mature leaves in comparison to mock, suggesting that GA can inhibit CK-mediated reduction of TOR activity. GA was previously reported to inhibit CK activity in leaf development upon co-treatment of both hormones at 100 µM (Fleishon et al., 2011).

FIGURE 5 *TOR* inhibition alters CK and GA pathway gene expression and reduces CK response in the meristem of young shoots. (a–h): Gene expression analysis of the indicated CK (a–d) and GA (e–h) pathway genes, with and without Torin2 (2 µM) treatment, was measured by RT-qPCR. 1:5000 DMSO in DDW served as Mock. Relative expression was calculated using the geometric mean of the gene copy number obtained for three reference genes, and normalised to the expression following Mock treatment. Developing leaves consisted of 3-week-old shoot apexes with six youngest primordia, mature leaves were the fifth leaf of 6-week-old plants. The following normaliser genes were used: for developing leaves: *RPL8* (Solyc10g006580), *EXP* (Solyc07g025390), and *CYP* (Solyc01g111170), and for mature leaves, *RPL8*, *CYP*, and *Actin* (Solyc11g005330). Analysis was conducted on six individual plants. Boxplots represent inner quartile ranges (box), outer quartile ranges (whiskers), median (line in box). Asterisks indicate significant differential regulation upon Torin2 treatment in Welch's *t*-test comparing each gene, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ns, non-significant. (a and b) Tomato response regulators (TRRs) and CK oxidases (CKXs). (c and d) Iso-pentenyl transferases (IPTs) and lonely-guy CK activating enzymes (LOGs). (e and f) GA20, GA2, and GA3 oxidases. (g and h): GA biosynthesis upstream enzymes (KA, KO, and KAO) and responsive genes (GAST1 and PRO). (i–k) *S. lycopersicum* cv. M82 3-week-old seedlings expressing VENUS driven by the cytokinin-responsive promoter TCSv2 were treated with Torin2 (2 µM) or Mock (1:5000 DMSO in DDW) for 48 h. (i) Typical Mock treated and Torin2 treated shoots are depicted. Images captured under identical conditions. The meristem (m), second (P2) third (P3) and fourth (P4) youngest leaf primordia are indicated. Bar: 1000 µM. TCSv2-driven total Venus fluorescence in the meristem (i and j) or leaf primordia (P1–P4) (i and k) was measured as corrected total fluorescence (CTF), in images captured under identical conditions. Boxplots represent inner quartile ranges (box), outer quartile ranges (whiskers), median (line in box), all points shown. Asterisks indicate significant TCSv2-driven signal reduction upon Torin2 treatment in an unpaired two-tailed *t*-test, N > 7, *$p < 0.05$. ANOVA, analysis of variance; CK, cytokinin; DMSO, dimethyl sulfoxide; GA, gibberellin; SEM, standard error of mean; TOR, Target of Rapamycin.
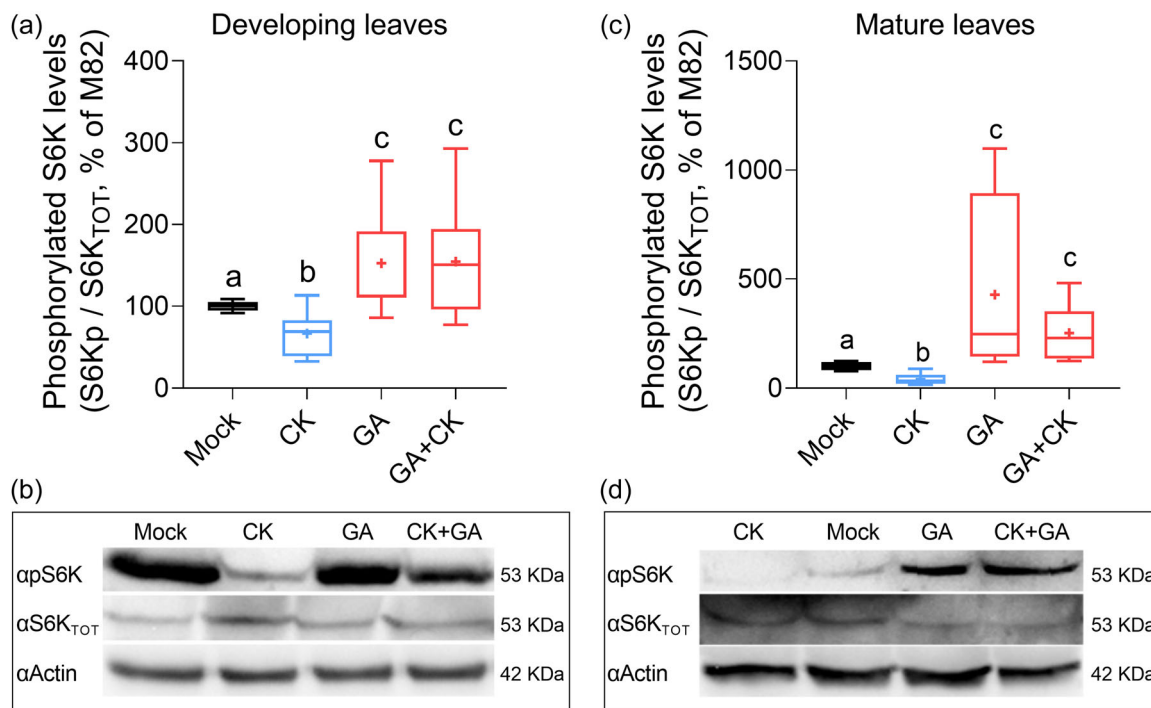
FIGURE 6 CK and GA affect TOR activation. *Solanum lycopersicum* cv. M82 6-week-old plants were treated with Mock (10 μM NaOH), 100 μM of the CK 6-benzylaminopurine (6-BAP), or 100 μM of GA3. 24 h after treatment, total cellular proteins were prepared from developing (a and b) and mature (c and d) leaves. Developing leaves consisted of 3-week-old shoot apexes with six youngest primordia (10–12 plants per biological repeat), and the fifth leaf of six individual 6-week-old plants was used for mature leaves. TOR activation was expressed as the ratio between phosphorylated S6K and total S6K, detected using specific antibodies. Actin was detected as an additional control. Experiment was repeated two independent times. (a and b) For developing leaves, each experiment consisted of four biological repeats of 7–10 plants each, $N = 8$. (c and d) For mature leaves, each experiment consisted of three biological repeats of three individual plants each, $N = 6$. Boxplots represent inner quartile ranges (box), outer quartile ranges (whiskers), median (line in box), mean ("+" sign). Different letters indicate statistically significant differences among samples in a Mann–Whitney U test, (a) $p < 0.045$, (c) $p < 0.0019$. ANOVA, analysis of variance; CK, cytokinin; GA, gibberellin; TOR, Target of Rapamycin.

