New Phytologist

Research

Moniliophthora perniciosa, the causal agent of witches' broom disease of cacao, interferes with cytokinin metabolism during infection of Micro-Tom tomato and promotes symptom development

Juliana L. Costa[1]*, Daniele Paschoal[1,2]*, Eder M. da Silva[1], Jamille S. Silva[1,2], Rafael M. do Carmo[1], Esther Carrera[3], Isabel López-Díaz[3], Mônica L. Rossi[1], Luciano Freschi[4], Piotr Mieczkowski[5], Lazaro E. P. Peres[2], Paulo J. P. L. Teixeira[2,5] and Antonio Figueira[1]

[1]Centro de Energia Nuclear na Agricultura, Universidade de São Paulo, Av. Centenário 303, Piracicaba, SP 13400-970, Brazil; [2]Escola Superior de Agricultura "Luiz de Queiroz", Universidade de São Paulo, Av. Pádua Dias 9, Piracicaba, SP 13418-900, Brazil; [3]Instituto de Biología Molecular y Celular de Plantas, Universitat Politècnica de València (UPV), Consejo Superior de Investigaciones Científicas (CSIC), Ingeniero Fausto Elío s/n, Valencia 46022, Spain; [4]Instituto de Biociências, Universidade de São Paulo, R. do Matão 321, São Paulo, SP 05508-090, Brazil; [5]University of North Carolina at Chapel Hill, Chapel Hill, NC 27599-7264, USA

Author for correspondence
Antonio Figueira
Email: figueira@cena.usp.br

Received: *30 January 2021*
Accepted: *25 March 2021*

New Phytologist (2021) **231:** 365–381
doi: 10.1111/nph.17386

Key words: cytokinin inhibitor, cytokinin oxidase, fruit locule number, hormonal imbalance, M82, Micro-Tom, tRNA-isopentenyl transferase, witches' broom disease.

Summary

- *Moniliophthora perniciosa* causes witches' broom disease of cacao and inflicts symptoms suggestive of hormonal imbalance. We investigated whether infection of the tomato (*Solanum lycopersicum*) model system Micro-Tom (MT) by the Solanaceae (S)-biotype of *Moniliophthora perniciosa*, which causes stem swelling and hypertrophic growth of axillary shoots, results from changes in host cytokinin metabolism.
- Inoculation of an MT-transgenic line that overexpresses the Arabidopsis *CYTOKININ OXIDASE*-2 gene (*35S::AtCKX2*) resulted in a reduction in disease incidence and stem diameter. RNA-sequencing analysis of infected MT and *35S::AtCKX2* revealed the activation of cytokinin-responsive marker genes when symptoms were conspicuous.
- The expression of an *Moniliophthora perniciosa tRNA-ISOPENTENYL-TRANSFERASE* suggests the production of isopentenyladenine (iP), detected in mycelia grown *in vitro*. Inoculated MT stems showed higher levels of dihydrozeatin and *trans*-zeatin but not iP. The application of benzyladenine induced symptoms similar to infection, whereas applying the cytokinin receptor inhibitors LGR-991 and PI55 decreased symptoms.
- *Moniliophthora perniciosa* produces iP that might contribute to cytokinin synthesis by the host, which results in vascular and cortex enlargement, axillary shoot outgrowth, reduction in root biomass and an increase in fruit locule number. This strategy may be associated with the manipulation of sink establishment to favour infection by the fungus.

Introduction

Pathogens have developed the ability to alter hormone signalling to promote virulence and/or induce susceptibility by altering host physiology (Spallek *et al.*, 2018; Han & Kahmann, 2019). Cytokinins are responsible for regulating several mechanisms concerning plant growth and development, including the maintenance and differentiation of root and stem meristems, cell division, vascular differentiation, delay of senescence, and source-sink relationships (Werner & Schmülling, 2009). More recently, the role of cytokinins in plant abiotic and biotic stress responses has been demonstrated (Cortleven *et al.*, 2019).

Natural plant cytokinins include N^6-(Δ^2-isopentenyl)adenine (iP), *trans*-zeatin (tZ), *cis*-zeatin (cZ), and dihydrozeatin (DHZ). Cytokinins are derived from substitutions at the N^6-terminus of an adenine or from the degradation of transfer RNAs (tRNAs) (Sakakibara, 2006). In brief, isopentenyl transferases (IPTs) either catalyse the linking of an isopentenyl to adenosine diphosphate (ADP) or adenosine triphosphate (ATP) or metabolize tRNAs, producing iP and cZ, respectively. tZ is then derived from the hydroxylation of iP by cytochrome monooxygenase (Kiba *et al.*, 2013). DHZ is considered to be derived from the reduction of tZ (Martin *et al.*, 1989), but new evidence suggests that DHZ biosynthesis occurs independently from the other pathways (Hošek *et al.*, 2020). The endogenous level of cytokinins is regulated by inactivation via conjugation or

*These authors contributed equally to this work.

degradation by cytokinin oxidases (CKXs) (Sakakibara, 2006). Histidine kinase (HK) receptors sense cytokinin and self-phosphorylate to transduce a signal to response regulators (RRs) by histidine phosphotransfer proteins (Argueso *et al.*, 2009).

Cytokinins may act as positive regulators of pathogen virulence (Chanclud & Morel, 2016). Symptoms of *Rhodococcus fascians* infection in Arabidopsis are primarily dependent on cytokinin perception through the receptors ARABIDOPSIS HISTIDINE-KINASE-3 (AHK3) and CYTOKININ RESPONSE-1 (CRE1)/AHK4 (Pertry *et al.*, 2009). *Rhodococcus fascians* synthesizes iP, tZ and cZ and their 2-methylthio derivatives, and inoculation of an Arabidopsis *ahk3/ahk4* mutant did not result in any symptoms (Pertry *et al.*, 2009). Fungal pathogens can produce cytokinin or cytokinin-like molecules to manipulate hosts (Walters & McRoberts, 2006; Chanclud & Morel, 2016). In *Claviceps purpurea*, there is overlap between the *de novo* and tRNA-IPT pathways for the synthesis of iP and tZ required for fungal colonization (Hinsch *et al.*, 2016). Similarly, in the rice pathogen *Magnaporthe oryzae,* mutation of the *tRNA-IPT* gene (*CKS1*) increased the plant defence response and decreased the amount of sugars and amino acids available for fungal use, suggesting a role for cytokinins as pathogen effectors (Chanclud *et al.*, 2016).

Witches' broom is a major disease of cacao (*Theobroma cacao* L.) that causes severe yield losses in cacao producing countries of South America (Marelli *et al.*, 2019). The disease is caused by the peculiar basidiomycete pathogen *Moniliophthora perniciosa*. The pathogen can infect only active meristematic tissues, causing symptoms in shoots, flower cushions and pods. Drastic shoot swelling with abnormal hypertrophic growth of axillary shoots ('brooms') is the most remarkable symptom, but infection of flower cushions can cause the reversion to a vegetative shoot and the production of parthenocarpic pods, all potentially associated with host hormone imbalance (Melnick *et al.*, 2012; Teixeira *et al.*, 2014). The overall debilitation of trees by intense shoot flushing, together with pod rot, is considered the major cause of crop losses (Purdy & Schmidt, 1996).

Pathogens can be categorized according to their lifestyles: biotrophs or hemibiotrophs depend on host living tissue, but hemibiotrophs eventually switch to a necrotrophic stage that kills host cells to feed from the dead tissue (de Silva *et al.*, 2016). *Moniliophthora perniciosa* is an unusual hemibiotroph with a peculiar long biotrophic phase (over 30 d) during which significant alterations of host morphology and physiology occur (Teixeira *et al.*, 2015). The germinating spores develop as monokaryotic mycelia to intercellularly colonize living tissues (Sena *et al.*, 2014). After a few weeks, the pathogen hyphae become dikaryotic by forming a short hyphal connection between adjacent cells (clamp connection), which enables the binuclear state of the fungal cells. Dikaryotization is triggered by an undetermined factor, while the cacao tissues become necrotic (Sena *et al.*, 2014).

Because the symptoms of *Moniliophthora perniciosa* infection of cacao imply a hormonal imbalance, the roles of various classes of hormones, including auxins (Krupasagar & Sequeira, 1969) and gibberellins (Dudman & Nichols, 1959; Mondego *et al.*, 2008; Teixeira *et al.*, 2014), have been long investigated, but without a sound association established. Although cytokinins are obvious candidates to induce witches' broom symptoms, this class of hormones used to be difficult to detect. Only one study reported the detection of tZ-riboside in cacao 'green brooms' via enzyme-linked immunosorbent assay (ELISA) (Orchard *et al.*, 1994). Therefore, the pathogenic strategies by which *Moniliophthora perniciosa* interferes with host hormone balance are still poorly understood.

Cacao, as a perennial tree species, is a challenging system to investigate, and its interaction with *Moniliophthora perniciosa* involves two nonmodel organisms (Teixeira *et al.*, 2014). *Moniliophthora perniciosa* can infect many hosts, particularly Solanaceae species, inducing shoot symptoms similar to those in cacao (Lisboa *et al.*, 2020). Isolates that infect cacao are considered to be from the C-biotype, whereas those that can infect Solanaceae species are classified as the S-biotype (Pierre *et al.*, 2017). We previously demonstrated that inoculation of the tomato (*Solanum lycopersicum*) cultivar Micro-Tom (MT) with an isolate of the S-biotype of *Moniliophthora perniciosa* induces typical symptoms of cacao infection at the biotrophic stage (e.g. stem swelling with outgrowth of axillary shoots), and we proposed using MT as a model system (Deganello *et al.*, 2014). Here, we demonstrate that the S-biotype of *Moniliophthora perniciosa* produces cytokinins *in vitro* and interferes with the cytokinin balance in MT plants to induce typical witches' broom disease symptoms, plus a reduction in root biomass and an increase in the number of tomato fruit locules. This strategy may be associated with the manipulation of sink formation in favour of fungal infection and metabolism.

