Cytokinin inhibits fungal development and virulence by targeting cellular trafficking

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Abstract

Cytokinin (CK) is an important plant developmental regulator, having activities in many aspects of plant life and its response to the environment. CKs are involved in diverse processes in the plant, including stem-cell maintenance, vascular differentiation, growth and branching of roots and shoots, leaf senescence, nutrient balance and stress tolerance. In some cases, phytopathogens secrete CKs. It has been suggested that to achieve pathogenesis in the host, CK-secreting biotrophs manipulate CK signaling to regulate the host cell cycle and nutrient allocation. CK is known to induce host plant resistance to several classes of phytopathogens from a handful of works, with induced host immunity *via* salicylic acid signaling suggested to be the prevalent mechanism for this host resistance.

Here, we show that CK directly inhibits the growth, development, and virulence of fungal phytopathogens. Focusing on *Botrytis cinerea (Bc)*, we demonstrate that various aspects of fungal development can be reversibly inhibited by physiological concentrations of CK. We also found that CK affects both budding and fission yeast in a similar manner. Investigating the mechanism by which CK influences fungal development, we found that CK affects cellular trafficking in *Bc*, lowering endocytic rates and endomembrane compartment sizes, likely leading to reduced growth rates and arrested developmental programs. Mutant analyses in yeast confirmed that the endocytic pathway is a CK target.

Our work uncovers a remarkably conserved role for a plant growth hormone in fungal biology, suggesting that co-evolution between pathogen and host resulted in fascinating molecular adaptations on fundamental processes in eukaryotic biology.

Importance

Cytokinins (CKs), important plant growth/ developmental hormones, have previously been associated with host disease resistance. Here, we demonstrate that CK directly inhibits the growth, development, and virulence of *B. cinerea* (*Bc*) and many additional phytopathogenic fungi. Molecular and cellular analyses revealed that CK is not toxic to *Bc*, but rather, *Bc* likely recognizes CK and responds to it, resulting in cell cycle and individual cell growth retardation, via downregulation of endocytic trafficking. Mutant analyses in yeast confirmed that the endocytic pathway is a CK target. Our work demonstrates a conserved role for CK in yeast and fungal biology, suggesting that co-evolution between fungal pathogens and plant hosts caused molecular adaptations in cellular trafficking, a fundamental process in eukaryotic biology.

Introduction

Cytokinins (CKs) are a class of extensively studied plant hormones, well known for their involvement in various aspects of plant life (1–4). CKs are involved in many physiological processes such as stem-cell control, vascular differentiation, senescence, chloroplast biogenesis, seed development, growth and branching of root, shoot and inflorescence, leaf senescence, as well as in nutrient balance and stress responses (5–8). Some findings have suggested a role for CKs in fungal pathogenesis (9–12). In some cases, plant pathogens can secrete CKs, or induce CK production in the host plant. It has been suggested that to achieve pathogenesis in the host, CK-secreting biotrophs or hemibiotrophs manipulate CK signaling to regulate the host cell cycle and nutrient allocation (13). Exogenous application of CK was reported to reduce plant infection of powdery mildew (10, 14), smut fungi (15), and viruses (16). Conidia, mycelia of some fungi, and germinating uredospores of *Puccinia graminis* and *P. recondite* have been shown to accumulate CK, manipulating CK signaling to regulate host plant cell cycle (17, 18).

CK has also been shown to promote resistance to plant pathogens that do not secrete CK. High levels of CKs were found to increase the plants' resistance to bacterial and fungal pathogens (19–24). Different mechanisms have been suggested for this enhanced resistance. In Arabidopsis, it was suggested that CK-mediated resistance functions through salicylic acid (SA) dependent mechanisms (21). An additional study suggested that CK signaling enhances the contribution of SA-mediated immunity in hormone disease networks (25). However, in tobacco, an SA-Independent, phytoalexin-dependent mechanism was suggested (22). We recently reported that CK induces systemic immunity in tomato (*Solanum lycopersicum*), promoting resistance to fungal and bacterial pathogens

(23, 24) including *Botrytis cinerea*, in an SA and ethylene dependent mechanism.

B. cinerea (grey mold), *Sclerotium rolfsii* (collar rot), and *Fusarium oxysporum* f. sp. *lycopersici* (fusarium wilt) are widespread fungal plant pathogens that infect hundreds of plant species and cause huge losses every year (26–28). More than 50% of these losses occur in field-grown cultivated crops if synthetic/chemical management is not applied (29). Therefore, large amounts of chemical pesticides are widely required for the management of fungal pathogens. However, the extensive use of synthetic/chemical pesticides results in serious environmental pollution (30), and expedites the appearance of pesticide resistant pathogens. To overcome this issue, there is a need to use chemicals that are natural, safe, and nontoxic to manage plant fungal disease.

Given that CK can induce plant immunity and restrict phytopathogen growth in certain cases, direct effects of CK against phytopathogens are an intriguing possibility. A direct effect of CK on bacterial pathogens was ruled out in previous works (23, 25). Interestingly, CK did not affect germination and elongation of germ tubes, but strongly inhibited appressorium formation of *Erysiphe graminis*, an obligate biotrophic powdery mildew causing barley pathogen (14). Another work described an increase in germination of conidia of two *Erysiphe* powdery mildew pathogens, *E. graminis* and *E. cichoracearum*, in the presence of CK (31). Fungal pathogens of the species *Erysiphe* are known to produce CKs. High levels of CK was also reported to inhibit mycelial growth and pathogenesis of fungi in canola (9).

In this work, we investigate the direct effects of CK on fungal plant pathogens, demonstrating that CK directly inhibits the growth, development, and virulence of fungal plant pathogens. *B. cinerea* (*Bc*) growth, sporulation, and spore germination were all inhibited by plant-physiological CK concentrations, in the absence of a host plant. We

found similar effects in a variety of plant pathogenic fungi. Molecular and cellular analyses revealed that CK is not toxic to *Bc*, but rather, *Bc* likely recognizes CK and responds to it, resulting in cell cycle and individual cell growth retardation. CK reduced *Bc* virulence in a reversible manner, confirming that no irreversible harm was caused to fungal development. Interestingly, we also found that CK affects two additional fungi from the ascomycota division that are not phytopathogens, but rather, yeasts. Both budding and fission yeast were inhibited by CK. We have defined a fundamental effect of CK in fungal biology that is preserved across fungal species and in yeast, pointing to CK affecting a fundamental cellular process. Indeed, we found that CK affects cellular trafficking in *Bc*, lowering endocytic rates and endomembrane compartment sizes, likely underlying the reduced growth rates and arrested developmental programs. Mutant analyses in yeast confirmed that the endocytic pathway is a CK target. Our work uncovers a novel, remarkably conserved role for a primary plant growth hormone in fungal biology, demonstrating that co-evolution between pathogen and host resulted in fascinating molecular adaptations on fundamental processes in eukaryotic biology. In time, this may hold promise for the development of CKs as antifungal agents in specific cases.

Results

Cytokinin directly inhibits *B. cinerea*, *S. rolfsii*, and *F. oxysporum* f. sp. *lycopersici*

In order to examine a possible direct effect of CK on fungal tomato pathogens, three fungi with varied lifestyles and infection modes were selected. *B. cinerea (Bc)*, an airborne necrotrophic spore producing ascomycete that does not produce CK, that causes gray mold disease in >1400 hosts (32); *S. rolfsii (Sr)*- a soilborne necrotrophic basidiomycete that does not produce spores and does not produce CK, that causes southern blight disease in

hundreds of hosts (33); and *F. oxysporum* f. sp. *lycopersici (Fol)*, a soilborne necrotrophic ascomycete that has the ability to produce CK (34), and causes fusarium wilt disease in a host specific manner (35). The effects of different CK concentrations and derivatives on the growth of *Bc*, *Sr* and *Fol* mycelia are shown in **Fig. 1**. The cyclic synthetic CK 6-BAP (**Fig. 1a-i**), the natural cyclic CKs Zeatin and Kinetin (**Fig. 1h**), and the synthetic bacterial derived non-cyclic CK Thidiazurn (TDZ, **Fig. 1h**)- all inhibited the growth of *B. cinerea*. 6-BAP (6-benzyl amino purine) inhibited the growth of *Bc* in a dose-dependent manner in concentrations ranging from 1 µM to 100 µM. Approximately 50% inhibition was observed at 100 µM (**Fig. 1h**). *Sr* did not show growth reduction at 1 µM but growth inhibition at 10 µM and 100 µM was similar to *Bc* (**Fig. 1i**). The least effect of 6-BAP was observed on *Fol* (**Fig. 1j**). LC/MS Hormonal measurements in mature tomato leaves demonstrate that they can contain close to 100 ng/g active CKs (**Fig. S1**), as was previously reported for tomato leaves (36, 37). This amount roughly corresponds, depending on the CK derivative, to to 500 nM, which can exert some, though not maximal inhibitory effect on the growth of the indicated fungal tomato pathogens.