3 | DISCUSSION

The TOR pathway senses many different inputs such as changes in cellular energy status, hormone levels, light, and abiotic or biotic stresses, to regulate growth, metabolism, transcription, and translation (Dobrenel et al., 2016). Various reports have suggested that TOR balances between growth and defense responses in plants (Caldana et al., 2019; De Vleesschauwer et al., 2018; Margalha et al., 2019; Ryabova et al., 2019). Previous work, for example, indicated that in plants, TOR acts as a molecular 'switch' at the intersection of growth and defense, and activates cell proliferation and plant growth at the expense of defense (De Vleesschauwer et al., 2018). Consistent with this, we have recently reported that plant immunity and defense responses are enhanced upon TOR downregulation in tomato (Marash et al., 2022).

3.1 | A TOR-CK/GA circuit mediates plant immunity

Despite the extensive research on the role of GA signalling in plant growth and development, there has been limited study on its role in

plant defense responses (Bari & Jones, 2009; Wang et al., 2013). Previous works in Arabidopsis show that treatment with GA increases resistance to (hemi)biotrophic bacterial pathogens, but reduces resistance to necrotrophic pathogens (Navarro et al., 2008), whereas in rice, it increases resistance to necrotrophic pathogens, and reduces resistance to (hemi)biotrophic pathogens (de Vleesschauwer et al., 2012, 2016; Qin et al., 2013; Yang et al., 2008). This implies that the effect of GA on plant immunity depends on both the host plant, and the identity of the pathogen involved (De Bruyne et al., 2014). As shown here (Figure 4), GA likely affects plant immunity in a similar manner in tomato and *Arabidopsis*. Our data suggest that immunity mediated by CK or GA requires inhibition of TOR activity for execution. This could also explain why TOR inhibition did not further enhance defense responses or *Bc* resistance when combined with CK application (Supporting Information: Figure S8). In tomato, TOR inhibition (Marash et al., 2022) or high endogenous CK levels (Gupta et al., 2020), promote pathogen resistance in an SA-dependent manner. Thus, a possible mechanism that could explain our results is that TOR and CK signalling pathways coordinately regulate plant defense responses through the modulation of SA. We hypothesise that the cross-talk between TOR and CK signalling could be involved in the ability of plants to modify growth

and developmental programmes upon pathogen attack, and thus enable a faster activation of plant defense responses. Conversely, the result that TOR downregulation decreased *Bc*-sensitivity in high GA (or low CK) genotypes or upon exogenous GA treatment, whereas it had no significant effect in low GA (or high CK) genotypes, suggests that TOR and the CK/GA pathways might share signalling components.

3.2 | TOR mediates the leaf developmental programme

Leaf development relies on the balance between GA, which promotes differentiation, and CK, which promotes morphogenesis (Hay et al., 2005; Jasinski et al., 2005; Yanai et al., 2005). Thus, CK and GA have a partially antagonistic role in leaf development (Bar et al., 2016; Fleishon et al., 2011). Generally, GAs are considered differentiation-promoting hormones which help to complete developmental programmes and regulate the achievement of final organ forms. GA shortens the morphogenetic stage of leaf development by promoting differentiation (Shwartz et al., 2016). The termination of the juvenile phase is associated with an increase in the levels of endogenous GA. This suggests that GAs promote the transition from a juvenile- or developing- state, to an adult- or differentiated- state (Andrés et al., 2014). On the other hand, CKs are known to alter leaf development and morphology (Hay & Tsiantis, 2010; Shani et al., 2010; Werner et al., 2003), and are regarded as factors that promote "juvenility" by promoting morphogenesis and delaying differentiation and senescence (Shwartz et al., 2016). Dividing tissues in the leaf have the highest levels of cytokinin, while bioactive gibberellins peak at the transition zone between the division and expansion zone (Nelissen et al., 2012).