Materials and Methods

Plant materials

All experiments used MT, and some experiments were repeated with M82 as a nonminiature control cultivar. MT and transgenic lines and mutants associated with hormone perception or response introgressed into the MT background (Table 1; Carvalho *et al.*, 2011) were used in a screening experiment. We inoculated MT-*ARR5::uidA* (GUS) to determine cytokinin signalling after infection (D'Agostino *et al.*, 2000). Seeds from all genotypes were germinated in a 1 : 1 mixture of substrate : expanded vermiculite (Carvalho *et al.*, 2011). The plants were grown in a growth chamber at 25°C, a 14 h photophase and 80% humidity.

Isolates of *Moniliophthora perniciosa* and spore production

Dry brooms from the Tiradentes isolate were obtained from naturally infected *S. lycocarpum* plants from Tiradentes (21°11′9.4″ S 44°19′15.8″W), MG, Brazil. Basidiomata of the isolates WMA5 and APS1 were obtained by the pie-dish method (Pierre *et al.*, 2017). Pilei from basidiomata produced after exposure of the brooms or the pie-dishes to alternating wet–dry cycles (12 h) were placed over storage buffer (16% glycerol; 0.01 M 2-(*N*-morpholino)ethanesulphonic acid (pH 6.1); 0.01% Tween 20) to collect spores (Frias *et al.*, 1995) for storage in liquid nitrogen.

Table 1 Transgenic lines and mutants associated with hormone perception and response introgressed into Micro-Tom (MT) tomato (*Solanum lycopersicum* L.).

Mutants	Hormonal class	Description/phenotype
diageotropica	Auxin	Reduced auxin sensibility. Cyclophilin defective protein (Oh *et al*., 2006).
entire	Auxin	Increased auxin sensibility. *La2922* defective protein, involved in auxin transcription pathway (Zhang *et al*., 2007).
epinastic	Ethylene	Increase production of ethylene. Unknown gene function (Fujino *et al*., 1988).
Never ripe	Ethylene	Low ethylene sensibility. Ethylene receptor defective protein (Wilkinson *et al*., 1995).
procera	Gibberellin	Hypersensitive to gibberellin. Loss of function of DELLA protein repression domain (Bassel *et al*., 2008).
Transgenic lines		
35S::PS	Jasmonic acid	Increased levels of jasmonic acid. Overexpression of *PROSYSTEMIN*, positive regulator of jasmonic acid signalling pathway (McGurl *et al*., 1994).
35S::nahG	Salicylic acid	Low levels of salicylic acid. Expression of *NAHG* gene, which catalyses the conversion of salicylic acid into catechol (Brading *et al*., 2000).
35S::AtCKX2	Cytokinin	Low levels of cytokinin. Overexpression of *AtCKX2* gene (Werner *et al*., 2003; Pino *et al*., 2010).

The basidiospore concentration was estimated in a Neubauer chamber.

Inoculation of MT, mutants and transgenic lines for their response to *Moniliophthora perniciosa*

The shoot apices and axillary buds of 17-d-old tomato seedlings of all genotypes were inoculated with 70 µl of 10^6 basidiospores in 0.3% agar–water (w/v) and kept in a humid chamber for 48 h. Control plants were treated with 0.3% agar–water. Symptoms were evaluated at 5, 15, 25 and 35 d after inoculation (dai). Disease symptoms were visually determined, and the stem diameter was measured between the first and second leaves with a digital pachymeter. Root dry weight was determined at 45 dai. The number of locules per fruit was determined at 35 dai. Data on disease incidence were transformed into $\sqrt{x}+1$ values for statistical analysis. The first experiment tested the isolates Tiradentes, WMA5 and APS1 in a completely randomized design ($n=66$). Significant differences were determined by one-way analysis of variance (ANOVA), and the means were compared by the Tukey test using Statistical Analysis System. SAS (SAS Institute, 1999). The genotype screening was conducted in a completely randomized design with nine genotypes × two inoculation conditions as factorials ($n=40$), and the data were subjected to two-way ANOVA; means were compared by the Tukey test using SAS.

Histological analysis

Stems from inoculated MT or *35S::AtCKX2* plants and the respective noninoculated controls were collected at 35 dai. The stem fragments between the first and second leaves (symptomatic or not) were sectioned using a microtome (SM200R; Leica, Heidelberg, Germany). The stem sections were then clarified in 50% sodium hydroxide (NaOH) and stained with 0.05% toluidine blue. The sections were visualized under a stereomicroscope with apochromatic optics (S8AP0; Leica). The areas of the pith, xylem, phloem and cortex were quantified using IMAGEJ (Schneider *et al*., 2012) ($n=3$). The estimated area for each tissue was subjected to one-way ANOVA; means were compared by the Tukey test using SAS.

Histochemical assay of *ARR5:uidA* expression in response to infection

The line MT-*ARR5*::*uidA*, which contains the reporter gene *uidA* (GUS) under the control of the cytokinin-responsive promoter *ARR5* (D'Agostino *et al*., 2000), was inoculated or not as described. Whole-plant samples were collected at 24 h after inoculation (hai) ($n=5$). Plant tissues were placed into GUS buffer (Jefferson *et al*., 1987), subjected to vacuum for 5 min, incubated at 37°C for 12 h, and then washed with 70% ethanol until tissue clarification. The samples were then observed under a stereomicroscope with apochromatic optics (S8AP0; Leica).

RNA extraction and RNA-sequencing analysis

Total RNA was extracted using TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA). Five biological replicates of stems from inoculated or noninoculated MT or *35S::AtCKX2* plants were collected at 5, 10, 20, and 30 dai. RNA-sequencing (RNA-seq) analysis was conducted at the High-Throughput Sequencing Facility at the University of North Carolina, Chapel Hill, NC, USA. Libraries were constructed from messenger RNA (mRNA) using 1 µg of total RNA via a KAPA Stranded RNA-seq kit for Illumina platforms (Kapa Biosystems, Wilmington, MA, USA). The insert size was approximately 200–300 bp, and dual adapters (2D, TruSeq RNA adapter plate, Illumina, San Diego, CA, USA) were used. Each pool was sequenced in one lane of the flow cell in single-end 50 bp reads using a HiSeq 4000 sequencer (Illumina). The read quality was evaluated using FASTQC (Andrews, 2010), and adapters were removed using Trimmomatic (Bolger *et al*., 2014). The cleaned reads were mapped against the reference tomato genome ITAG3.1 (Sato *et al*., 2012) using HISAT2 (Kim *et al*., 2015). Reads were counted with the featureCounts function from the subread package (Liao *et al*., 2013). The reads that mapped to exons were used for differential expression analysis. A one-way experimental design was adopted to compare five libraries from inoculated MT plants vs those of noninoculated plants at each time point using a generalized linear model implemented in EDGER (Robinson *et al*., 2010). The Benjamini–Hochberg method (false discovery rate, FDR) was applied to correct the *P*-values after performing multiple comparisons (Benjamini & Hochberg, 1995). Genes with an

FDR ≤ 0.01 (1%) and a fold-change ≥ 2 were considered differentially expressed. We defined a set of 148 cytokinin marker genes whose expression was significantly induced in tomato leaves at 2 and 24 h after treatment with 5 μM benzyladenine (BA) (Shi *et al.*, 2013). We used ComplexHeatmap v.2.0.0 (R v.3.5.3) to construct heatmaps based on Euclidean distances and to perform a hierarchical cluster analysis to categorize the cytokinin marker genes by their expression profiles. RNA-seq reads and the count matrix are available at the National Centre for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) under the accession number GSE153345.

Hormone quantification

Samples of *Moniliophthora perniciosa* mycelia and stem tissues of inoculated MT and *35S::AtCKX2* plants were used to estimate cytokinin (iP, tZ, DHZ), indole-3-acetic acid (IAA), salicylic acid (SA), and jasmonic acid (JA) levels. Stem sections between the first and second leaves were collected at 5, 10, 20 and 30 dai ($n = 5$ per time point), frozen, and ground in a cryogenic grinder (Cryo Mill; Retsch, Haam, Germany). Mycelia of the Tiradentes isolate grown in MYEA media (30 g l^{-1} agar; 30 g l^{-1} malt extract; 5 g l^{-1} yeast extract; pH 6.8) for 15 d were frozen, dried and ground. Hormones were extracted as described by Martínez-Bello *et al.* (2015). Briefly, the samples were homogenized in 80% methanol with 1% acetic acid, the solution of which contained deuterated hormones as internal standards. The mixture was then mixed for 1 h at 4°C and incubated at −20°C overnight. The extracts were filtered through an Oasis-HLB cartridge (Seo *et al.*, 2011). The eluate was then dissolved in 5% acetonitrile : 1% acetic acid and injected into a UPHL chromatograph (Accucore RP-MS column 2.6 μm, 50 × 2.1 mm; Thermo Fisher Scientific). The hormones were separated using a 5–50% acetonitrile gradient containing 0.05% acetic acid at 400 μl min^{-1} for 14 min. Hormone quantification was performed using a Q-Exactive mass spectrometer (Q-Exactive, Orbitrap detector, Thermo Fisher Scientific) in conjunction with internal standards, calibration curves and the Xcalibur 2.2 SP1 build 48 Tracefinder programs. The experiment was conducted in a completely randomized design at four time points × two inoculation conditions ($n = 5$). A second analysis was conducted comparing the MT and *35S::AtCKX2* genotypes, with or without inoculation (2 × 2 factorial) at 30 dai. The data were subjected to two-way ANOVA, and the means were compared by the Tukey test using SAS.