To examine the breadth of this direct inhibition phenomenon, we tested *in vitro* growth of additional phytopathogenic fungi in the presence of 100µM 6-BAP or the control Adenine. Our results show that CK directly inhibits mycelial growth of fungal pathogens from several different classes (ascomycetes, basidiomycetes) and different lifestyles (hemibiotrophs, necrotrophs) (**Fig. S2**). All classes of phytopathogenic fungi tested were inhibited by CK (**Fig. S2a**), however, the level of inhibition also differed significantly among them, with *Fusarium* spp. showing the least inhibition (**Fig. S2b**). The phylogeny is detailed in **Fig. S2c**, and does not indicate that the ability to be inhibited by CK is specific to any particular class or taxon.

We have previously reported that CK reduces tomato disease by inducing immunity (24). Fungal pathogens with different lifestyles have different infection and pathogenesis strategies, and host plants employ different protection mechanisms to resist different types of fungal pathogens. In order to examine the ability of CK to reduce disease caused by fungal pathogens which are directly inhibited by CK, we treated tomato plants with 100 µM CK (6-BAP) 24 hours prior to pathogen inoculation. **Fig. S3** details the effect of CK in tomato disease caused by different pathogens. 6-BAP pre-treatment significantly decreased disease levels caused by the necrotrophic fungal pathogens *Bc* , as we previously reported (24), and *Sr*. Upwards of 50% disease reduction in tomato plants was observed with *Bc* and *Sr* as compared to control (**Fig. S3 a,b**). However, no disease reduction was observed with *Fol* (**Fig. S3c**). Pre-treatment with 6-BAP causes significantly lower disease levels of *Bc* and *Sr* as compared to control, whereas no effect of 6-BAP was observed in plants inoculated with *Fol*.

Cytokinin inhibits *B. cinerea* sporulation, spore germination, and germ tube elongation

Pursuant to the direct effect of CK on fungal mycelia growth, we studied its effect on additional aspects of fungal development, *viz*., sporulation, spore germination, and germ tube elongation. In samples treated with 100 nM CK, there were approximately 50% less spores produced as compared to mock (**Fig. 2a,b**). The effect of CK on spore germination was even more pronounced (**Fig. 2c,d**). Less than 50% of the spores germinated with 100 nM CK. Interestingly, though less spores germinated in the presence of 6-BAP, the spores that did germinate had accelerated germ tube growth in 100 nM 6-BAP, and inhibited germ tube growth 100 µM 6-BAP (**Fig. 2c,e**). After 8 hours of growth with 100 µM 6-BAP, the germ tube length was 50% of the control (**Fig. 2e**), correlating with ~50% inhibition in mycelial growth (**Fig. 1a,b,h**).

Cytokinin reversibly attenuates *B. cinerea* virulence

Is direct CK inhibition of phytopathogenic fungi reversible? Or does CK irreversibly harm fungal development? Furthermore, since *Bc* is inhibited in CK concentrations normally found within plant leaves (see **Fig. 1**), how does CK affect fungal virulence? To answer these questions, we conducted two types of disease assays. In the first case, we harvested *Bc* spores from fungi grown with or without CK, normalized the spore count to 10^5 spores/mL, and used equal amounts of spores to infect tomato leaves. When spores harvested from mycelia grown with 6-BAP were used for infecting tomato leaves, no reduction in lesion size was observed as compared with spores grown without CK (**Fig. 3a**). In the second case, we harvested spores from mycelia grown without CK, mixing half the spores with CK just prior to tomato leaf infection. When spores were treated with CK prior to infection, there was approximately 40% reduction in lesion area (**Fig. 3b**), comparable to disease reduction observed when treating plants with CK (**Fig. 1l**).

Examining the expression of virulence genes in *Bc* treated with CK revealed that 6-BAP differentially down-regulated the expression levels of virulence genes in *Bc* (**Fig. 3c**). All tested genes related to virulence viz., *BcPG1* (endopolygalacturonase), *BcXynl*, *BcXyn10A* and *BcXyn11C* (Xylanases), *BcPME1* (pectin methylesterase), *BcPLC1* (phospholipase C), *BcBMP1* (mitogen-activated protein (MAP) kinase) and *BcPLS1* (tetraspanin) were expressed at significantly lower levels upon growth in the presence of 100 µM of 6-BAP. In addition, the expression of *Bchex*, which encodes the principle protein of the woronin body, was markedly down-regulated upon 6-BAP treatment. The woronin body is known to be involved in fungal membrane integrity (38) .

Cytokinin is not toxic to *B. cinerea*

Why is CK inhibiting fungal growth and development? The disease assay results (**Fig. 3**) suggested that CK is not irreversibly harming fungal development. Spores generated from fungus grown in the presence of CK were able to infect tomato leaves normally, once removed from the CK containing environment, indicating that the spores themselves were normal, and that disease reduction stems from a reduction in spore formation, spore germination, and germ tube growth (**Fig. 3**). To confirm that CK is not killing fungal cells, we examined fungal cellular leakage in the presence of CK. Application of 100 µM 6-BAP led to no significant leakage of cellular contents of *Bc*. As shown in **Fig. 4**, no significant leakage of nucleic acids and proteins were observed after 24 h treatment with 100 µM 6-BAP, when compared to the control. Additionally, no change in electrical conductivity was observed upon 24 hours of 100 µM 6-BAP treatment (**Fig. 4)**. These results confirm that CK is not toxic to *Bc*.

Cytokinin affects cell morphology and DNA replication

Since we observed that CK inhibits sporulation, spore germination, and hyphal growth, we examined the morphology and relative DNA quantity of *Bc* cells grown with and without CK (**Fig. 5**). Using calcofluor white staining, we observed a significant reduction in the cell size upon CK (100 µM 6-BAP) treatment. Cell size was reduced to 25% in comparison to mock, with cells appearing much smaller and rounder (**Fig. 5a,c**). Similarly, the distance between the last two septa from the hyphal tip were reduced to about 30% of normal length (**Fig. 5d**). Quantifying the number of septa present in individual hyphae from discrete germinated spores demonstrated that 8 hours after germination, CK grown hyphae produced less than half the number of septa when compared with mock grown hyphae (**Fig.**

5e), indicating that cell replication is inhibited. Concurrently, we quantified hoescht staining in mock and CK grown mycelia. Hoescht binds to DNA and has been used before to estimate DNA content in live cell nuclei (39). CK grown cells were stained by Hoescht less than 50% of the amount of stain observed in Mock grown cells. Images were taken under identical conditions. Reduction in Hoescht staining coupled with lower rates of cell replication (estimated by septa formation) can indicate that CK may be inhibiting mitosis in *Bc*.

Cytokinin inhibits endocytosis in *B. cinerea*

Why does CK directly inhibit growth and virulence of fungal pathogens from different classes (ascomycetes, basidiomycetes), different lifestyles (hemibiotrophs, necrotrophs), different modes of plant infection (air borne, soil borne, vascular or not) and different host target organs (leaves, stems, roots)? Of note is that us and others have found that CK has no direct inhibitory effect on bacterial pathogens (23, 40). Our results demonstrate that CK inhibits fungal growth and development (**Figs. 1,2,5**). We hypothesized that CK affects a fundamental cellular process relevant to most fungi; a process that is crucial to execute the fast growth occurring in hyphal tips (41), growth that requires membrane remodeling (42). Us and others (24, 43) have previously shown that CK can influence cellular trafficking in plants. We therefore examined the effect of CK on endocytosis in *Bc*. **Fig. 6** shows that 6-BAP inhibits endocytosis of the endocytic tracer FM-4-64, which is routinely used in fungi (44), reducing the amount of endocytic vesicles by more than 50% (**Fig. 6a,b**). Interestingly, low levels of 6-BAP also cause an increase in the size of the vesicles containing endocytic tracer, suggesting a possible impact on membrane function and/ or

fission of vesicles (**Fig. 6c**), and possibly correlating with increased hyphal growth achieved with 100 nM CK (**Fig. 2e**).

Inhibition of endocytosis abolishes *B. cinerea* CK sensitivity

To further examine the effect of CK on endocytosis inhibition in *Bc*, we examined combined effects of CK and endocytosis inhibition using butanol treatments. 1-butanol inhibits phospolipases (45, 46), which have been linked to both growth and virulence in *Bc* (47) (see also **Fig. 3**), and was used as an endocytosis inhibitor in previous works, including in fungi (48–50). We examined CK-mediated growth inhibition of both mycelia area and dry weight, in the presence or absence of the endocytosis inhibitor 1-butnaol, as well as its structural analog 2-butanol, which does not inhibit endocytosis (49). **Fig. 7** demonstrates that upon endocytosis inhibition with 1-butanol, CK no longer affects fungal growth, while CK-mediated growth inhibition is retained in the mock and 2-butanol treatments. (**Fig. 7 a,b**). At 0.4%, 2-butanol did not inhibit fungal growth. At 0.8%, fungal growth inhibition was observed with 2-butanol, though to a significantly lesser degree than that observed with 1-butanol (**Fig. 7a**).