It has been suggested that active growth and cell-cycle progression are required for the formation of the hormonal axis, involving auxin and cytokinin, which is required for organ formation and patterning during plant development (Du et al., 2018). Accordingly, several studies reported that TOR plays a role during leaf development in *Arabidopsis*. For example, *TOR* downregulation has been shown to result in the production of smaller leaves with fewer cells (Caldana et al., 2013) whereas *TOR* overexpression results in the production of bigger leaves with larger cells (Deprost et al., 2007). Likewise, mutation in *AtLST8*, a member of the TOR complex, results in a reduction in the number of leaves and in leaf size (Moreau et al., 2012), and mutation in *AtRAPTOR1B*, another component of the TORC1 complex, stalls leaf initiation (Anderson et al., 2005). By contrast, we did not observe any significant phenotypic alterations in the tomato WT M82 cultivar upon *TOR* silencing. This could be ascribed to the different inhibition methods used, or the different plant species. While TOR inhibition might affect the translation of the ectopically expressed proteins in transgenic lines, given similar results achieved with several mutants, this would be unlikely to explain our results, however, it should also be noted that TOR knockout is lethal, and our developmental analyses are limited by use of the VIGS

system. Improved systems to comprehensively study plant development upon TOR inhibition will no doubt emerge in the future.

Our work indicates that TOR is required for the developmental response to hormonal signals. Response to exogenous GA treatment, as well as the patterning of leaf organs programmed by CK and GA, were perturbed by TOR inhibition. These findings agree with a previous report demonstrating that mutants in *raptor*, a protein in the TOR complex, were less sensitive to exogenous GA treatment (Zhang et al., 2018). The increased leaf complexity in lines with a high CK/GA ratio, and decreased complexity in lines with a low CK/GA ratio, were both partially rescued to WT M82 levels as a result of *TOR* silencing (Figure 3). In general, TOR silencing partially rescued a variety of aberrant leaf developmental phenotypes (Figure 3 and Supporting Information: Figure S7). This brings forth the notion that TOR mediates signals from additional hormones, or that TOR is responsible for the reduction to practice of a variety of cues and signals generated by the balance and cross-talk of several developmental hormones (Greenboim-Wainberg et al., 2005; Israeli et al., 2021). Thus, TOR supports hormonal signal output, resulting in the typically observed leaf phenotypes. When TOR is inhibited, the signalling output from the aberrant hormonal balance in developmental mutants is no longer supported by TOR, resulting in milder phenotypes. These phenotypic changes could indicate that CK and GA distribution and/or signalling are altered in response to *TOR* silencing, or that factors which execute organ patterning downstream of hormonal cues are dependent on TOR status. It is therefore possible that TOR is required for the execution of a variety of cues that are integrated to form a cohesive leaf developmental programme. Notably, TOR inhibition reduced CK signalling and increased GA signalling in developing leaves, and promoted CK signalling and reduced GA signalling in mature leaves (Figure 5). Due to the lethality of complete TOR inhibition, and the limitations of the VIGS system, we were not able to assess a full leaf developmental time course upon TOR inhibition. However, we would expect TOR inhibition early in leaf development to result in precocious differentiation and simpler leaves, while TOR inhibition in late development could potentially lengthen the morphogenetic window.

The TOR pathway is involved in the regulation of translation and ribosome biogenesis in mammals and plants, and therefore its activity is tightly regulated (Pereyra et al., 2020). Interestingly, proteome analysis of CK activity in *Arabidopsis* demonstrated extensive differential regulation of ribosomal proteins in response to CK (Brenner and Schmülling, 2012). In another proteomic study, the functional classification 'Ribosome biogenesis' was found to be strongly differential in response to CK depletion or overproduction (Černý et al., 2013). Thus, the molecular mechanisms underlying the effect of CKs on leaf development and morphology could potentially be mediated by changes in translational processes due to differential regulation of ribosomal proteins. (Horiguchi et al., 2011) demonstrated that ribosomal proteins play a key role in *Arabidopsis* leaf development, supporting this notion. Thus, it is likely that TOR is involved in the execution of CK-mediated signals, in both defense

and development, by its direct or indirect regulation of proteins required for the execution of these processes.

3.3 | Immunity to *B. cinerea* depends on developmental status

The phenomenon in which developing leaves show stronger resistance than developmentally mature leaves (Figure 1 and Supporting Information: Figure S2) has been described as "leaf-stage associated disease resistance" (Berens et al., 2019; Develey-Rivière & Galiana, 2007; Xu et al., 2018). (Zhang & Chen, 2009), for example, showed that as tomato leaves age, they become more susceptible to diseases caused by *B. cinerea* and *Fusarium oxysporum*. In *Arabidopsis*, it was proposed that this resistance is due to a higher accumulation of SA in young leaves (Zeier, 2005). Our results suggest that high CK or low GA signals prevent the increase in *B. cinerea* susceptibility that normally occurs with developmental stage progression. As young organs have a higher CK/GA ratio than mature organs, and since the CK/GA balance, rather than the content or signal of each individual hormone, was reported to execute leaf developmental functions (Fleishon et al., 2011; Shani et al., 2006; Shwartz et al., 2016), it is possible that leaf developmental stage-related *B. cinerea* susceptibility may also depend on the CK/GA balance. This idea corresponds to previous work demonstrating that altered GA levels were not able to prevent the age-related decrease in JA-mediated immunity (Mao et al., 2017). Therefore, disease resistance related to developmental status could potentially be attributed to the CK/GA balance, and further, be mediated by TOR in a manner similar to that we observed for leaf developmental processes. Thus, it emerges from our results that TOR activity supports GA-mediated processes and reduces disease resistance, while a decrease in TOR activity supports CK-mediated processes and increases disease resistance.