Exogenous application of 6-benzyladenine (BA) or the cytokinin receptor inhibitors LGR-991 and PI-55

LGR-991 (6-(2,5-dihydroxybenzylamino)purine) and PI-55 (6-(2-hydroxy-3-methylbenzylamino)purine), which are synthetic cytokinin inhibitors that block AHK3 and AHK4, respectively (Spíchal *et al.*, 2009; Nisler *et al.*, 2010), were kindly provided by Professor Lukás Spíchal. Stock solutions of 1 M BA dissolved in dimethyl sulphoxide (DMSO) and 20 mM solutions of LGR-991 and PI-55 were diluted in lanolin (99 : 1) heated to 55°C. A preliminary experiment tested increasing doses of LGR-991 or PI-55 (50, 100 or 200 μM) applied to MT plants ($n = 15$) with or without inoculation, as described previously. Briefly, 12 μl of 20 mM BA or 12 μl of 100 μM LGR-991 or PI-55 was applied to the shoot apices and axillary buds of MT plants ($n = 15$), previously inoculated with *Moniliophthora perniciosa*, every 10 d after the first application, while control plants received DMSO on 99 : 1 lanolin. Similarly, 12 μl of 20 mM BA or 12 μl of 100 μM PI-55 was applied to M82 plants ($n = 10$), with identical controls. The stem diameter between the first and second leaves was evaluated at 15, 25, and 35 d after application (daa) of either compound, whereas the root biomass and number of locules per fruit were determined at 55 daa for the BA experiment. Both experiments were completely randomized, and the data were subjected to one-way ANOVA. The means were compared by Tukey's test using Sas. Stem fragment samples from the BA experiment using MT were collected at 35 dai for histological analysis ($n = 3$) as described.

Results

Infection of MT by *Moniliophthora perniciosa* reduces root biomass and increases the number of fruit locules

We previously characterized the infection of MT by the Tiradentes isolate of the S-biotype of *Moniliophthora perniciosa* (Deganello *et al.*, 2014). Here, APS1 and WMA5 (Pierre *et al.*, 2017) plus Tiradentes were evaluated for their aggressiveness, which was estimated by the disease incidence assessed at 40 dai, changes in stem diameter, root biomass, and fruit locule number of the infected MT plants (Fig. 1). Plants inoculated with the Tiradentes isolate showed a significantly higher disease incidence than those inoculated with APS1 or WMA5 (Fig. 1a). The increase in stem diameter in inoculated plants occurred more prominently after 20 dai, and plants inoculated with Tiradentes presented a more pronounced increase in stem diameter than those inoculated with the other two isolates (Fig. 1b).

Plants inoculated with Tiradentes (Fig. 1g) and WMA5 (Fig. 1f) presented significantly less root biomass (Fig. 1c) than did the controls (Fig. 1d). We also noticed a change in fruit morphology, in which there was a significant increase in the number of locules per fruit of inoculated MT compared with those produced by noninoculated plants (Supporting Information Fig. S1c–e). Uninfected MT predominantly produced fruits with two or three locules (mean of 3 locules per fruit, ranging from 2 to 5; Fig. S1a,b). The fruits of MT inoculated with Tiradentes had on average 4.7 locules $fruit^{-1}$ (ranging from 2 to 10), which was significantly more than those of plants inoculated with APS1 (4.2 locules per fruit, ranging from 3 to 9) or WMA5 (3.9 locules per fruit, ranging from 2 to 8) (Fig. S1a). Since Tiradentes was shown to be more aggressive, all subsequent experiments were conducted using this isolate.

Inoculation of hormone mutants and transgenic lines with *Moniliophthora perniciosa* suggests the involvement of cytokinin in symptom development

To identify the classes of hormones that might be involved in pathogenesis during *Moniliophthora perniciosa* infection of

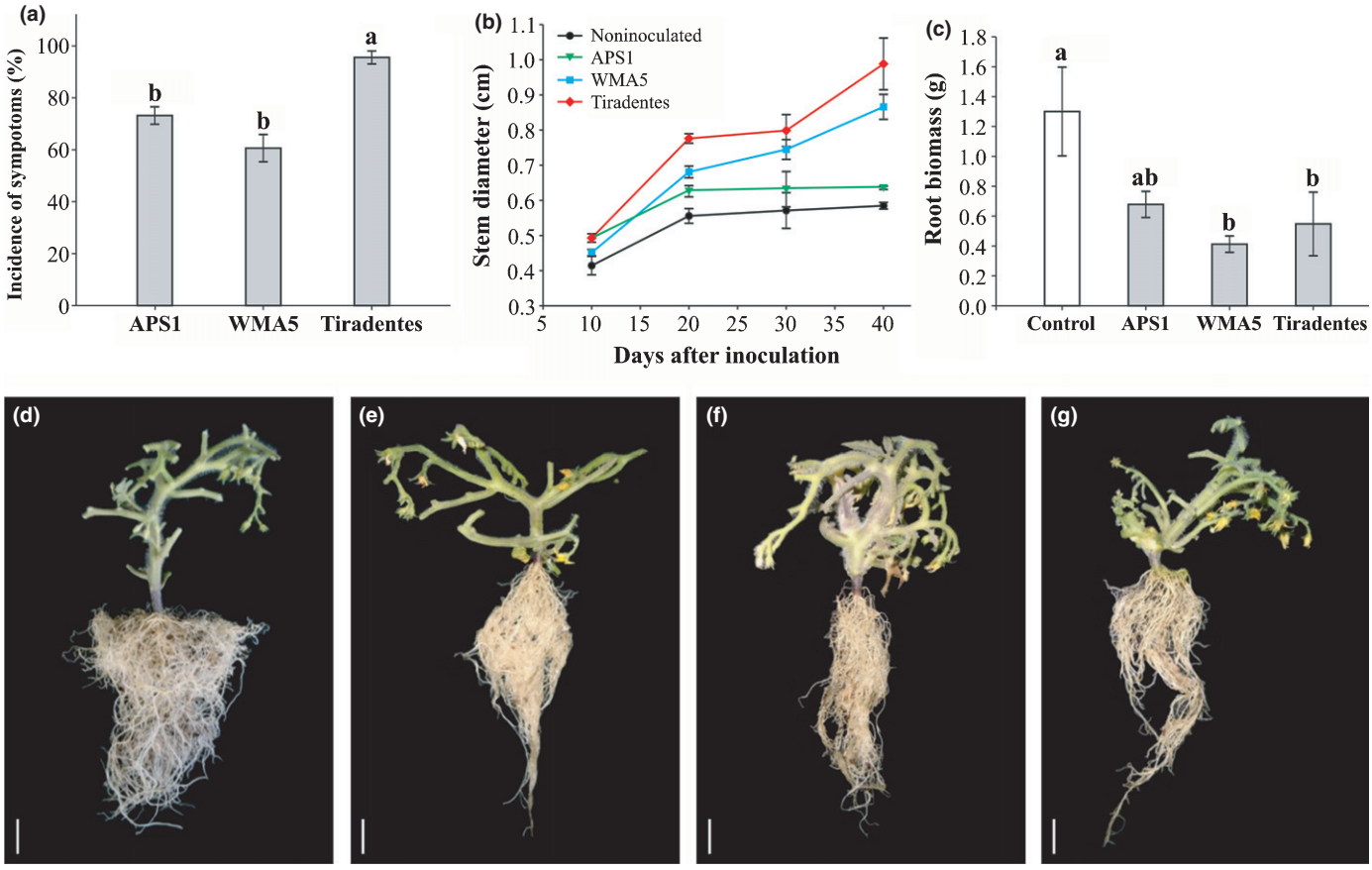

Fig. 1 Response of Micro-Tom (MT) tomato (*Solanumlycopersicum*) plants to inoculation by three isolates of the S-biotype of *Moniliophthoraperniciosa*. (a) Incidence of symptoms (%) in inoculated MT plants by isolates APS1, WMA5 and Tiradentes at 40 d after inoculation (dai) ($n = 66$: 6 replicates of 11-plant plots). (b) Stem diameter (cm) between the first and second leaves of inoculated plants compared to noninoculated at 10, 20, 30 and 40 dai ($n = 66$). (c) Root biomass (g dry weight) of inoculated MT plants compared to noninoculated at 45 dai ($n = 30$). The error bars represent SE. Statistically significant differences were determined by one-way ANOVA, followed by the Tukey test at 5% probability; different letters indicate statistically significant differences. (d) Representative image of noninoculated MT plant at 45 dai with leaves removed. (e) MT plant inoculated with APS1 at 45 dai with leaves removed. (f) MT plant inoculated with WMA5 at 45 dai with leaves removed. (g) MT plant inoculated with Tiradentes with leaves removed at 45 dai. Scale bar represents 2 cm (d–g).

tomato, we screened five mutants (*diageotropica*, *entire*, *epinastic*, *Never ripe*, and *procera*) and three transgenic lines (*35S::AtCKX2*, *35S::PS* and *35S::nahG*) with altered synthesis or perception of hormones, all in the MT genetic background (Table 1). Inoculation with *Moniliophthora perniciosa* significantly increased the stem diameter of all genotypes compared with that of the respective noninoculated control plants at 35 dai (Fig. 2a). Compared with all the other inoculated genotypes, the inoculated *35S::AtCKX2* plants presented the smallest increase in stem diameter. In addition, the genotypes that contrasted in terms of their auxin perception, *diageotropica* and *entire*, exhibited contrasting responses, with significant decreases or increases in stem diameter, respectively, in relation to those of inoculated MT plants (Fig. 2a). Regarding disease incidence, inoculated *35S::AtCKX2* was the only genotype with a significant reduction in incidence (Fig. 2b). Therefore, *35S::AtCKX2* seems to prevent symptom development.