Cytokinin reduces yeast growth

Given our observations that CK can affect as fundamental a cellular process as endocytosis, we examined inhibitory roles for CK in the growth of *Saccharomyces cerevisiae*, a budding yeast, and *Schizosaccharomyces pombe*, a fission yeast, the growth of which more closely resembles that of fungal hyphae. Growth curves were generates by measuring OD_{600} over time, as previously described (51). We observed that CK inhibits the growth of *S. cerevisiae* (**Fig. 8a,b**), and *S. pombe* (**Fig. S2a,b**) in a dose-dependent manner. We found

that *S. pombe* was more strongly inhibited. Interestingly, trans zeatin was previously reported not to affect *S. pombe* cell division (52).

Cytokinin inhibits endocytosis in yeast

To examine whether growth inhibition is mediated by endocytosis in yeast, as we found for *B c*, we conducted endocytic assays in *S. cerevisiae* (**Fig. 9**) and *S. pombe* (**Fig. S3**). Yeast cultures were grown overnight, diluted to an OD_{600} of 0.2, and grown for a further six hours with or without CK. Cultures were then stained with FM4-64 (Vásquez-Soto *et al.*, 2015). Confocal microscope images were acquired using a Zeiss LSM780 confocal microscope. CK reduces cell size, internalization of FM4-64, endosome size, and endosome density in both *S. cerevisiae* (**Fig. 9a-d**) and *S. pombe* (**Fig. S3a-e**), indicating that endocytosis is a universal mechanism through which CK exerts its effect.

In parallel, the effect of CK on the growth of *S. cerevisiae* endocytic mutants was examined. Genes known to be involved in endocytosis, the absence of which is known not to be lethal, were selected. *S. cerevisiae* mutant strains knockout by disrupting in the indicated genes were generated as described in the methods section. Mutations in *YPT31*, a Rab/GTPase known to be involved in vesicular trafficking (54), *SSA1*, known to be involved in clathrin vesicle uncoating (55), *VPS1*, a dynamin-like GTPase known to be required for vacuolar sorting, cytoskeleton organization and endocytosis (56, 57) and *SPO14*, a phospholipase D protein required for sporulation (58), were generated and examined. Three out of the four generated mutants, *ypt31*Δ, *ssa1*Δ, and *vps1*Δ, exhibited a partial rescue in CK mediated inhibition (**Fig. 10a-c,e,f**), growing significantly better in the presence of 300 and 500 μM 6-BAP than the wild type (WT) strain. The *spo14*Δ mutation did not rescue CK-mediated growth inhibition (**Fig. 10d-f).**

Discussion

As reported previously by us and others, CK was shown to promote fungal disease resistance in plants (21, 24). Direct effects of CK on fungal growth and development have not been investigated in depth in plant-free systems, and although a few studies have discussed some effects of CK on fungal pathogen growth (9, 10), no mechanisms for CK antifungal activity have yet been reported. The present study was performed to examine the direct inhibitory effect of CK on different classes of fungal pathogens. We focused our efforts on three fungal pathogens, *Bc*, *Sr* and *Fol*, with varied lifestyles and infection modes. *Bc*, an airborne necrotrophic spore producing ascomycete, does not produce CK (32). *Sr*- a soilborne necrotrophic basidiomycete, does not produce spores and does not produce CK. *Fol*, a soilborne necrotrophic ascomycete, has the ability to produce CK (34). Our results demonstrate that CK exhibits inhibitory activity against *Bc*, *Sr* and, to a lesser degree, *Fol*, *in vitro*.

We found that CK treatment strongly inhibits *Bc* and *Sr*, and inhibits *Fol* more weakly (**Fig. 1l-n**). It has been demonstrated that pathogenic fungi of the *Fusarium* species complex are able to produce CKs endogenously, which might be the reason for its tolerance towards exogenous CK (34). Interestingly, varying levels of inhibitory activity were observed for CK in many phytopathogenic fungi (**Fig. S1**).

Examination of possible effects of CK on *Bc* development revealed that CK attenuates *Botrytis* sporulation, spore germination and germ tube elongation (**Fig. 3**), with differential effects of CK concentration on different processes. Interestingly, genes involved in the regulation of growth, conidiation, germination, virulence, and pathogenicity, in *Bc*, were strongly inhibited by CK treatment (**Fig. 2**). *BcBMP1*, *BcPME1* , *BcPLS1*, *BcXyn*, *BcPLC1* and *Bchex* were previously found to be involved in plant infection processes and defined

as essential determinants for *Bc* pathogenicity (47, 59–64). All virulence genes assayed exhibited statistically significant down-regulation upon CK treatment (**Fig. 2c**), suggesting that interference with the expression of virulence genes may also partly contribute to the inhibitory effects of CK against *Bc*. Inhibition of growth and spore germination together with downregulation of virulence genes in *Bc* could account for disease attenuation in tomato. This mechanism could complement previously reported plant induced resistance mechanisms being activated by CK, which, as reported by us and others (21, 24, 65), induces plant immunity even in the absence of a pathogen.

Damage in cell membrane integrity in fungi usually leads to the release of nucleic acids and proteins (66–68). CK showed no effect on leakage of nucleic acids and proteins from *Bc* after treatment, and no change was observed in media conductivity of *Bc* after CK treatment, indicating that cell membrane permeability remained unchanged. Taken together, these results suggest that CK is not toxic to fungi. CK can thus be defined as possessing fungistatic, and not fungicidal, activity.

To understand the mode of action by which CK inhibited the growth of *Bc*, we examined cell morphology and DNA replication after CK application. CK strongly reduced cell area, distance between septa, and likely, mitosis. Cell division/septum formation is dependent on the signals generated during cell extension and growth, and nuclear division. Reduced cell growth and elongation effected by CK treatment correlates with the lesser number of septa in the treated cells. Hoescht staining revealed there is less DNA in CK treated cells, possibly due to inhibition of mitosis. Inhibition of cell division (septa formation) coupled with reduced DNA amounts, strongly support the notion that CK is inhibiting mitosis in fungal cells.

It has been previously suggested that certain classes of fungi possess CK receptors to be able to "sense plants", a trait posited to have been required for land colonization by fungi (69). We and others have previously demonstrated that plants activate CK signaling upon pathogen attack, and that CK serves as a cue to activate defense responses (21, 24, 65) . A possibility arising from our work is that when plants sense the presence of phytopathogenic fungi, an additional reason for the activation of CK pathways is to promote CK biosynthesis, thereby inhibiting the growth of the potential fungal attacker. We have shown here that CK inhibits growth in different types of fungi from different classes (basidiomycetes, ascomycetes) and lifestyles (soilborne, airborne, hemibiotroph, necrotroph), and yeasts (**Figs. 1, 2, 5, 8, S1, S2**), and endocytosis in *Bc*, *S. cerevisiae*, and *S. pombe* (**Figs. 6, 7, 9, S3**). Chemically or genetically attenuating cellular trafficking in *Bc* (**Fig. 7**), or *S. cerevisiae* (**Fig. 10**) respectively, resulted in loss of CK mediated growth inhibition. It would seem that, in employing CK as a fungal pathogen inhibitor, plants have succeeded in managing to target a process so fundamental to growth, endocytosis, that this inhibition is preserved all the way to budding yeast.

CK inhibited the amount of endocytic vesicles in *Bc* (**Fig. 6**), *S. cerevisiae* (**Fig. 9**), and *S. pombe* (**Fig. S3**), indicating that it likely has an impact on membrane function and/ or fission of vesicles. Cell elongation, in particular hyphal elongation, requires continuous addition of new plasma membrane, proteins, and cell wall material at the hyphal tip. Cellular trafficking and endo/exocytosis regulate the amount of membrane transferred towards this cellular growth, tightly controlling the amount of cellular material required for plasma membrane extension (42). Inhibition of endocytosis by CK explains the reduced elongation and growth of *Bc* and yeast cells. Fission yeast may be more reliant on endocytosis for rapid cell elongation than budding yeast, and their growth resembles more

the growth of fungi than that of budding yeasts, possibly also explaining why fission yeast is more strongly inhibited by CK than budding yeast.