3.4 | Increased *B. cinerea* resistance in young leaves could be a result of decreased TOR activity

TOR's low activity in young leaves (Figure 1) could account for their enhanced *Bc* resistance. The observation that CK application results in a decrease in TOR activation whereas GA application results in an increase in in TOR activation (Figure 6) may provide mechanistic insight into how resistance is affected by developmental status. Disease resistance could be decoded by the mediation of developmental hormone signals through TOR, suggesting that disease resistance may be dependent on TOR activity as well as the relationships between hormonal signals. Additionally, this implies the existence of a feedback mechanism that balances between TOR activity and these hormonal pathways. It is noteworthy that previous work in *Arabidopsis* cell suspensions revealed that kinetin and auxin both induced the phosphorylation of AtS6k (Turck et al., 2004), whereas the activation of TOR in *triticum aestivum* was induced

during GA-triggered germination (Smailov et al., 2020) Our data demonstrate that the ability of CK to induce immunity is related to TOR status (Figure 1 and Supporting Information: Figure S2), and that increased CK leads to a reduction in TOR activity (Figure 6). Our results align with those obtained in wheat, and differ from those obtained in *Arabidopsis*, possibly due to the use of cell suspensions, or due to differences among plant hosts.

### 3.4.1 | The involvement of TOR in growth-defense tradeoffs

Phytohormones mediate both development and defense responses, likely serving as mediators of the tradeoff between growth and defense (Berry & Argueso, 2022). Recent studies demonstrating that growth and defense can be uncoupled, suggest that resource reallocation toward immunity is not the sole factor governing growth inhibition during defense (Campos et al., 2016; Kliebenstein, 2016).

Leaves become more susceptible to necrotrophic diseases with developmental ageing (Figure 1). This increased susceptibility appears to be largely influenced by the CK/GA ratio, which correlates with leaf developmental stage. It is tempting to speculate that this might be a mechanism by which old plants, in which the CK/GA ratio is very low, die and allow for the allocation of resources to their offspring. Thus, the change in CK/GA ratio in mature plants is translated into defense hormonal outputs that modulate plant immunity.

Following our findings, we propose a hypothetical model by which TOR modulates CK and GA signalling and acts as a mediator of both developmental and defense processes, potentially regulating development-defense trade-offs (Figure 7). Developing leaves have low TOR activity (Brunkard et al., 2020; Figure 1), a relatively high CK to GA ratio (Shwartz et al., 2016), and are resistant to *Bc* (Figure 1). Mature differentiated leaves have higher TOR activity, a lower CK to GA ratio, and are more sensitive to *Bc* (Figure 1). Our results suggest that developmental-status-related resistance could depend on processes by which TOR transduces signals derived from the CK/GA balance. In mature leaves under standard growth conditions, TOR activity is high (the CK/GA ratio is relatively low), and morphogenesis is largely concluded. The "price" for this is relative disease susceptibility. When pathogens attack, CK increases and GA decreases (Meldau et al., 2012), and TOR becomes less active (Margalha et al., 2019), promoting disease resistance. The "price" for this disease resistance could be an arrest of growth, until the pathogen is vanquished.

TORs involvement in both development and defense poises TOR as a prime regulator of tradeoffs between these two important aspects of plant life. Here, we demonstrate that CK and GA can regulate TOR activity, while TOR is required for the interpretation of defense and developmental signals originating from the CK/GA balance. The interaction between TOR, GA, and CK could potentially help plants mediate growth and defense tradeoffs to adapt to the environment, with TOR sensing integrating environmental cues and stresses with plant hormonal balances, potentially allowing gradual
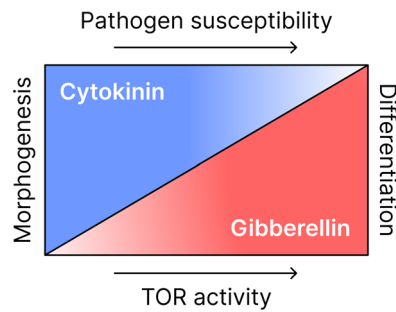
FIGURE 7 Model describing the interplay between the CK/GA balance and *TOR* in the regulation of leaf development and defense cues. Cytokinin promotes both morphogenesis and defense in leaves. Balanced CK/GA levels are required to achieve "normal" developmental patterning and disease resistance (Figure 2–3 and Supporting Information: Figure S2). In young leaves undergoing morphogenesis, CK signalling is high and TOR activity is low (Figures 1 and 5), resulting in decreased pathogen susceptibility (Figure 1 and Supporting Information: Figure S2). As leaves mature developmentally, CK signals are reduced and GA signals increase, the leaf morphogenetic potential declines as it differentiates, and TOR activity and pathogen susceptibility increase (Figures 1, 5 and Supporting Information: Figure S2). The CK/GA balance underlies both the morphogenetic potential and the disease susceptibility of the leaf. Inhibition of TOR results in rescue of altered CK/GA balances, partially restoring baseline developmental patterning and disease resistance (Figures 1–3, 5 and Supporting Information: Figures S6 and S7), and suggesting that TOR mediates these developmental and defense cues originating from the CK/GA balance. In the tradeoff between development and defense, high CK can cause downregulation of TOR (Figure 6), resulting in a shift towards defense (Figure 2 and Supporting Information: Figure S2), while high GA results in upregulation of TOR activity (Figure 6), and increased disease susceptibility (Figures 2 and 4). The switch between development and defense may be modulated by cross-talk between environmental sensing and TOR status.