There was no major difference in the number of locules in fruits from inoculated compared with noninoculated *35S::AtCKX2*; this was in contrast to the increase in the number of locules per fruit in the inoculated MT plants (Fig. S2). The low incidence of disease, less stem swelling and the absence of an infection effect on the number of locules per fruit suggest that *35S::AtCKX2* exhibits some tolerance to infection. Since *35S::AtCKX2* expresses a cytokinin oxidase, symptom development after infection by *Moniliophthora perniciosa* may be associated with increased levels of cytokinins, and the number of locules per fruit appears to be associated with alteration in the cytokinin balance.

To investigate which tissues correlated with the increase in stem diameter of inoculated plants, we performed a histological analysis of cross-sections of MT and *35S::AtCKX2* stems. We observed that the pathogen promoted a significant increase in the cell number and size of the pith (Fig. 3b,c,e–g), xylem (Fig. 3b,c, e,f,h), phloem (Fig. 3b,c,e,f,i), and cortex (Fig. 3b,c,e,f,j) in both genotypes compared with those of the respective noninoculated control plants (Fig. 3a,d,g–j). Compared with the inoculated *35S::AtCKX2*, the inoculated MT plants presented an increased area of pith (Fig. 3g), xylem (Fig. 3h), phloem (Fig. 3i), and cortex (Fig. 3j). Inoculation with *Moniliophthora perniciosa* may

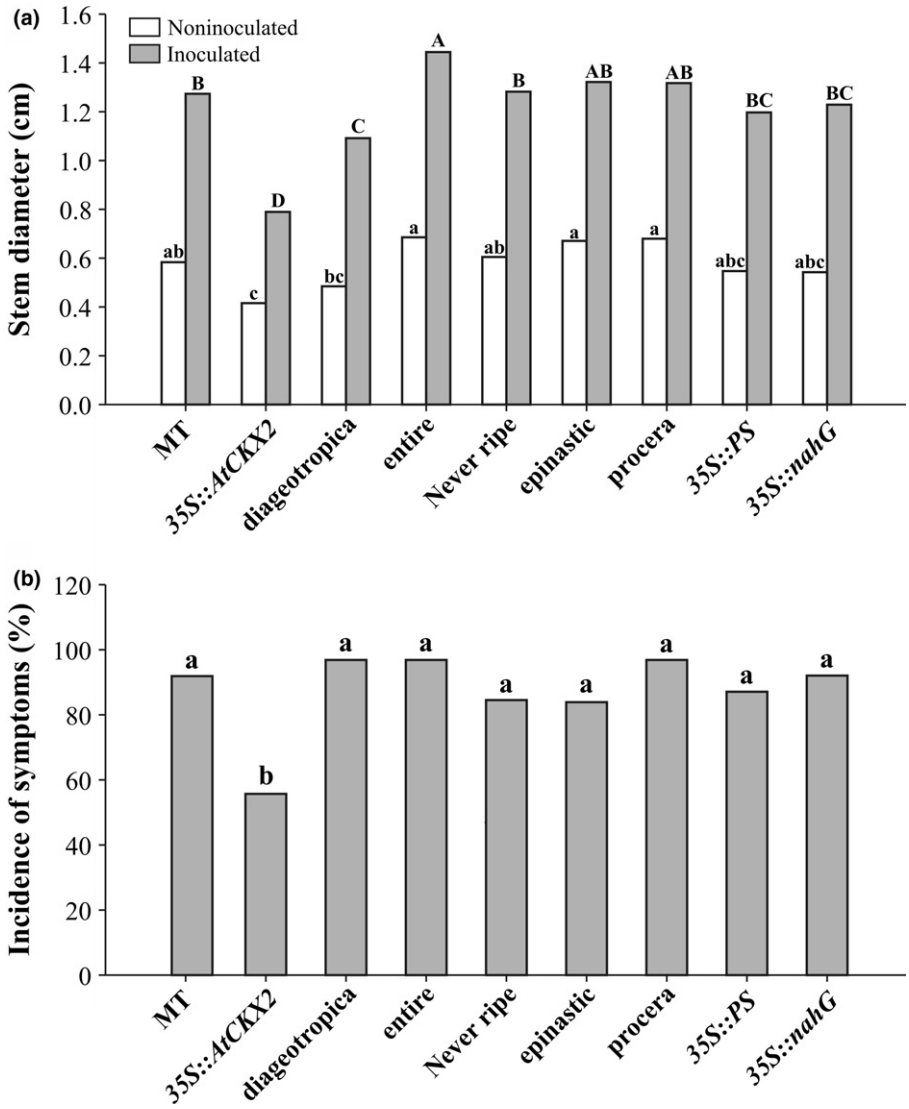

Fig. 2 Screening of mutants and transgenic lines associated with hormone metabolism or perception in the Micro-Tom (MT) tomato (*Solanumlycopersicum*) background for response to inoculation by the Tiradentes isolate of the S-biotype of *Moniliophthoraperniciosa*. (a) Mean of stem diameter (cm) between the first and second leaves of plants from mutants (*diageotropica*, *entire*, *Neverripe*, *epinastic* and *procera*) and transgenic lines (*35S::AtCKX2,35S::PS* and *35S::nahG*) introgressed into MT, inoculated with the Tiradentes isolate of the S-biotype of *Moniliophthoraperniciosa* compared with noninoculated control plants. Statistically significant differences were determined by two-way ANOVA, followed by the Tukey test ($P < 0.05$) ($n = 40$); different uppercase letters indicate significant mean differences among the inoculated genotypes, and lowercase letters indicate significant mean differences among noninoculated genotypes. Inoculation significantly increased stem diameter for all genotypes. (b) Incidence of symptoms (%) estimated by the rate of inoculated plants expressing visible symptoms ($n = 40$: 4 replicates of 10-plant plots); different letters indicate significant differences among inoculated genotypes.

promote hyperplasia and hypertrophy of vascular tissues, leading to stem thickening through the action of cytokinin and, possibly, auxin.

Moniliophthora perniciosa infection of *ARR5::GUS* indicates cytokinin signalling

We inoculated the MT-*ARR5::GUS* line (D'Agostino *et al.*, 2000) with *Moniliophthora perniciosa* basidiospores. We detected differential cytokinin signalling in response to infection in inoculated *ARR5::GUS* plants at 24 hai (Fig. 4g–l), compared to that in noninoculated plants (Fig. 4a–f). Increased differential histochemical expression of GUS was detected in the roots (Fig. 4g), hypocotyls (Fig. 4h), stems (Fig. 4i), leaf veins (Fig. 4j,k), and shoot apices (Fig. 4l) in the inoculated plants compared with the controls (Fig. 4a–f). The differential cytokinin signalling in response to infection corroborates the increase in cytokinin levels/signalling during pathogenesis.

RNA-seq analysis revealed differentially expressed marker genes associated with cytokinin metabolism

We performed RNA-seq analysis to verify the expression profiles of genes associated with cytokinin metabolism in stems by comparing infected MT plants with noninoculated controls. For this, we evaluated a set of cytokinin marker genes previously defined as being induced in MT leaves after BA treatment (Shi *et al.*, 2013). Hierarchical clustering based on the gene expression profiles grouped these marker genes into seven clusters, each with a similar expression pattern during infection (Fig. 5a). The annotation and cluster classification of the 148 marker genes are given in Supporting Information Table S1; all differentially expressed genes are presented in Table S2. Clusters 1, 2 and 5 contained upregulated cytokinin marker genes compared to those in noninoculated plants, particularly at 20 and 30 dai (Fig. 5a). *CYTOCHROME-P450* (Solyc02g094860), orthologous to Arabidopsis cytokinin hydroxylase that converts N^6-