In the evolutionary war against plant pathogens, plants appear to have succeeded in creating a 1-2 mechanism for utilization of CK, previously believed to be only a "developmental" hormone. 1- The plant senses a pathogen and activates its CK response, leading to immunity signaling , which culminates in increased immunity and systemic pathogen resistance; and 2- the activation of CK response leads to the generation of increased CK levels, which inhibit the growth and development of fungal pathogens by targeting their endocytic machinery. Future work will reveal how CK manipulates endocytosis in fungi, and whether this fungistatic activity can be agriculturally adapted.

Materials and Methods

Pathogen growth conditions

Botrytis cinerea (strain Bcl16), *Sclerotium rolfsii* (70), *Fusarium oxysporum* f. sp. *lycopersici* (strain 4287) were cultivated on potato dextrose agar (PDA) at 22 ± 2 °C for *B. cinerea*, 26 ± 2 °C for *S. rolfsii* and 28 ± 2 °C for *F. oxysporum* for 5-7 days. Pathogen isolates were kindly gifted by Yigal Elad, David Ezra and Shay Covo.

Mycelial growth assay

To study the effect of CK on mycelial growth of pathogenic fungi, 6-BAP (6-Benzylaminopurine, Sigma-Aldrich) and adenine (Sigma-Aldrich) were dissolved in 10mM NaOH and 1M HCl, respectively and added to PDA media at a concentration gradient of 0, 1, 10 and 100 uM. *B. cinerea*, *S. rolfsii*, and *F. oxysporum* mycelial plugs (5 mm) taken ~1cm from the edge of a fresh plate were placed at the center of PDA plates and incubated under the above mentioned growth conditions. Adenine is commonly used

as a control due to its structural similarity to cyclic CKs (2). The effect of 6-BAP on the growth of all phytopathogenic fungi was studied similarly.

Cytokinin level measurement

To analyze the active CKs levels in mature tomato (cv. M82) leaves, phytohormone extraction was performed according to the following: 200-450 mg fresh ground tissue powder was transferred to a 2ml tube containing 1ml extraction solvent (79% isopropyl alcohol: 20% Methanol: 1% acetic acid) mixture supplemented with 20 ng of deuterium-labelled internal standards (IS, Olomouc, Czech Republic). The tubes were incubated at 4°C for 1 h on shaker and centrifuged at 14,000 g for 15 min at 4°C. The supernatant was transferred to 2ml tubes and 500 µl of extraction solvent was added to the pellet and the extraction steps were repeated twice. The combined extracts were evaporated using speed-vac at room temperature and dried samples were dissolved in 200 µl 50 % methanol and further filtered with 0.22 µm cellulose syringe filter. A volume of 5–10µl was injected for each analysis. LC–MS-MS analyses were conducted using a UPLC-Triple Quadrupole MS (WatersXevo TQMS). Separation was performed on Waters Acuity UPLC BEH C18 1.7µm 2.1x100 mm column with a Van Guard precolumn (BEH C18 1.7µm 2.1x5mm). 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 0.3ml/min, and the column temperature was kept at 35 °C. Acquisition of LC–MS data was performed using Mass Lynx V4.1 software (Waters). Quantification was done using isotope-labeled internal standards (IS). Solvent gradients and MS-MS parameters are detailed in supplemental Table 1.

Plant pathogenesis assays

To study the effect of CK on *Bc* pathogenicity, spores were harvested in 1 mg ml^{-1} glucose and 1 mg ml^{-1} K_2HPO_4 and filtered through sterile cheesecloth. Spore concentration was

adjusted to 10^6 spores ml^{-1} using a Neubauer chamber. Solution of 6-BAP (100 µM) with Tween 20 (100 µl l^{-1}) was sprayed onto three-week-old plants 24 hours before pathogen inoculation. Mock plants were sprayed with the abovementioned solution of NaOH and Tween 20. Each tomato leaflet was inoculated with two droplets of 10 µl spore suspension (71). The disease symptom was measured after five days of pathogen inoculation. Lesion diameter was measured by ImageJ software.

Fol culture was grown in KNO_3 media (containing yeast nitrogen base, sucrose, KNO_3 and distilled water) at 28 °C for 5 days. The spore suspension used for inoculation was applied at a concentration of 10^6 spores ml^{-1} (72). Two week old tomato plants treated with 100 µM 6-BAP through foliar spray and inoculated using the root dip method (73, 74) with the pathogen spore suspension after 24 h of 6-BAP application. As control, non-inoculated tomato plants were used. The disease index (DI) was calculated after three weeks of pathogen infection by 0–5 scale as follows: 0, no symptoms; 1, ≥ 2% (healthy plant); 2, 3–30% (slight disease); 3, 31–60% (moderate disease); 4, 61–90% (severe disease), and 5, ≤ 91% (dead plant).

For *Sr*, tomato plants were soil drenched with 100 µM concentration of 6-BAP one week (alternate days) prior to infection. After one week, 3-4 sclerotia of *Sr* were placed on the soil, ~2cm from the plant stem. Mass culture of sclerotia of *Sr* was done on PDA media. First symptoms appeared one week after inoculation, and ultimately developed into severe rot and wilted the plants. Mock plants were drench with the abovementioned solution of NaOH and Tween 20. The disease index (DI) was calculated by using the above mentioned 0–5 scale.

B. cinerea sporulation and spore germination measurement

To study the effect of sporulation, *Bc* plug (5 mm) from the fresh plate was placed at the center of PDA plates containing 0, 0.1 and 100 µM 6-BAP for five days at 22 ± 2 °C. Since *Bc* spores are black while the mycelium is white, plates were analyzed for black color, i.e., spore formation, in four independent experiments of at least 3 replicates. For spore germination, *B. cinerea* spores at a concentration of 10^6 spores ml^{-1} were incubated in potato dextrose broth (PDB) containing 0 and 100 µM 6-BAP at 22 ± 2 °C for 8 hours. Thereafter, conidia were washed twice in sterile water (1 ml), centrifuged at 12,000 rpm for 5 min, and the pellet was re-suspended in 100 µL of sterile water. A 10 µL sample was analyzed under the microscope. The percentage of sporulation, spore germination and the length of germ tubes were measured using ImageJ software.

***B. cinerea* virulence assessment**

To examine the effect of cytokinin on fungal virulence, we grew *Bc* spores in PDB with 0 and 100 µM 6-BAP in a rotary shaker at 180 rpm and 22 ± 2 ℃ for 24 hours. The total RNA was isolated using Tri reagent (Sigma-Aldrich) according to the manufacturer's instructions. RNA (3µg) was used to prepare cDNA using reverse transcriptase (Promega, United States) and oligodT 15. qRT-PCR was performed on a Step One Plus Real-Time PCR system (Thermo Fisher, Waltham, MA, United States) with Power SYBR Green Master Mix protocol (Life Technologies, Thermo Fisher, United States). For virulence analysis, we selected the following genes: *BcBMP1* (mitogen-activated protein (MAP) kinase), *BcPME* (pectin methylesterase), *BcPLS1* (tetraspanin), *BcPLC1* (phospholipase C), *BcPG1* (endopolygalacturonase), *Bchex* (Woronin body protein), *BcXynl*, *BcXyn10A* and *BcXyn11C* (Xylanases) (38, 67, 75). The primer sequences for each *gene* detailed in Supplementary Table 2. *B. cinerea* ubiquitin-conjugating enzyme E2 (ubce) (76) was used as internal reference gene and relative gene expression levels were calculated using the 2^{-}

$^{\triangle\triangle Ct}$ method (77). At least four independent biological replicates were used for analysis. Each target gene was measured in triplicate and the mean Ct value was calculated.

Measurement of cellular leakage and electrolytes

Bc were treated with 6-BAP (0 and 100 µM) in PDB and incubated on a rotary shaker at 180 rpm for 24h at 22 °C. The mycelia were subsequently centrifuged and the aqueous solutions were used for measurement of the leakage of nucleic acids and proteins. The concentration of the proteins released from the cytoplasm was measured according to Bradford method [74]. The absorbance of the supernatant was measured at 595 nm using UV/VIS spectrophotometer. Leakage of proteins was quantified according to Bradford (78). The release of nucleic acids in various treatments was measured by detecting the optical density at 260 nm. Electrical conductivity was measured using a conductivity meter (EUTECH instrument con510) after 24 h.

Cell elongation and DNA content

To examine the cell morphology of *Bc* spores treated with 6-BAP, 1 g l^{-1} calcofluor white M2R (Sigma-Aldrich) was used. Cells were cultured in PDB with or without 6-BAP (100 µM) at 22 °C for 16 h. The cells were then collected and stained with calcofluor white M2R at room temperature for 15 min. Additionally, for septa counting after 8 hours of CK co-cultivation, growing hyphae from germinating spores were stained with 0.1% toluidine Blue O (Sigma-Aldrich) and visualized under a light microscope.