shifts between growth and defense as a mechanism regulating plant robustness and survival under changing environments and pathogen loads. TOR mediation of the CK/GA balance likely occurs through the activity of as-of-yet-to-be-discovered target proteins. These targets potentially participate in tissue-dependent trade-offs between growth/development and defense. The relationship between TOR, GA, and CK appears to involve complex feedback mechanisms based on mutual regulation between these pathways. It will be interesting to investigate how different metabolic states and biotic and abiotic stresses alter the cross-talk between CK, GA, and TOR in the future.

4 | MATERIALS AND METHODS

4.1 | Plant material and growth conditions

Plants were grown in soil (Green 332; Even-Ari Green) in a growth chamber set to long-day conditions (16/8 light/dark) at 24°C, or in a greenhouse under natural day length conditions.

The genotypes used in this study are detailed in the Table 1. Promoter line selection and hormone content are explained below.

4.1.1 | Lines with altered GA signalling

The *N'*-terminal region of DELLA proteins contains the DELLA domain, which is required for the interaction with the GID1 receptor. Gain-of-function (GOF), dominant mutations in the DELLA domain block the interaction between DELLA and GID1 and prevent DELLA degradation (Locascio et al., 2013; Murase et al., 2008). The C'-terminal region of DELLA interacts with- and represses- multiple growth-promoting transcription factors (Locascio et al., 2013; Sun et al., 2012). Loss-of-function (LOF), recessive mutations in DELLA's C'-terminus are linked to constitutive GA responses (Achard et al., 2006, 2008; Nir et al., 2017). DELLA proteins promote Jasmonic Acid (JA) signalling and repress salicylic acid (SA) biosynthesis and signalling, promoting susceptibility to biotrophs and resistance to necrotrophs. A DELLA loss-of-function mutant (quadruple *della* mutant, lacking four out of the five *Arabidopsis* DELLA proteins) for example, was shown to be partially insensitive to gene induction by JA, whereas a DELLA GOF mutant *gai* (constitutively active dominant DELLA mutant) was shown to be more sensitive (Navarro et al., 2008). There is only one DELLA protein in tomato (Livne et al., 2015). We used the GA deficient mutant *ga20ox3* and the DELLA gain-of-function line *pFIL»GFP-PROΔ17* as lines with low GA signalling, and the DELLA loss-of-function mutant *procera^ΔGRAS* as a line with high GA signalling. The gain-of-function transgenic line *pFIL»GFP-PROΔ17* expresses the stable DELLA mutant protein *PROΔ17*, which lacks the DELLA domain. GA responses are constitutively suppressed in this line, resulting in a severe GA-deficient phenotype and GA insensitivity (Nir et al., 2017). *pro^ΔGRAS* is a *procera* null mutant that lacks the entire C'-terminal region of DELLA and exhibits enhanced GA responses (Livne et al., 2015). The GA-deficient mutant *ga20ox3* was generated using CRISPR/CAS9 essentially as described in (Israeli et al., 2019), using the same constructs and methodology. gRNA primers are detailed in Supporting Information: Table S1.

4.1.2 | Lines with altered CK signalling

We used the high endogenous CK content genotype *pBLS » IPT7*, which overexpresses the CK biosynthesis gene *ISOPENTYL TRANSFERASE 7 (IPT7)* and the low CK content genotype *pFIL» CKX3*, which overexpresses the CK degrading enzyme *CKX OXIDASE3 (CKX3)* (Shani et al., 2010). We also used the increased CK sensitivity and decreased GA sensitivity mutant *clausa*. CLAUSA (*CLAU*) is a MYB transcription factor that promotes the transition from morphogenesis to differentiation by negatively affecting CK signalling and promoting GA signalling (Bar et al., 2016; Israeli et al., 2021).

TABLE 1 Plant genotypes used in this study.