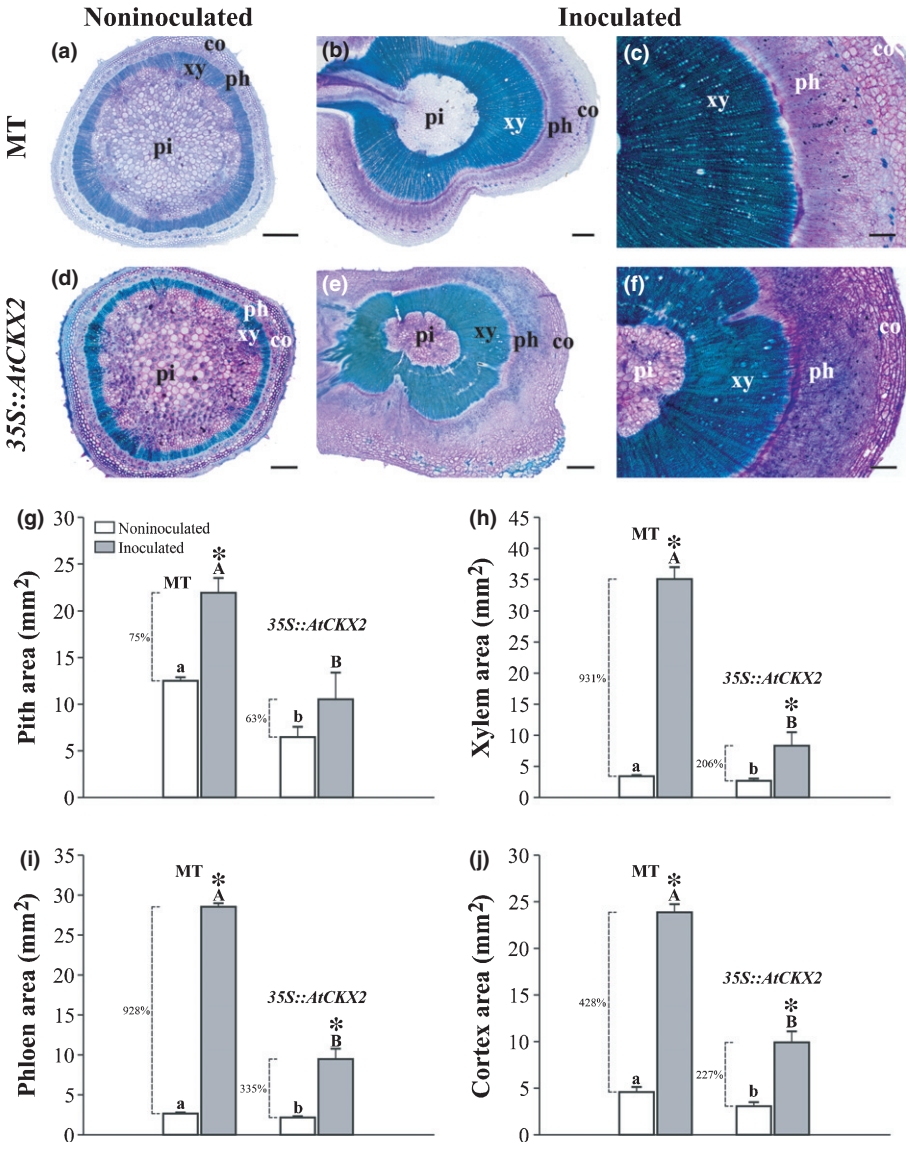

Fig. 3 Histology of stem cross-sections stained in 0.05% toluidine blue, with estimated tissue areas from Micro-Tom (MT) tomato (*Solanumlycopersicum*) and the transgenic line *35S::AtCKX2* inoculated or not with the Tiradentes isolate of the S-biotype of *Moniliophthoraperniciosa*. (a) noninoculated MT control; (b) and (c) inoculated MT; (d) noninoculated *35S::AtCKX2*; (e) and (f) inoculated *35S::AtCKX2*; areas expressed in mm^2 of stem cross-section of inoculated MT and *35S::AtCKX2* plants compared with noninoculated controls of the tissues: (g) pith; (h) xylem; (i) phloem; (j) cortex. The error bars represent SE. Statistically significant differences were determined by two-way ANOVA, followed by the Tukey test ($P < 0.05$) ($n = 3$); different uppercase letters indicate significant mean area differences between inoculated MT and *35S::AtCKX2* genotypes; lowercase letters indicate significant mean differences between noninoculated MT and *35S::AtCKX2* genotypes. Asterisks indicate significant difference between inoculated and noninoculated plants within the same genotype (MT or *35S::AtCKX2*). pi: pith, xy: xylem, ph: phloem, co: cortex. Bars: (a), (b) and (e): 1 mm; (c), (d) and (f): 0.5 mm.

isopentenyladenosine-5′-monophosphate (iPRMP) to tZ-riboside-monophosphate (Takei *et al.*, 2004; Kiba *et al.*, 2013), belongs to Cluster 1 (Fig. 5a). Purine permeases (Solyc02g071080; Solyc02g071100) and cytokinin oxidase/dehydrogenase-like genes (Solyc04g016430: orthologous to *AtCKX1*; Solyc01g088160) are members of Cluster 2. Upregulation of *CKXs* may indicate a feedback response to high levels of cytokinins, as previously observed in cacao shoots (Teixeira *et al.*, 2014) and flower cushions (Melnick *et al.*, 2012) infected by *Moniliophthora perniciosa*. Cluster 5 contains induced marker genes that encode cytokinin RRs (Solyc02g071220: orthologous to Arabidopsis ARR5 and ARR6-type-A; Solyc03g113720: orthologous to ARR4 and ARR3-type-A; Solyc05g006420: no orthologue in Arabidopsis but groups with type-ARRs) and a histidine-kinase (Solyc04g008110) orthologous to Arabidopsis AHK4 (Fig. 5a; Table S1). Clusters 4 and 7 comprise genes that encode an RR (Solyc06g048930: tomato RR16,17 orthologous to Arabidopsis ARR16 and ARR17), cytokinin-response factor (Solyc08g081960: orthologous to Arabidopsis CRF1, CRF2, CRF3, and CRF4), and adenine phosphoribosyltransferase-like (Solyc08g079020), whose product is known to inactivate iP to isopentenyladenosine-5′-monophosphate (Hošek *et al.*, 2020).

The expression profiles of Clusters 1, 2 and 5 are presented as box-plots, highlighting the effect of MT plant infection on each group of genes (Fig. 5b). Importantly, genes in Clusters 1, 2 and 5 were not strongly activated in inoculated *35S::AtCKX2* plants (Fig. 5b, last column), indicating that the responses after inoculation in MT are cytokinin-dependent.

We also detected the strong downregulation of two tomato genes encoding tRNA-dimethylallyltransferase (*tRNA-IPTs*) (Solyc09g064910.1; Solyc01g080150.3) in inoculated MT plants at 10 and 30 dai in relation to those in noninoculated controls (Fig. S3). However, the same *tRNA-IPT* gene (Solyc09g064910.1) was 30-fold less repressed in inoculated *35S::AtCKX2* at 30 dai, whereas the other *tRNA-IPT* (Solyc01g080150.3) was 5- and 15-fold less repressed at 10 and 20

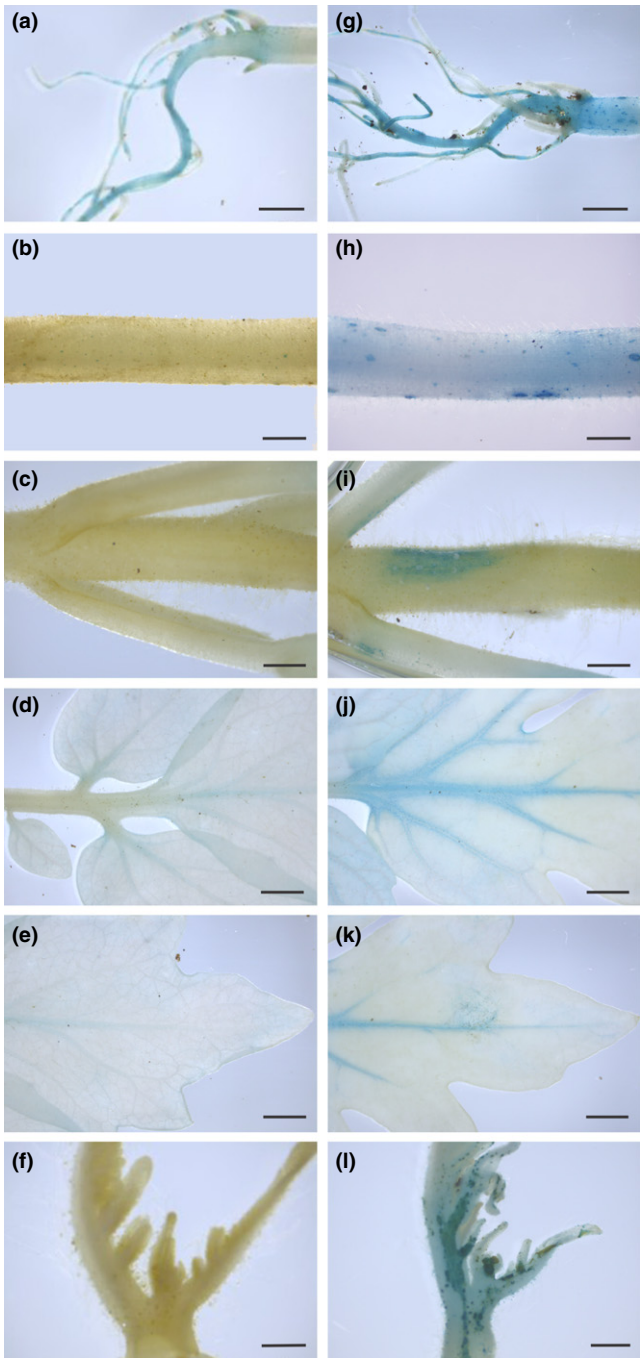

Fig. 4 Histochemical assay of *ARR5::uidA* (GUS) Micro-Tom (MT) tomato (*Solanumlycopersicum*). Histochemical assay of *ARR5::uidA* (GUS) of noninoculated control plants: (a) root, (b) hypocotyl, (c) stem, (d) petiole and leaf veins, (e) leaf veins, (f) shoot apex; and of plants inoculated with the Tiradentes isolate of the S-biotype of *Moniliophthoraperniciosa* at 24 h after inoculation (hai) (g) root, (h) hypocotyl, (i) stem, (j) petiole and leaf veins, (k) leaf veins, and (l) shoot apex. Samples were stained in GUS buffer for only 12 h at 37°C followed by clarification using 70% ethanol. Bars: (a), (d), (e), (g), (j) and (k): 2 mm; (b), (c), (h), (i), (l): 1 mm; (f): 0.5 mm.

dai, respectively, than in inoculated MT (Fig. S3). The difference between inoculated MT and *35S::AtCKX2* suggests that the inhibition of tomato *tRNA-IPT* expression is not needed in *35S::AtCKX2* plants with low cytokinin levels.

Notably, we detected the expression of an *Moniliophthora perniciosa tRNA-IPT* (MP02226) gene in our RNA-seq analysis of infected MT stems (Fig. S4). Mycelia of the C-biotype grown *in vitro* express this *tRNA-IPT* (MP02226) gene (Fig. S4), whereas analysis of the 'WBD Transcriptome Atlas' (Teixeira *et al.*, 2014) indicates that the C-biotype expresses MP02226 during the biotrophic infection of cacao (Fig. S4).