For DNA replication**,** a stock solution of 1.5 mg ml^{-1} Hoechst 33342 (Sigma-Aldrich) dye was prepared in water. The stock solution was further diluted 1:100 using water, and applied to *Bc* spores cultured in PDB with or without 6-BAP (100 µM) for 16 h at 22 °C. The botrytis samples stained with hoechst were plated on microscope slides, and the slides were incubated in the dark for 1 h in a humid chamber at room temperature before microscopic visualization (79). Nuclei and septa were visualized with either a light or a

fluorescence Olympus microscope, using the 4′,6-diamidino-2-phenylindole (DAPI) channel, and the images were analyzed using Fiji-ImageJ. Cell size and length (septal distance) were measured using the area measurement tool. Septa were also counted using the counter tool. DNA staining was assessed using the mean intensity measurement tool.

B. cinerea endocytosis

To measure endocytosis in *Bc* hyphae, the culture was grow in PDB with or without 6-BAP (100nM, 100 µM) for 16 h at 22 °C, after which the cells were collected and stained with 25 mM FM4-64 on a glass coverslip. We acquired confocal microscopy images using a Zeiss LSM780 confocal microscope equipped with a C-Apochromat 40×/1.2 W Corr M27 or 63×/1.15 Corr Objective. FM-4-64 images were acquired with a 514nm excitation laser (4% power), with the emission collected in the range of 592–768 nm. Images of 8 bits and 1024 × 1024 pixels were acquired using a pixel dwell time of 1.27, pixel averaging of 4, and pinhole of 1 airy unit. Image analysis (18-24 images per treatment collected in three independent experiments) was conducted with Fiji-ImageJ using the raw images and the 3D object counter tool and measurement analysis tool (80).

Budding (*Saccharomyces cerevisiae*) and fission (*Schizosaccharomyces pombe*) yeast growth

Wild-type haploid yeast strains were grown over night at 30°C. *S. cerevisiae* cells were grown in synthetic defined (SD) medium and *S. pombe* cells were grown in edinburgh minimal medium (EMM), without (Mock) or with the addition of indicated concentrations of CK (6-BAP). *S. cerevisiae* and *S. pombe* were diluted to an OD_{600} of 0.01. 200 µL of cells were plated in 96 well plates and incubated at 30°C for 25h (*S. cerevisiae*) or 45h (*S. pombe*), with continuous shaking. OD_{600} was measured using a TecanTM SPARK 10M plate reader. Experiment was repeated three times with similar results. WT strains of *S. cerevisiae* and *S. pombe* were kind gifts from Martin Kupiec and Ronit Weisman.

Budding (*S. cerevisiae*) and fission (*S. pombe*) yeast endocytosis

Saccharomyces cerevisiae was grown overnight in YPD media, then the culture was diluted (OD_{600} = 0.2) and incubated for 6 hours in YPD (mock) or YPD supplemented with 300 µM 6-BAP. *Saccharomyces pombe* was grown overnight in YE. The cultures were then diluted (OD_{600} = 0.2) and incubated for 6 hours in YE media (mock) or media supplemented with 100 µM 6-BAP. Cell cultures were collected by centrifugation at 5,000 rpm for 4 minutes, and re-suspended in fresh growth medium. FM4-64 staining was performed as described (53). Cells were incubated with 24 µM FM4-64 (Invitrogen) for 30 min at 4°C. Subsequently, the FM4-64 containing medium was replaced with fresh medium, and cultures was incubated for 15 minutes at 28°C. To observe FM4-64 distribution, 5 µl of the suspensions were placed on a slide and live confocal imaging was performed. Confocal microscopy images were acquired using a Zeiss LSM780 confocal microscope equipped with Objective LD C-Apochromat 63×/1.15 Corr. Acquisition settings were designed using an excitation laser wavelength of 514 nm (4% power). The emission was then collected in the range of 592–768 nm. Images of 8 bits and 1024 × 1024 were acquired using a pixel dwell time of 1.27, pixel averaging of 4 and pinhole of 1 airy unit. Bright field was acquired using the T-PMT (transmitted light detector). Image analysis was performed using Fiji-ImageJ with the raw images (80), endosome count and size measurements were performed with the 3D Object counter tool and pixel intensity was measured using the measurement analysis tool.

Construction of budding yeast mutant strains

The *YPT31, SSA1, VPS1 and SPO14* genes were disrupted in wild-type yeast strain (BY4741) via homologous recombination using PCR fragments amplified from the plasmid pFA6a-KanMX6 as a template with suitable primers. Gene replacement was

validated by PCR with suitable primers. Primer sequences are provided in Supplemental Table 3.

Data analysis

All data is presented as the average ±SEM. We analyzed the statistical significance of differences between two groups using a two-tailed t-test, with additional post hoc correction where appropriate, such as FDR calculation with Holm-Sidak correction in growth curve assays, and Welch's correction for t-tests between samples with unequal variance. We analyzed the statistical significance of differences among three or more groups using analysis of variance (ANOVA). Regular ANOVA was used for groups with equal variances, and Welch's ANOVA for groups with unequal variances. Significance in differences between the means of different samples in a group of 3 or more samples was assessed using a post-hoc test. The Tukey post-hoc test was used for samples with equal variances, when the mean of each sample was compared to the mean of every other sample. The Bonferroni post-hoc test was used for samples with equal variances, when the mean of each sample was compared to the mean of a control sample. The Dunnett post-hoc test was used for samples with unequal variances. Statistical analyses were conducted using Prism8™.

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Author contributions

Conceptualization: MB, RG. Design: RG, LP, GA, DL, TY, MB. Methodology & experimentation: RG, LP, GA, DL, NK. Analysis: RG, LP, GA, DL, UG, MB. Manuscript: RG, LP, GA, DL, UG, MB.

The authors declare no competing 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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Figure Legends

Fig. 1. Direct effect of cytokinin on fungal growth and disease.

(a)-(f) *B. cinerea, S. rolfsii and F. oxysporum* were cultured on potato dextrose agar (PDA) plates in the presence of 100 µM 6-Benzylaminopurine (6-BAP). **(g)** Quantification of results from 4-6 biological repeats ±SE, including adenine (Ade, 100 µM) as a control,

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Fig. 6. Cytokinin inhibits FM-4-64 endocytosis in growing *B. cinerea* hyphae.

B. cinerea (*Bc*) was cultured in PDB liquid broth in the presence of 100 nM or 100 µM CK (6-Benzylaminiopurine) for 16 hours. **(a)** FM-4-64 endocytic vesicles in *Bc* hyphae. **(b)** quantification of the amount of endocytic vesicles; **(c)** quantification of the average size of vesicles. Measurements were done using the counting tool of Fiji. Quantification of results from 3 biological repeats ±SE, b: N>18, c: N>136. Box-plot with all values displayed; line indicates median. Asterisks indicate significance in one-way ANOVA with a Tukey post hoc test, *p<0.05;**p<0.01; ns (non-significant).

Fig. 7. Inhibition of endocytosis abolishes *B. cinerea* cytokinin sensitivity

B. cinerea was cultured on 1/2 PDA plates (a) or in PDB liquid broth (b) in the presence or absence of 100 µM CK (6-Benzylaminopurine), the endocytosis inhibitor 1-butanol (v/v%), or control compound 2-butanol (v/v%) for 48 (a) or 96 (b) hours. **(a)** Mycelium area; measurements were done using Fiji. **(b)** Dry weight %. Quantification of results from 4 biological repeats ±SE, a: N>6, b: N>9. Boxplots are shown with inter-quartile-ranges (box), medians (black line in box), means (dot) and outer quartile whiskers, minimum to maximum values. Asterisks indicate significance in one-way ANOVA with a Dunnett post hoc test, **p<0.01; ****p<0.0001; ns (non-significant). Red letters in (a) indicate significance between the different treatments without CK in a one-way ANOVA with a Dunnett post hoc test, p<0.01.

Fig. 8. Cytokinin inhibits the growth of budding yeast.

(a) Wild-type *Saccharomyces cerevisiae* were grown over night at 30°C in minimal synthetic defined medium, treated with either 10uM NaOH (Mock) or with the addition of indicated concentrations of CK (6-Benzylaminopurine). Cells were incubated at 30°C for 25h, with continuous shaking. Average growth per time point for 3 experiments is presented, N=9. Blue color represents statistical significance from Mock treatment in a two-tailed t-test with Holm-Sidak correction, p<0.05. **(b)** Average growth (OD) at mid log phase (15h) in three independent experiments. Letters indicate significance in a one-way ANOVA with a post hoc Tukey test; p<0.0001. All points displayed; red lines indicate mean ±SE.