Genotype name	Source	Phenotype	References
Solanum lycopersicum cultivar M82		WT	
ga20ox3 null mutant Cas9 Knockout of the GA biosynthesis enzyme GA20OX3. M82 background line.	Prof. Naomi Ori	Short stature; increased leaf complexity.	This work
pTCSv2::3×VENUS Overexpression of VENUS driven by the synthetic two-component signalling sensor pTCSv2. M82 background line.	Bar lab	WT	Bar et al. (2016), Steiner et al. (2020)
pBLS»IPT7 ("*IPT*"): Transgenic overexpression of IsoPentenyl Transferase7 from the leaf *BLS* (Lifschitz et al., 2006) promoter. Elevated endogenous leaf levels of CK. M82 background line.	Prof. Naomi Ori	Highly complex rugose leaves; short stature; hirsutism; disease[n,b] resistance.	Bar et al. (2016), Gupta et al. (2020, 2021, 2022), Shani et al. (2010)
pFIL»CKX3 ("*CKX*")- Transgenic overexpression of Cytokinin Oxidase3 from the leaf *FIL* (Bonaccorso et al., 2012) *promoter*. Decreased endogenous leaf CK content. M82 background line.	Prof. Naomi Ori	Highly simple thin leaves; lack of hairy trichomes; disease[n,b] susceptibility.	Bar et al. (2016), Gupta et al. (2020, 2022), Shani et al. (2010)
clausa ("*clau*"): Recessive MYB TF mutant. High CK sensitivity coupled with low CK content; low GA sensitivity coupled with increased amounts of pre-active GAs. M82 background line.	Prof. Naomi Ori	Highly complex rugose leaves; hirsutism; disease resistance[n,b].	Bar et al. (2016), Gupta et al. (2020, 2022), Shani et al. (2010)
pFIL»GFP-PROΔ17: Transgenic overexpression of a mutated, unprocessed version of the DELLA TF Procera from the leaf *FIL* promoter. Low GA signal. M82 background line.	Prof. David Weiss	Complex leaves. Disease resistance[n,b] (this work).	Israeli et al. (2021), Nir et al. (2017)
procera: Recessive mutant in the DELLA TF Procera. ΔGRAS allele. High GA signal. M82 background line.	Prof. David Weiss	Simple leaves; tall stature. Disease susceptibility[n]/resistance[b] (this work; resistance[b] previously reported in *Arabidopsis*).	Livne et al. (2015), Navarro et al. (2008)

[n]Necrotrophic.

[b]Hemi/biotrophic.

4.2 | Rationale for genotype selection and reported CK and GA content in different genotypes

We used transgenic lines expressing genes of interest from the leaf specific promoters *FIL* and *BLS*. In tomato, the Arabidopsis *FIL* (filamentous flower) promoter drives expression throughout leaf primordia, starting from initiation (first plastochron), in initiating leaflets, and the abaxial side of the leaves. The *BLS* promoter drives expression later in leaf development, in primordia from about the fourth plastocrhon stage, and in young leaves (Lifschitz et al., 2006). Plants overexpressing *IPT* have been shown to contain increased levels of cytokinin many times, in various plant species (Márquez-López et al., 2019; Redig et al., 1996; Smigocki & Owens, 1988). Strongly increasing CK levels throughout the plant also led in some cases to undesirable phenotypes such as reduced apical dominance, increased lateralisation, late flowering, and infertility. Therefore, tissue-specific promoters were used to express *IPT* (Bartrina et al., 2011; Shani et al., 2010; Smigocki et al., 1993). This eliminated the undesired effects, and allowed plants to be viable and fertile, though mild effects of increased CK were occasionally observed in the non-targeted

organs as well. Plants overexpressing *CKX* have been shown to contain reduced levels of cytokinin many times, in various plant species. *CKX3* overexpression was specifically shown to cause a reduction in CKs in several works (Nishiyama et al., 2011; Reid et al., 2016). Reducing cytokinin levels with *CKX* overexpression led to stunting in Arabidopsis when expressed from the strong 35S promoter (Vercruyssen et al., 2011), but overexpression of *CKX3* in tomato had relatively minimal phenotypes under optimal conditions (Farber et al., 2016). The lines we used, which overexpress Arabidopsis *AtIPT7* or *AtCKX3* from the leaf-specific promoters *pFIL* and *pBLS*, in tomato cv M82, have normal early development and are viable (Shani et al., 2010). *pFIL»IPT7* is mostly infertile, which is why we used *pBLS»IPT7*, which has a milder phenotype, due to the expression being later in development, and normal fertility (Shani et al., 2010). *pFIL»CKX3* and *pFIL»GFP-PROΔ17* are both viable and fertile. We used lines with expression driven from the *FIL* promoter in both these cases because lines driven from the *BLS* promoter had mild to undetectable leaf phenotypes. Thus, *BLS* was used only in the case of IPT, to avoid pleiotropic effects. CK and GA content were previously analysed in the tomato *clausa* mutant. *clausa* is highly CK sensitive and GA

insensitive, and displays meristematic and leaf phenotypes similar to those of overexpression of *IPT*. In terms of hormonal content, *clausa* has a significant reduction in the content of many CK compounds, and a significant increase in several active GA precursors (Israeli et al., 2021). The *procera* mutant was reported to have decreased levels of the active gibberellin GA20 (Jones, 1987), indicating the presence of a feedback mechanism aimed at controlling the high increase in GA sensitivity displayed in this mutant, as is the case with CK content in the *clausa* mutant (Israeli et al., 2021). Hormonal content was not directly measured in *ga20ox3*, or, to the best of our knowledge, in *pFIL»GFP-PRO∆17*. While it is difficult to predict the actual hormonal content of different genotypes since feedback mechanisms are often present, we selected the different genotypes based on their developmental phenotypes which were as expected, mimicking phenotypes of high CK sensitivity/response and/or low GA sensitivity/response, as indicated.

4.3 | Leaf tissue collection

For leaves of different ages (Figure 1 and Supporting Information: Figure S2), leaves 3 (L3), 5 (L5), and 8 (L8) from 5 week-old-plants were selected for analysis as they possess significantly different morphogenetic windows in tomato (Shleizer-Burko et al., 2011), and are all formed before the formation of the inflorescence meristem from the shoot apical meristem in determinate *S. lycopersicum* cultivar "M82", meaning that they all represent vegetative growth.