Altogether, the expression data suggest that *Moniliophthora perniciosa* could synthesize cytokinins via the *tRNA-IPT* pathway, as observed for other fungi (Chanclud *et al.*, 2016; Hinsch *et al.*, 2016). A phylogenetic analysis showed the clustering of the *Moniliophthora perniciosa* tRNA-IPT sequence with those from other plant pathogens that use this pathway to produce cytokinins as an effector (Fig. S5). Increased cytokinin levels in infected plants may explain the downregulation of tomato *tRNA-IPT*s. Since *35S::AtCKX2* degrades cytokinins, *Moniliophthora perniciosa* may require increased cytokinins to establish pathogenesis.

MT plants inoculated with *Moniliophthora perniciosa* and fungal mycelia accumulate cytokinin

We measured the levels of iP, DHZ and tZ in infected MT and *35S::AtCKX2* plants (Fig. 6). Compared with the noninoculated plants, the inoculated MT plants showed significantly less iP at 5 dai and no significant differences thereafter (Fig. 6a). However, we detected a significant increase in tZ (Fig. 6b) and DHZ levels (Fig. 6c) after 10 dai in inoculated MT compared to those in noninoculated controls. Notably, we detected the production of iP but no other type of cytokinin by *Moniliophthora perniciosa* dikaryotic mycelia (Fig. 6a). It is possible that *Moniliophthora perniciosa* produces iP via the *tRNA-IPT* biosynthesis pathway, as described for other pathogens (Siddique *et al.*, 2015), and this cytokinin could be part of a mechanism of pathogenesis (Chanclud *et al.*, 2016; Morrison *et al.*, 2017). Noninoculated *35S::AtCKX2* plants presented lower levels of iP (Fig. 6a) and DHZ than noninoculated MT plants at 30 dai (Fig. 6c). Compared with noninoculated plants, inoculated *35S::AtCKX2* plants showed only a modest increase in iP and DHZ levels (Fig. 6a,c). Since *35S::AtCKX2* overexpresses a cytokinin oxidase, this genotype likely degrades the cytokinins produced by the plant and/or the pathogen, preventing the development of typical symptoms of infection.

Additionally, we detected a significant increase in IAA, SA, and JA levels in inoculated MT compared with levels in noninoculated MT after 10 dai, and after 5 dai for SA and JA (Fig. S6). Compared with noninoculated controls, inoculated *35S::AtCKX2* showed a significant decrease in IAA (Fig. S6a) and SA (Fig. S6b). We detected IAA (Fig. S6a) and SA (Fig. S6b) in dikaryotic mycelia of *Moniliophthora perniciosa*, supporting previous reports (Chaves & Gianfagna, 2006; Kilaru *et al.*, 2007).

Exogenous application of BA mimics infection symptoms, while cytokinin receptor inhibitors reduce symptoms in MT plants

To mimic the presumed changes in cytokinin levels after infection with *Moniliophthora perniciosa*, we applied BA at the

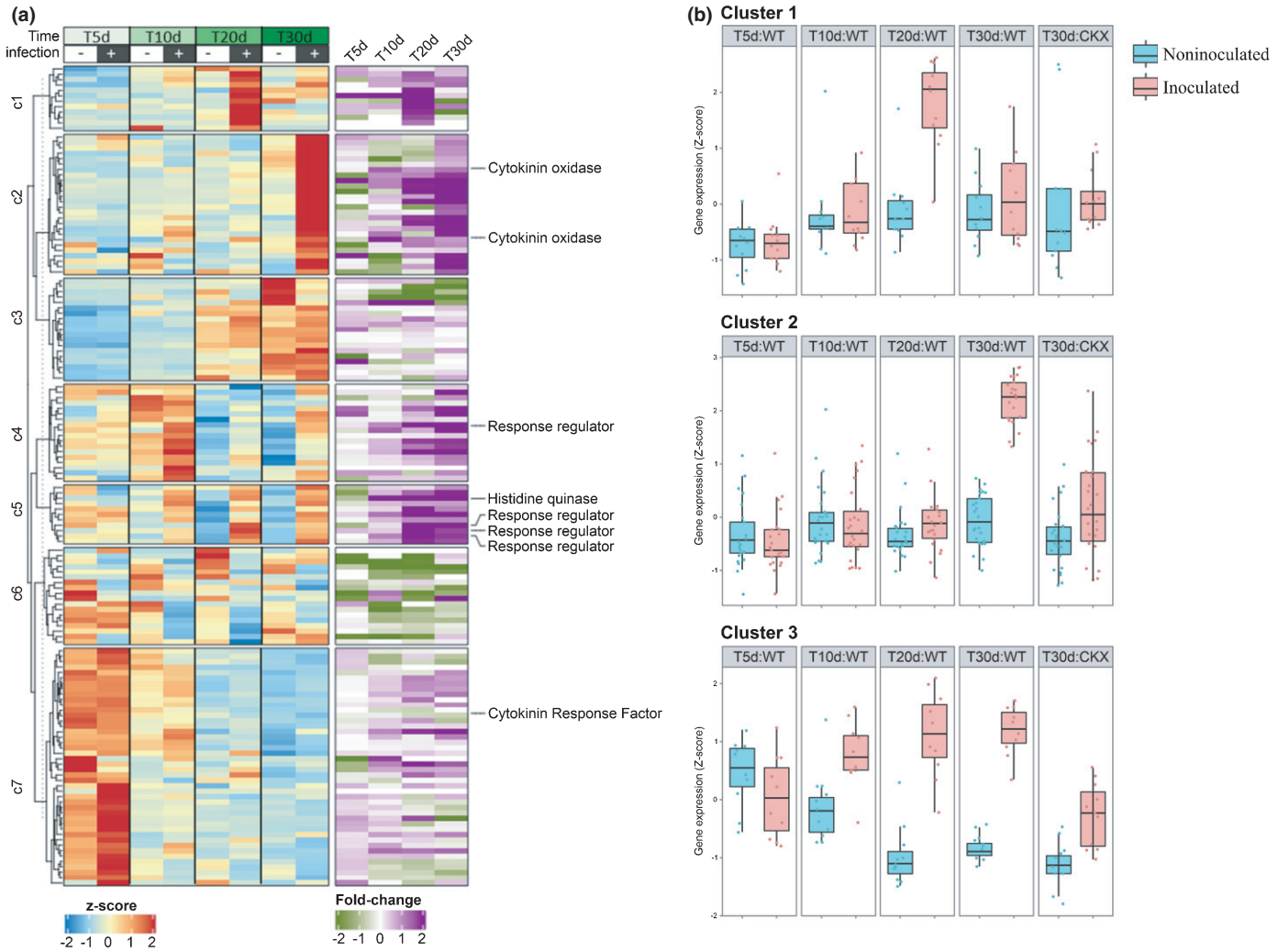

Fig. 5 Expression changes of tomato (*Solanumlycopersicum*) cytokinin marker genes in Micro-Tom (MT) after inoculation with the Tiradentes isolate of the S-biotype of *Moniliophthoraperniciosa*. (a) Heatmap of differentially expressed cytokinin marker genes (Shi *etal*., 2013) obtained via RNA-seq analysis of stems from infected MT compared to noninoculated MT at 5, 10, 20 and 30 d after inoculation (dai), with clustering analysis according to temporal expression profiles. Scale bar represents values in *z-score*-transformed RPKM values (at left) or fold-change (at right) ($n = 5$). The annotation for the highlighted genes (from the top to bottom of the heatmap) is as follow: Cluster 2 – cytokinin oxidase (Solyc04g016430 – orthologous to the Arabidopsis *AtCKX1*); cytokinin oxidase (Solyc01g088160); Cluster 4 – response regulator (RR) (Solyc06g048930 – Tomato RR 16,17 – orthologous to Arabidopsis ARR16 and ARR17); Cluster 5 – histidine kinase (Solyc04g008110 – orthologous to Arabidopsis AHK4, RR (Solyc02g071220 – type-A RR2 – orthologous to Arabidopsis ARR5 and ARR6 – type-A), RR (Solyc03g113720 orthologous to Arabidopsis ARR3 and ARR4 – type-A, RR (Solyc05g006420); Cluster 7: cytokinin response factor (Solyc08g081960 orthologous to Arabidopsis CRF1, CRF2, CRF3, and CRF4). (b) Gene expression pattern of the Clusters 1, 2 and 5 of infected MT compared to noninoculated control plants at 5, 10, 20 and 30 dai, and inoculated *35S::AtCKX2* compared to noninoculated at 30 dai (last column). Values are represented in z-score-transformed RPKM. Gene expression values are represented by the filled circles. In each boxplot, the middle line represents the median value, upper and lower bars correspond to the first and third quartiles, respectively, and whiskers represent 1.5 times the interquartile range. The complete annotation of the differentially expressed cytokinin marker genes can be found in Supporting Information Table S1. The complete list of all differentially expressed genes with their respective annotation can be found in Table S2.

equivalent site of inoculation of MT (Fig. 7) or M82 (Fig. S7) after inoculation or not with *Moniliophthora perniciosa*. Treatment of MT and M82 with BA induced symptoms similar to those in response to inoculation with the S-biotype, resulting in an increase in stem diameter at the site of treatment (Figs 7a,e, S7c,e) compared to that of noninoculated/mock-treated plants (Figs 7a,c, S7a,e). Application of BA to MT or M82 inoculated with *Moniliophthora perniciosa* promoted an even larger increase in stem diameter at the site of treatment and inoculation (Figs 7a, f, S7d,e) compared with that of inoculated plants without BA treatment (Figs 7a,d, S7b,e).