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S.cerevisiae yeast cells were grown overnight at 30°C in YPD medium, diluted (OD_{600} = 0.2) and incubated for 6 hours in YPD media (Mock) or media supplemented with 300 µM CK (6-Benzylaminopurine). Cells were incubated with 24 µM FM4- 64 (Invitrogen) at 4°C for 30 min. Subsequently, the FM4-64 containing medium was replaced with fresh medium and cultures was incubated at 28°C for 15 minutes. Confocal microscopy images were acquired using a Zeiss LSM780 confocal microscope. **(a)** cell size; **(b)** total internalized FM4-64 represented by pixel intensity; **(c)** endosome density. **(d)** Representative images, Bar, 10 µm. Box-plots with all values displayed; line indicates median. **a-c:** N>160. Image analysis was performed using Fiji-ImageJ with raw images collected from 3 independent biological experiments. Endosome count measurements were done with the 3D Object

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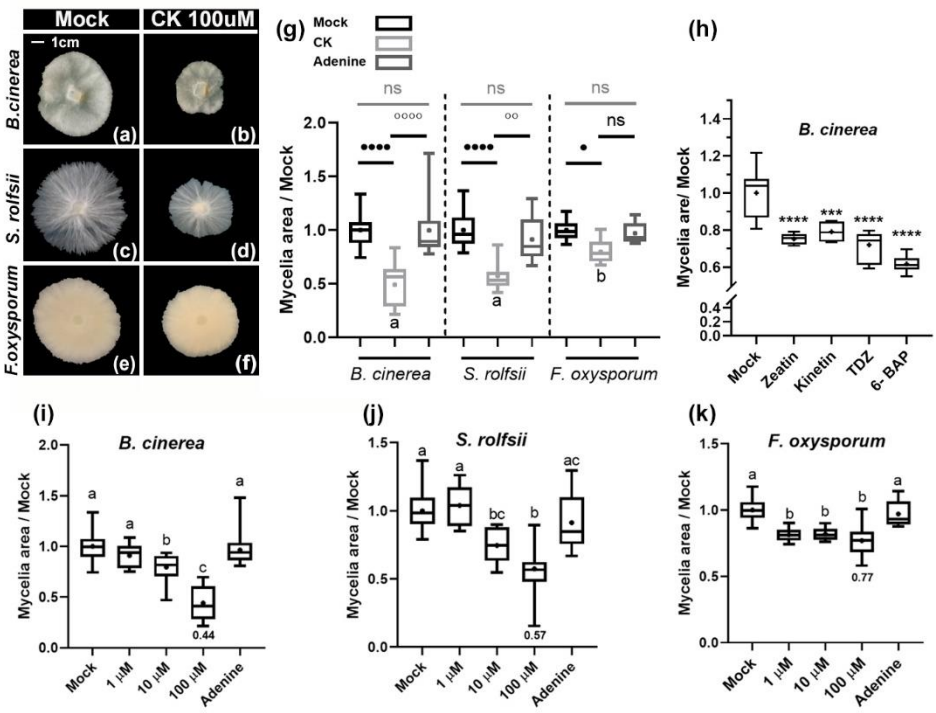
Fig. 10. Cytokinin mediated growth inhibition is partially rescued in budding yeast endocytic mutants.

S. cerevisiae Wild-type (WT, a-f), *ypt31Δ* (**a,e,f**), *ssa1Δ* (**b,e,f**), *vps1Δ* (**c,e,f**) and *spo14Δ* (**d,e,f**) were grown over night at 30°C for 25 h, in minimal synthetic defined medium, treated with either 10 µM NaOH (Mock) or with the addition of 300 µM (**a-e**) or 500 µM (**f**) CK (6-Benzylaminopurine).

(a-d) Average growth per time point for three experiments is presented, N=9. Asterisks indicate statistical significance of each mutant with CK compared to WT with CK, in a two-tailed t-test with Holm-Sidak correction, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

(e-f) Percentage of growth inhibition of each strain with 300µM CK (e) and 500 µM CK (f) as compared to mock treatment, at 13 h, in three independent experiments, N=9. Boxplots are shown with inter-quartile-ranges (box), medians (black line in box), and outer quartile whiskers, minimum to maximum values. Asterisks indicate significance in a one-way ANOVA with a post hoc Tukey test; *p<0.05; **p<0.01; ***p<0.001; ns (non-significant).



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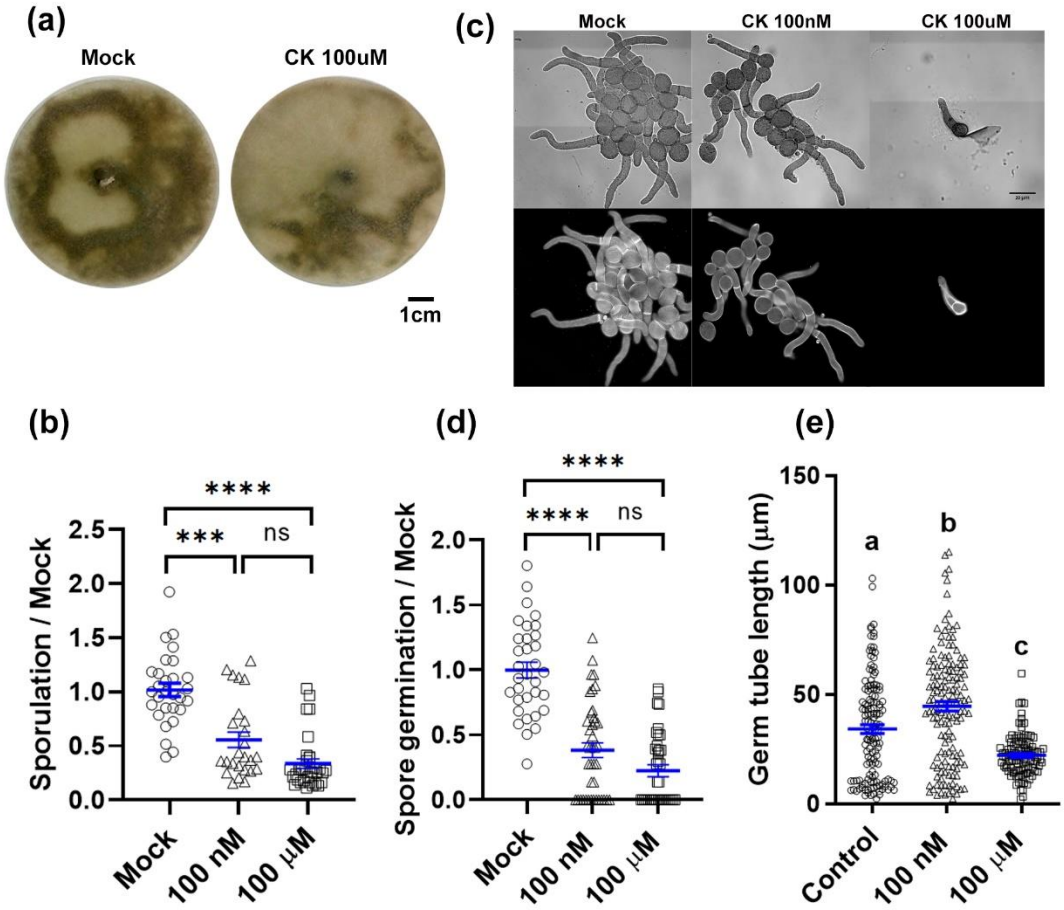

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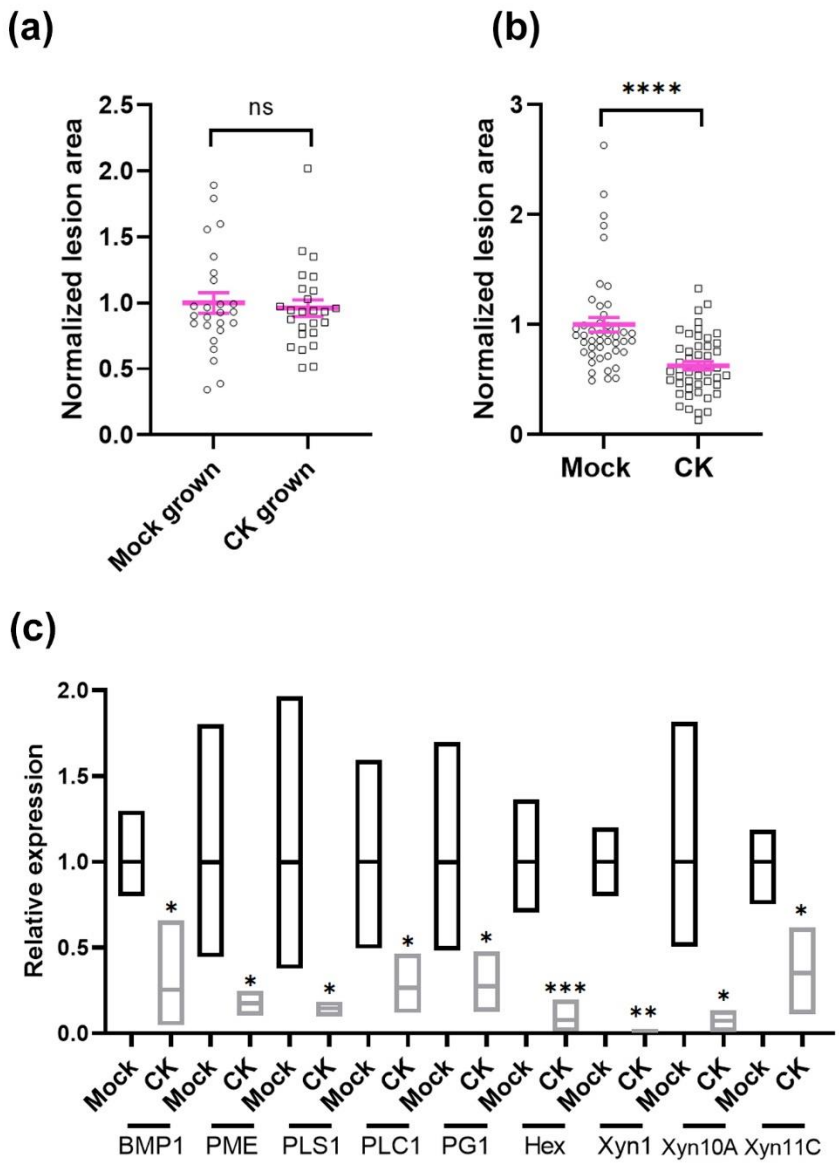