For comparison between developing and mature leaves (Figures 5–6 and Supporting Information: Figure S9), for developing leaves, shoots were analysed from 3-week-old plants, and for mature leaves, L5 was analysed from 6-week-old plants.

4.4 | Torin2 and WYE132 treatments

It was previously demonstrated that Torin2 and WYE132 are effective and specific inhibitors of TOR (Li et al., 2017; Marash et al., 2022; Montané & Menand, 2013). Torin2 (SML1224 Sigma-Aldrich) or WYE132 (PZ0321 Sigma-Aldrich), were applied to detached tomato leaves through the petiole for 24 h before pathogen inoculation (both inhibitors), defense response quantification (only Torin2), or RNA preparation (only Torin2). For both inhibitors, a 10 mM stock solution was prepared in concentrated DMSO (P0037 SIGMA-Aldrich) and diluted to 2 µM in water. Mock leaves were treated with water containing 1:5000 of DMSO. Torin2 was used in this study in a concentration of 2 µM based on previous studies (Marash et al., 2022; Ye et al., 2022).

4.5 | Hormone treatments

For CK treatment (Supporting Information: Figure S5), plants were sprayed with 100 µM 6-benzyl purine (6-BAP, Sigma-Aldrich) or Mock solution 24 h before analysis. The stock solution was prepared in NaOH and diluted with water. Similarly diluted NaOH in water served as Mock.

For GA treatment (Figure 4), GA_3 (Sigma-Aldrich) in the indicated concentrations was dissolved in ethanol and applied by spraying three times a week for 2 weeks. The stock solution was prepared in ethanol and diluted with water.

For assessing TOR activity following hormonal treatment (Figure 6), 10 µM 6-BAP or GA_3, or both, were sprayed on developing leaves (shoots of 3-week-old plants) or mature L5 from 6-week-old plants, 4 h before protein extraction. Mock treatments were dilute NaOH for CK, or ethanol with Tween 20 (100 µL/L) for GA, as described above. Hormonal treatments for assessing TOR activity were given at the relatively high concentration of 100 µM in accordance with previously published data indicating that exogenous CK treatment has only mild effects in tomato (Fleishon et al., 2011).

4.6 | Virus-induced gene silencing (VIGS)

VIGS was performed as previously described (Liu et al., 2002). The *SlTOR* silencing construct was generated as previously described in Marash et al. (2022). The TRV2:TOR construct, as well as an empty TRV RNA2 for control, and the pTRV1 vector were introduced into *A. tumefaciens* strain GV3101::pMP90. The cultures were adjusted to $OD_{600} = 0.2$ and TRV RNA1 was mixed in at a ratio of 1:1 with RNA2 (either empty or *TRV2:TOR*) in infiltration buffer, and infiltrated into cotyledons of 10-day-old seedlings. Analyses were subsequently conducted when plants reached 5-6 weeks of age, except in the case of *pTCSv2*::3X VENUS, which was imaged 2 weeks after silencing.

4.7 | Imaging of the CK-response synthetic promoter *pTCSv2*::3×Venus

Stable transgenic M82 tomato *pTCSv2*::3×VENUS seedlings that express VENUS driven by the synthetic two-component signalling sensor *pTCSv2* (Bar et al., 2016; Steiner et al., 2020) were treated with Torin2 at 3-weeks-old or VIGS silenced at 10-days-old, as detailed in the corresponding methods. VENUS expression was analysed 48 h after treatment or 2 weeks after VIGS 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 | Pathogenesis assays

Pathogenesis assays were conducted on the fifth leaf of 5–6-week-old plants. *Botrytis cinerea* (*Bc*) pathogenicity assays were performed as previously described (Gupta et al., 2020). Briefly, inoculum of *Bc* isolate Bcl16 was maintained on potato dextrose agar (PDA; Difco) plates in an incubator at 22°C. Agar discs with a diameter of 0.4 cm were then pierced from colony margins and used to inoculate

detached leaves. Inoculated leaves were kept in a humid chamber at 22°C under long-day conditions. Necrotic lesion size was measured 2–3 days post-inoculation using ImageJ.

Xanthomonas campestris pv. vesicatoria (*Xcv*) was grown at 28°C in Luria Bertani (LB) broth overnight supplemented with Rifampicin (10 µg/mL) (Sigma), diluted to a concentration of $OD_{600} = 0.0002$ in 10 mM $MgCl_2$, and used to pressure-infiltrate L5 of silenced plants with a 1-mL needleless syringe. Disease was assessed by measuring the water-soaked lesion area as previously described (Teper et al., 2018), 10 days after inoculation.

Oidium neolycopersici (*On*) was continuously maintained by randomly placing healthy plants alongside pre-infected plants in a dedicated chamber, and allowing them to be inoculated through air circulation. New plants were introduced every 2 weeks, upon which wholly covered chlorotic plants were discarded. For inoculation, diseased leaves with 80% On coverage were shaken above L5 of each plant. Two infected leaves were used for each individual plant. Disease severity was calculated based on the percentage of the leaf surface covered with powdery mildew symptoms 10-14 dpi.