We verified the pattern of tissue proliferation in the stems of MT treated with BA by histology (Figs S8, S9). BA caused an increase in xylem, phloem and cortex tissues (Figs S8c,d, S9) compared with those of noninoculated/mock-treated plants (Figs S8a, S9), similar to tissues from the stems of MT inoculated with *Moniliophthora perniciosa* (Figs S8b, S9). In turn, plants inoculated and treated with BA presented an even larger increase in

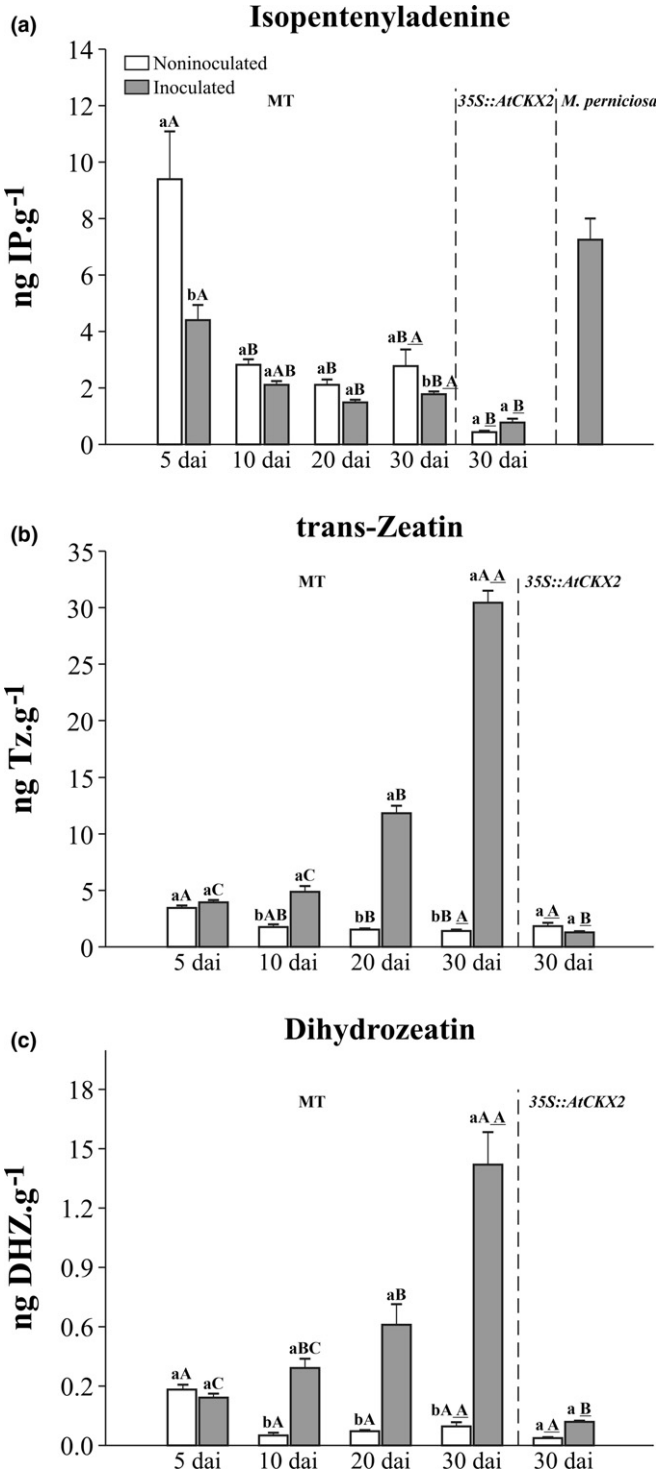

Fig. 6 Quantification of cytokinins in Micro-Tom (MT) tomato (*Solanumlycopersicum*) or *35S::AtCKX2* plants after inoculation with the Tiradentes isolate of the S-biotype of *Moniliophthoraperniciosa*, or in *invitro*-grown mycelia of the same *Moniliophthoraperniciosa* isolate. Quantification of cytokinins in MT plants occurred at 5, 10, 20 and 30 d after inoculation (dai), in *35S::AtCKX2* plants at 30 dai, or 15-day old *invitro* grown *Moniliophthoraperniciosa* mycelia. (a) Quantification of isopentenyladenine (iP) in ng g^{-1}; (b) *trans*-zeatin (tZ) in ng g^{-1}; (c) dihydrozeatin (DHZ) in ng g^{-1}. The error bars represent SE. Statistically significant differences were determined by two-way ANOVA, followed by the Tukey test ($P < 0.05$) ($n = 5$). Different lowercase letters indicate mean significant differences between inoculated or noninoculated plants within MT or *35S::AtCKX2* genotypes at each time point; different uppercase letters indicate mean significant differences among time points within the MT genotype at the same condition (inoculated or noninoculated); different underlined uppercase letters indicate mean significant differences between MT and *35S::AtCKX2* genotypes at the same condition (inoculated or noninoculated) at 30 dai.

phloem and cortex (Figs S8e,f, S9) than did noninoculated plants treated with BA (Figs S8c,d, S9) and inoculated and mock-treated plants (Figs S8b, S9).

The application of BA to inoculated MT or M82 led to a significant reduction in root biomass (Figs 7b, S7f). However, BA alone did not significantly decrease the root biomass compared with inoculation with *Moniliophthora perniciosa*. We also analysed the number of locules in fruits from MT treated or not with BA to verify whether the effects would resemble those of inoculated plants. The application of BA indeed increased fruit locule number, which varied from 1 to 6 locules $fruit^{-1}$ in BA-treated MT plants and from 2 to 8 locules $fruit^{-1}$ in MT inoculated with *Moniliophthora perniciosa* and treated with BA (Fig. S10).

To confirm whether cytokinin signalling is critical for symptom development in *Moniliophthora perniciosa*-infected plants, we tested two synthetic inhibitors of the cytokinin receptors, LGR-991 and PI-55 (Spíchal *et al.*, 2009; Nisler *et al.*, 2010). PI55 inhibits AHK4, blocking tZ action (Spíchal *et al.*, 2009), whereas LGR-991 inhibits both AHK4 and AHK3 (Nisler *et al.*, 2010). We applied LGR-991 or PI-55 to the shoot apices and axillary buds of MT plants that were previously inoculated (2 dai) or not (Fig. 8), as previously described (Roman *et al.*, 2016), via a dose tested in a previous experiment (Fig. S11). Treatment with PI55 or LGR-991 led to a significant decrease in stem diameter (Fig. 8g,h) compared with that of inoculated mock-treated MT plants (Fig. 8d,g,h). We repeated this experiment using M82 and PI55 (Fig. S12). PI55 led to a significant decrease in stem diameter at the site of treatment of inoculated M82 plants (Fig. S12d, e) compared with inoculated mock-treated M82 plants (Fig. S12b,e). Thus, plants with a limited cytokinin response presented less pronounced symptoms after inoculation with *Moniliophthora perniciosa* than mock-treated inoculated plants. These results corroborate the fundamental role of cytokinins during symptom development in *Moniliophthora perniciosa* infection.

Discussion

Witches' broom is a typical symptom of various plant diseases and is characterized by excess brush-like outgrowth of shoots (Agrios, 2005). Witches' broom symptoms have been associated with the production/induction of cytokinins by invading organisms (Kilaru *et al.*, 2007). In cacao, witches' broom disease has long been suggested to result from a hormone imbalance