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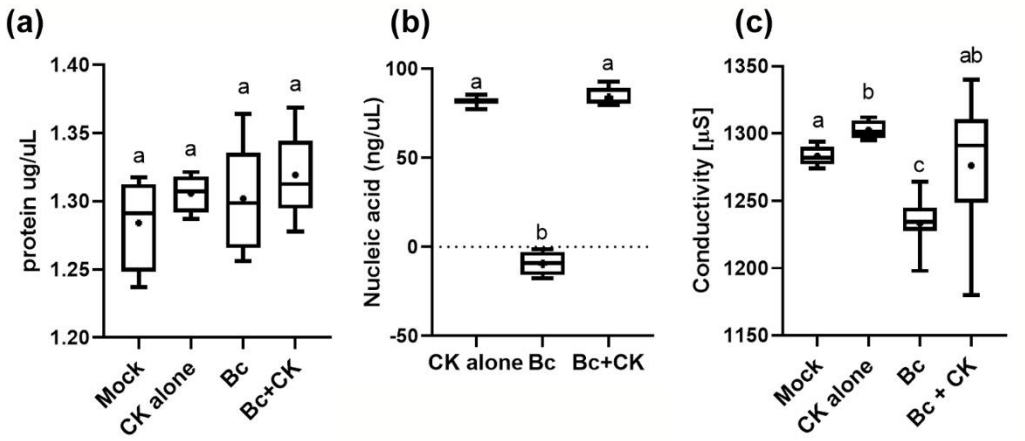

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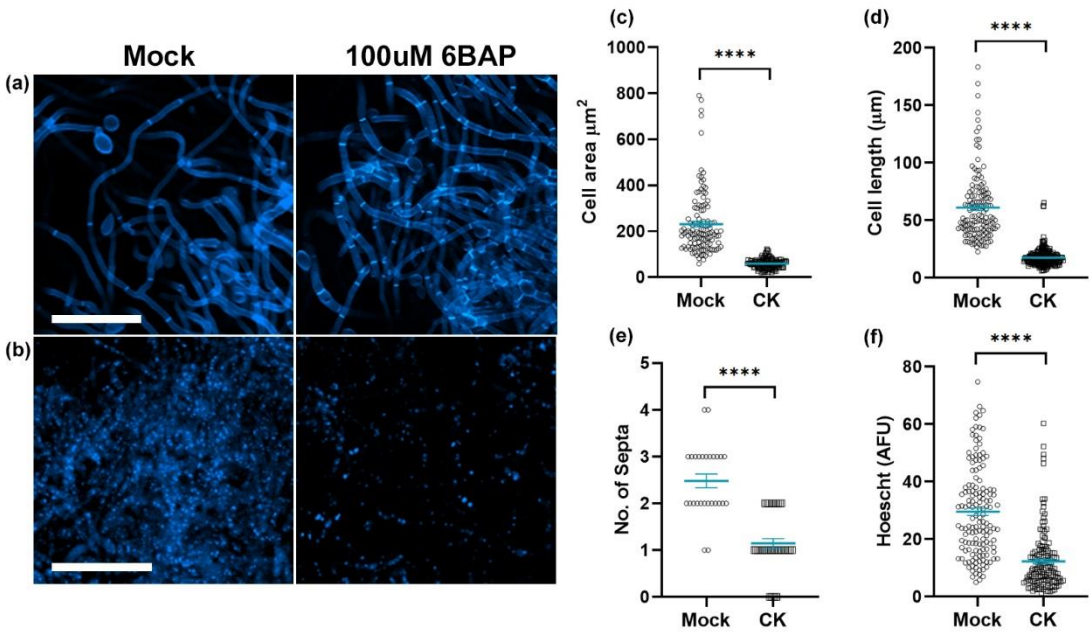

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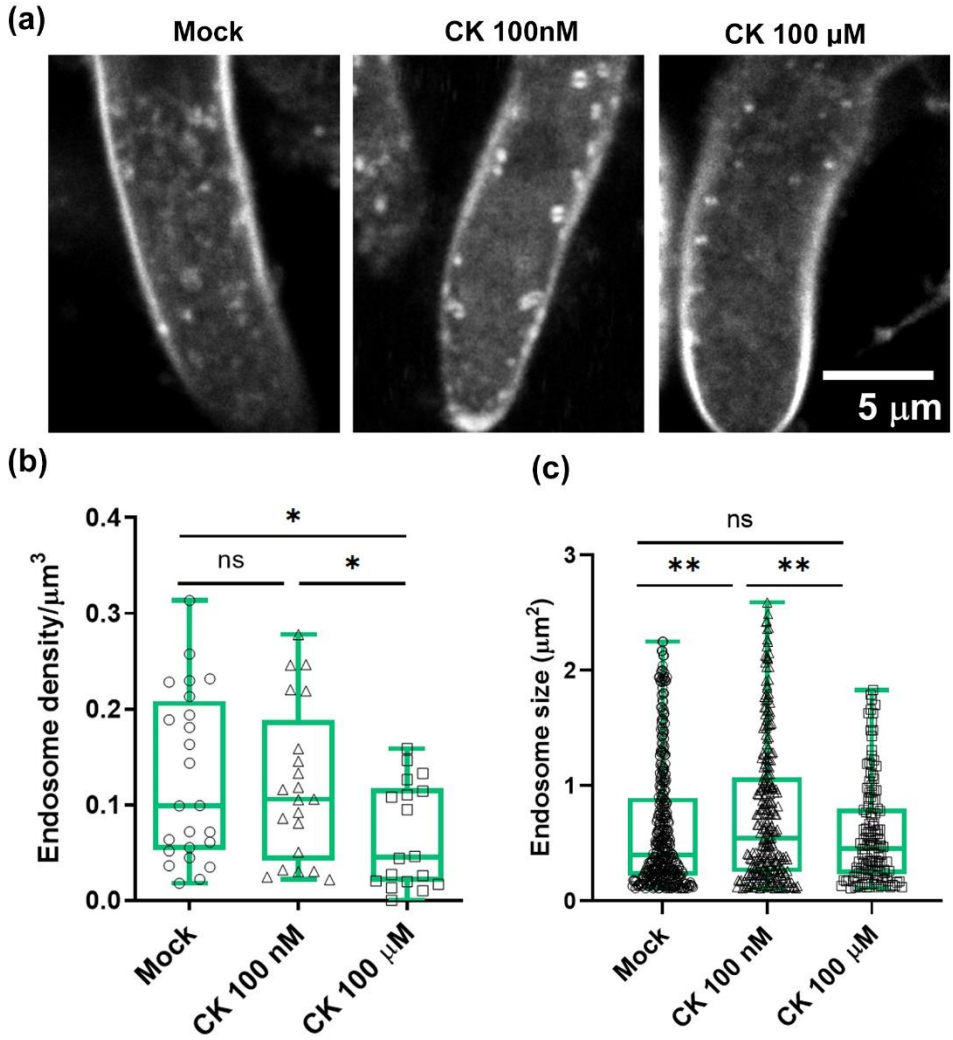