4.9 | ROS production measurement

Immunity assays were conducted on the fifth leaf of 5–6-week-old plants. ROS measurement was carried out as previously described (Anand et al., 2021; Leibman-Markus et al., 2017; Pizarro et al., 2018). 0.5 cm diameter leaf discs were collected, and each disc was incubated in 250 µL distilled water in a 96-well plate (SPL Life Science) at room temperature with gentle shaking. After 4 h, the water was removed and 50 µL of distilled water were added. Right before measurement, 100 µL of distilled water with or without 1 µM flg22 (PhytoTechLabs #P6622) were added. Light emission was measured using a luminometer (GloMax® Discover, Promega).

4.10 | Ion leakage (conductivity) measurement

Immunity assays were conducted on the fifth leaf of 5–6-week-old plants. Conductivity was measured as described (Anand et al., 2021; Leibman-Markus et al., 2017; Pizarro et al., 2018). 0.9 cm diameter leaf discs were harvested and washed with distilled water for 3 h in a 50 mL tube. For each sample, five discs were placed in a 10-flask with 1 mL of distilled water, with 2 µM Torin2, 6-BAP, or DMSO, for 48 h at room temperature with gentle shaking. After incubation, 1.5 mL of distilled water were added to each sample, and conductivity was measured using a conductivity meter (AZ® Multiparameter pH/Mv/Cond./Temp Meter 86505).

4.11 | RNA extraction and RT-qPCR

For RNA extraction, either five 0.9 cm diameter leaf discs were harvested from L5 of 6-week-old plants (Figure 5b,d,f,h) or whole

shoots of developing leaves (m+6) of 3-week-old seedlings with four true leaves (Figure 5a,c,e,g) were used. Isolation of total RNA was performed according to the TRI reagent (Sigma-Aldrich) procedure, with application of DNAse (EN0521 ThermoFisher) to remove genomic DNA. 1 µg of RNA was used for cDNA synthesis using Maxima reverse transcriptase (ThermoFisher). RT-qPCR assays were conducted with Power SYBR Green Mix (Life Technologies), using specific primers (Supporting Information: Table S1) in a Rotor-Gene Q machine (Qiagen). Standard curves were achieved by dilutions of one cDNA sample. Relative expression was quantified by dividing the expression of the relevant gene by the geometric mean of the expression of the following normaliser genes: for developing leaves: *RPL8* (Solyc10g006580), *EXP* (Solyc07g025390), and *CYP* (Solyc01g111170), and for mature leaves, *RPL8*, *CYP*, and *Actin* (Solyc11g005330). All primer pairs had efficiencies in the range of 0.97-1.03. All the primers used for RT-qPCR are listed in Supporting Information: Table S1. Assayed genes were selected based on their activity and predicted expression pattern, see Supporting Information: Table S2.

4.12 | Protein purification and western blot analysis

For protein purification from mature leaves, whole tomato leaves (Figure 6c,d) were harvested from 6-week-old plants with nine true leaves, and ground in liquid nitrogen. 150 mg ground tissue was used. For developing leaves (Figure 6a,b), 25 mg of tissue was collected from shoots of developing leaves (m+6) of 3-week-old seedlings with four true leaves. Each sample of developing leaves was composed of a total of 10–12 different plants. The tissues were then ground in liquid nitrogen with three volumes of extraction buffer (100 mM MOPS pH 7.6, 100 mM NaCl, 40 mM ß-MeOH, 5% SDS, 10% glycerol, 4 mM EDTA, 2 mM PMSF, and phosphatase inhibitor (Sigma)), boiled for 5 min at 95°C and centrifuged at 10 000 rpm for 10 min, to remove cell debris. Samples of equal volume were separated on 15% SDS acrylamide gels, transferred to nitrocellulose membranes (Protran, #10401380), stained with Ponceau red as loading and transfer control, and blocked with 3% skimmed milk in Tris-buffered saline (TBS) with 1% Tween20 for 1 h at room temperature with gentle shaking. Membranes were probed with Anti-S6K1 p-Thr449 polyclonal antibody (AB-ab207399, Abcam,1:750), Anti-S6K1/2 (GRS-AS121855, Agrisera, 1:500), or Anti-ACTIN (GRS-AS132640, Agrisera, 1:1000) overnight at 4°C. IgG HRP-conjugated goat-anti-rabbit (AB-ab205718, Abcam, 1:10 000) was used as a secondary antibody. Chemiluminescence was observed using Elistar Supernova as substrate (Cyanagen, #XLSE2) and images of protein bands were acquired and quantified using the Alliance UVITEC software. Phosphorylation status of S6K1 was tested 4 h after GA and CK treatment based on a previous study quantifying the decrease in TOR activity upon inhibition (Upadhyaya et al., 2020).

4.13 | Statistical analysis

All data are presented as average ±SEM, or as boxplots showing minimum to maximum values, with the box representing inner quartile ranges and the whiskers representing outer quartile ranges. Data sets were analysed for normality using the Shapiro–Wilk test. For non-Gausian distributed samples, differences between two groups were analysed for statistical significance using a Mann–Whitney U test, and differences between three groups or more were analysed using Kruskal–Wallis ANOVA with Dunn's post hoc test. For normally distributed samples, differences between two groups were analysed for statistical significance using a two tailed t-test, with Welch's correction for samples with unequal variances, where appropriate. Differences among three groups or more were analysed for statistical significance using one-way 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 was assessed using a post hoc test. Tukey's or Bonferroni's tests were employed for samples with equal variances, and Dunnett's test was employed for samples with unequal variances. All statistical analyses were conducted using Prism9™.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

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 is available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.