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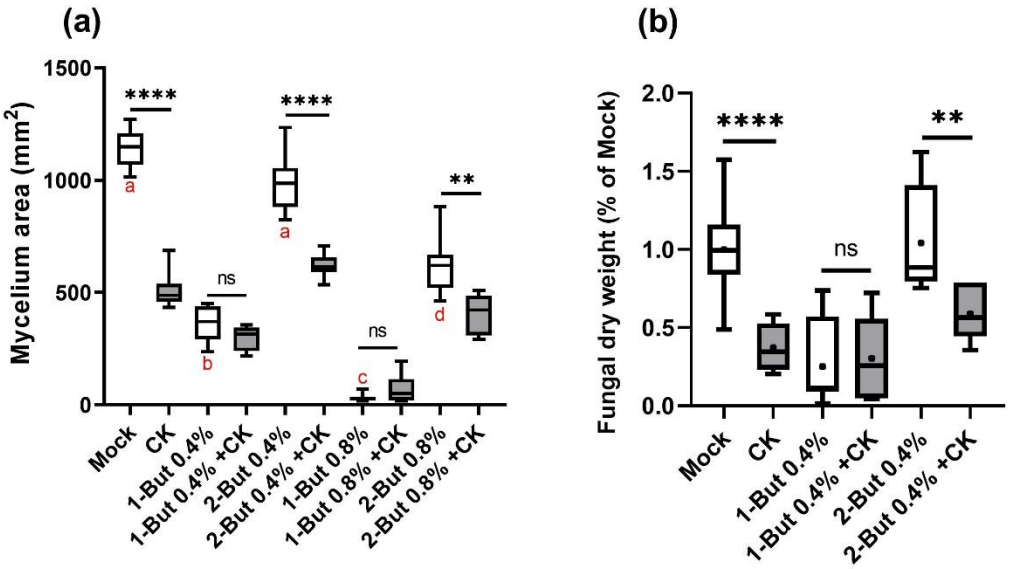

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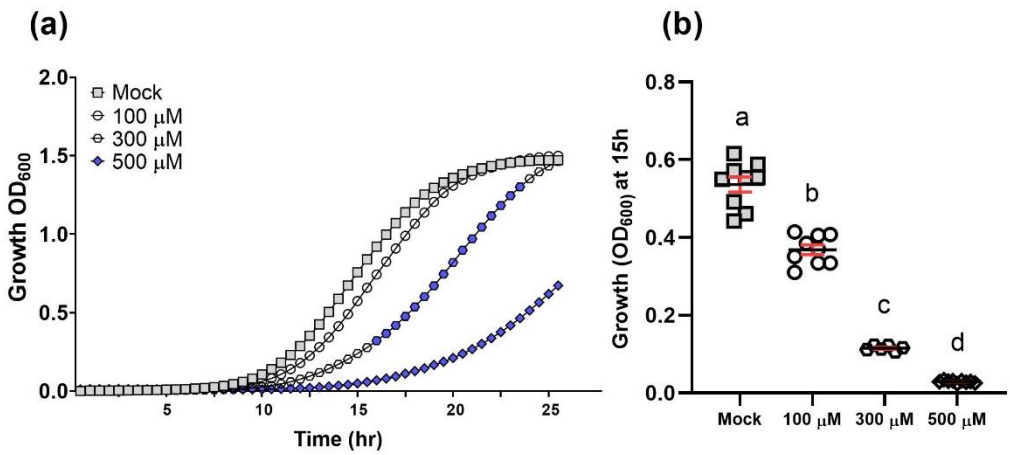

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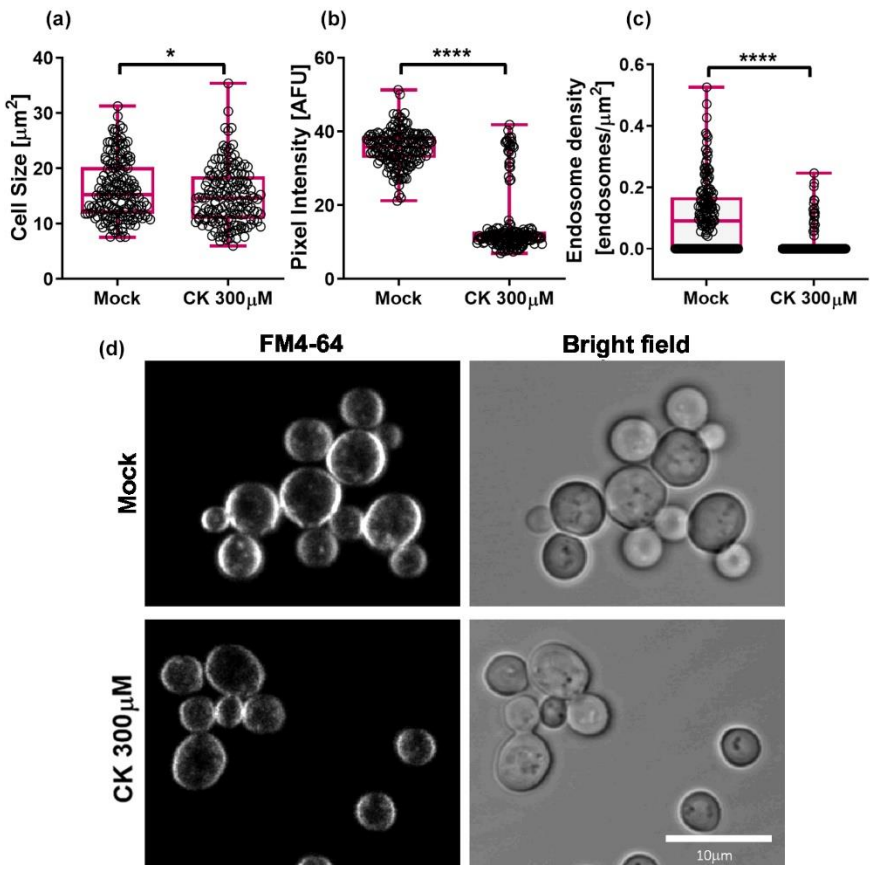

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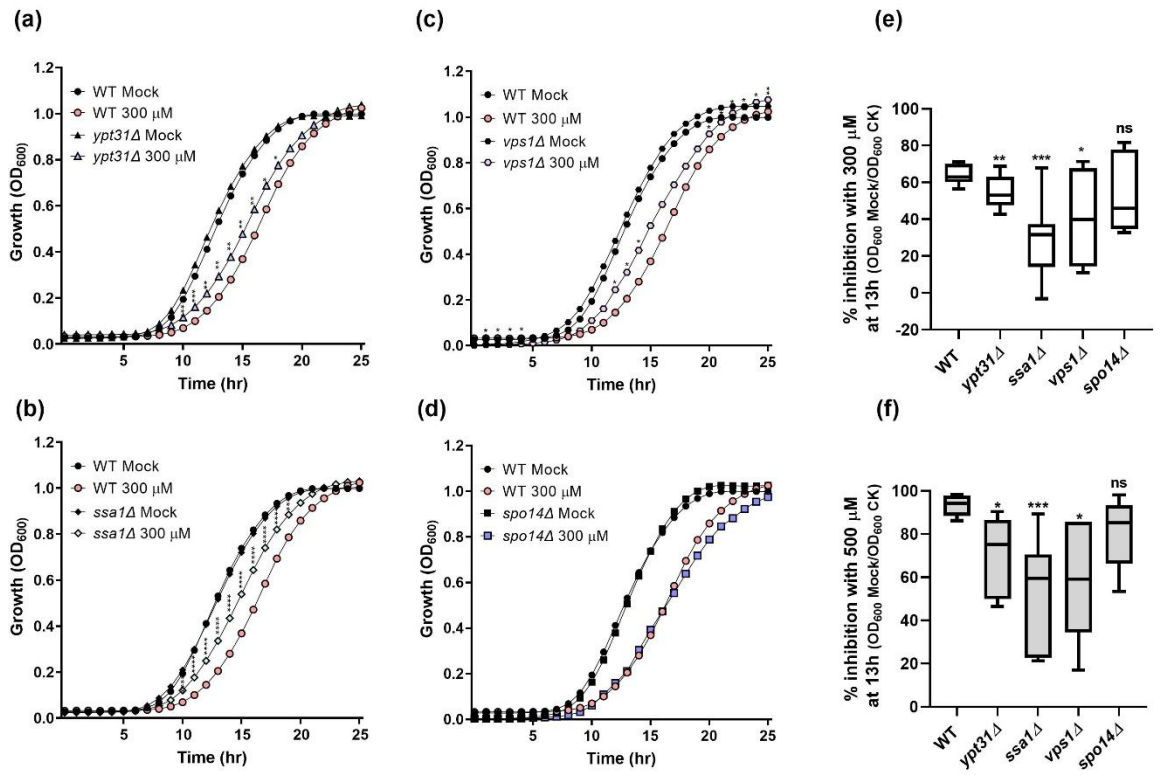

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