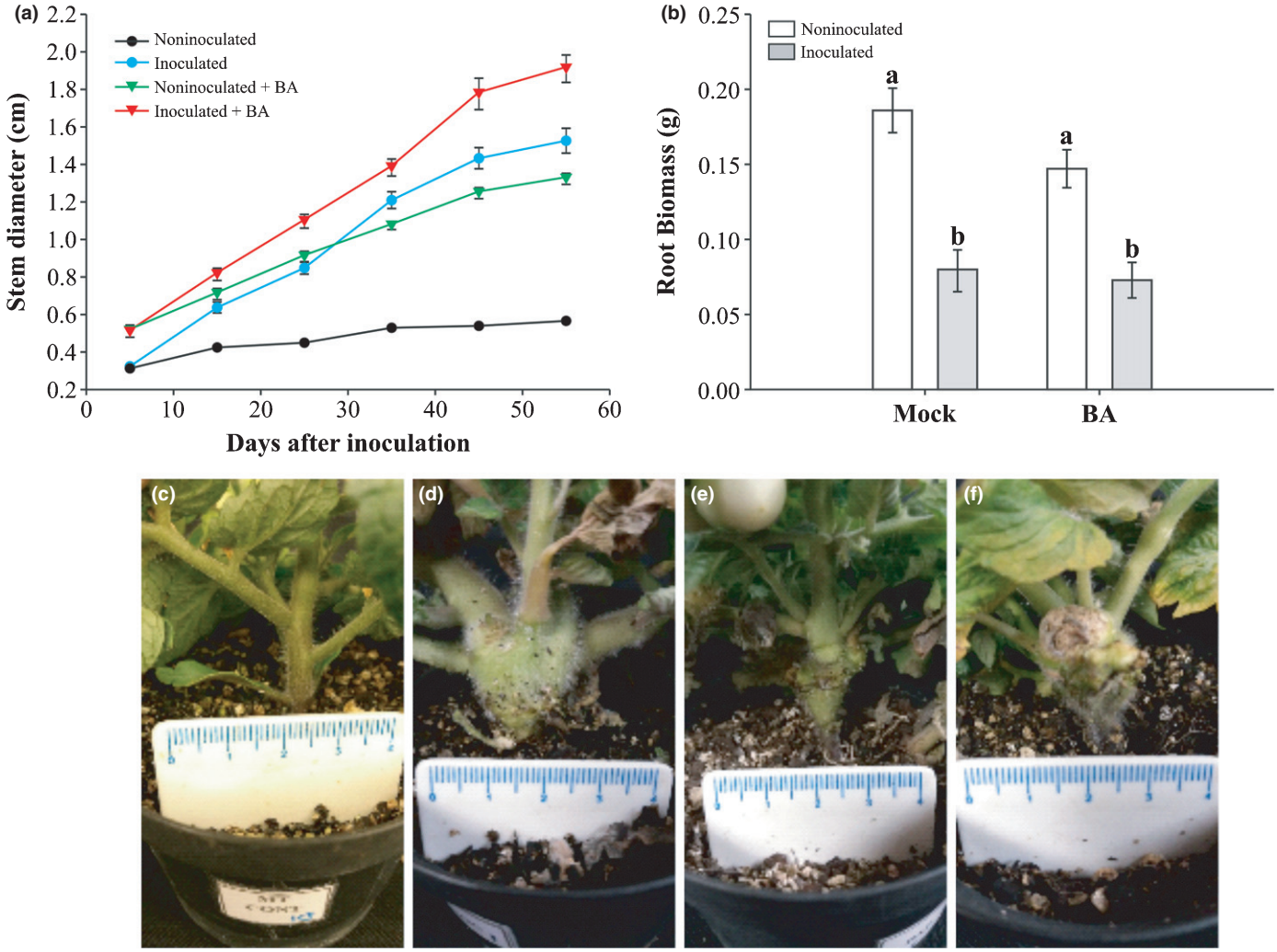

Fig. 7 Effect of topical application of 20 mM 6-benzyladenine (BA) in Micro-Tom (MT) tomato (*Solanumlycopersicum*) shoot apices and axillary buds, inoculated or not with the S-biotype of *Moniliophthoraperniciosa*. (a) Stem diameter (cm) between the first and second leaves of inoculated MT plants treated or not with 20 mM BA, or noninoculated plants treated or not with 20 mM BA, at 5, 15, 25, 35, 45 and 55 d after inoculation (dai). (b) Root biomass (g dry weight) of inoculated MT, treated or not with 20 mM BA, or noninoculated MT plants treated or not with 20 mM BA at 55 dai. The error bars represent SE. Statistically significant differences were determined by one-way ANOVA, followed by the Tukey test ($P < 0.05$) ($n = 15$); different letters indicate significant differences among the four treatments. (c) Image of a noninoculated, mock-treated MT plant. (d) MT plant inoculated, but not treated with BA. (e) MT plant noninoculated, but treated with 20 mM BA. (f) MT plant inoculated plant and treated with 20 mM BA, with the development of a gall-like structure.

(Dudman & Nichols, 1959; Krupasagar & Sequeira, 1969). Some cacao-infected tissues have shown increases in ethylene (Scarpari *et al.*, 2005), IAA and SA (Chaves & Gianfagna, 2006; Kilaru *et al.*, 2007). Transcriptomic analysis of infected tissues revealed the upregulation of cacao genes related to the auxin response together with biosynthesis and response to gibberellins and ethylene and cytokinin degradation (Teixeira *et al.*, 2014). The accumulation of transcripts associated with cytokinin oxidation suggests the occurrence of a feedback response to altered levels of cytokinins, but there has been no direct evidence of cytokinin accumulation (Melnick *et al.*, 2012; Teixeira *et al.*, 2014). One study detected small quantities of tZ-riboside in infected cacao (Orchard *et al.*, 1994). In addition, abscisic acid, IAA, JA, and SA have been detected in the mycelia of the C-biotype of *Moniliophthora perniciosa* (Kilaru *et al.*, 2007). Therefore, the specific roles of hormones during *Moniliophthora perniciosa* infection remain elusive. Here, we used the MT model to provide evidence of the involvement of cytokinins during the pathogenesis of *Moniliophthora perniciosa*. We propose a hypothetical model to explain the role of cytokinins, possibly produced by the fungus or by the host in response to the fungus, in inducing symptom development and pathogenesis (Fig. S13).

We previously characterized the infection of MT by the S-biotype of *Moniliophthora perniciosa*, particularly the abnormal swelling of stems, similar to that of cacao infected by the C-biotype during the biotrophic phase (Deganello *et al.*, 2014). Here, we demonstrated additional symptoms in infected MT, such as a decrease in root biomass and an increase in the number of fruit locules. We speculate that the reduction in roots in infected MT could also be derived from hormone imbalance,

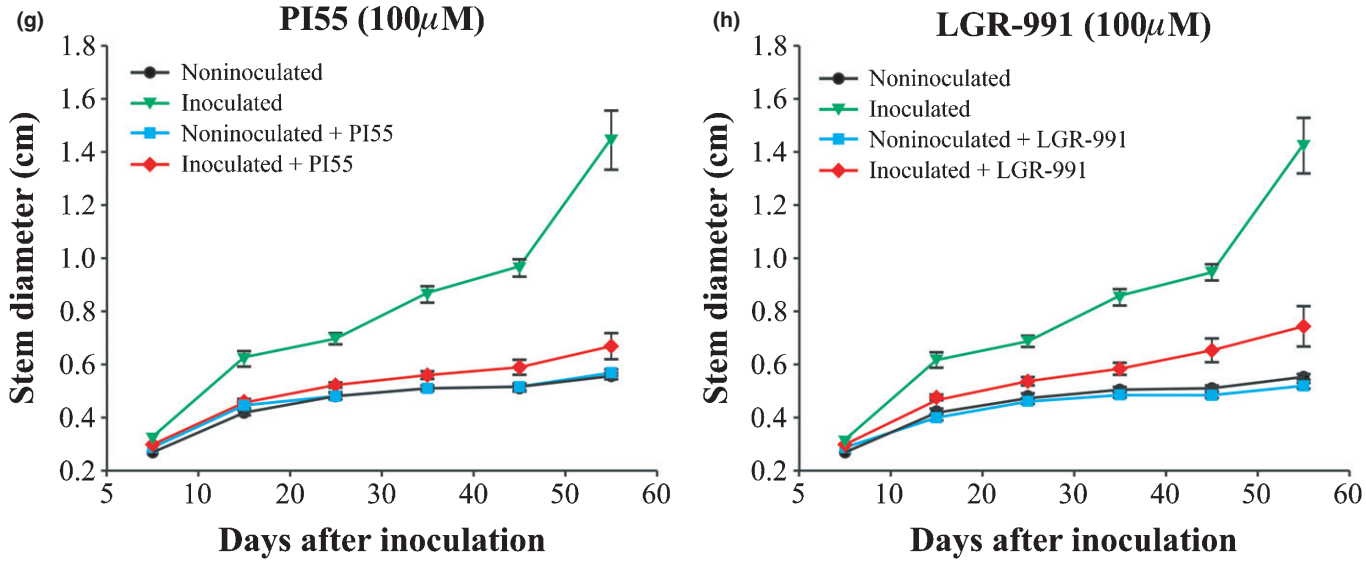

Fig. 8 Application of the cytokinin receptor inhibitors PI55 and LGR-991 in Micro-Tom (MT) tomato (*Solanumlycopersicum*) shoot apices, inoculated or not with the S-biotype of *Moniliophthoraperniciosa*. (a) Noninoculated and mock-treated plant; (b) noninoculated plant treated with 100 μM LGR-991; (c) noninoculated plant treated with 100 μM PI55; (d) inoculated with the S-biotype of *Moniliophthoraperniciosa* and mock-treated plant; (e) plant inoculated with the S-biotype of *Moniliophthoraperniciosa* treated with 100 μM LGR-991; (f) plant inoculated with the S-biotype of *Moniliophthoraperniciosa* and treated with 100 μM PI55. Mean stem diameter (cm) between the first and second leaves of MT plants, inoculated or not, and treated or not with (g) 100 μM LGR-991; (h) 100 μM PI55 at 5, 15, 25, 35, 45 and 55 d after inoculation ($n = 30$). The error bars represent SE.

considering the critical roles of cytokinins and auxins in root development (Ioio *et al.*, 2008). The accumulation of cytokinins and auxins at the site of infection might affect the amount and distribution of these hormones to other organs, affecting proper root development. Another possibility is the systemic presence of the fungus in the roots; however, the plants were inoculated at the shoot apex, and *Moniliophthora perniciosa* tends to remain confined to symptomatic tissues (Calle *et al.*, 1982). Alternatively, a potential change in sink strength by infection may lead to a lack of energy for root growth. Infection of white spruce by mistletoe caused an increase in localized host cytokinins that alter the balance of carbon distribution of the tree to favour the parasite (Logan *et al.*, 2013). Considering the fundamental role of roots, the determination of the factors associated with root reduction is critical. The decrease in root biomass might be the cause of the overall debilitation of infected cacao trees, which may severely affect cacao yield (Marelli *et al.*, 2019). The effect of witches´ broom disease on cacao roots remains to be determined.

The number of fruit locules in tomato is controlled by at least three genes *fasciated*, *locule number*, and *excessive floral organs* (Muños *et al.*, 2011; Xu *et al.*, 2015; Yuste-Lisbona *et al.*, 2020), all associated with the expression of *CLAVATA3* (*CLV3*) and *WUSCHEL* (*WUS*). Meristem maintenance is coordinated by *WUS*, which regulates *CLV3* expression, which in turn activates a signalling cascade to repress *WUS* transcription as part of a feedback loop (Somssich *et al.*, 2016). Studies in Arabidopsis have suggested the regulation of *WUS* by cytokinins in the shoot stem-cell niche (Gordon *et al.*, 2009), axillary meristem initiation (Wang *et al.*, 2017), and flower development (Lindsay *et al.*, 2006). Thus, we speculate that the accumulation of cytokinins at the inflorescence meristem of infected MT can contribute to the increase in locule number. Accordingly, the inoculation of *35S::AtCKX2* did not affect the number of locules, whereas the application of BA to MT increased the number of locules per fruit.

Our screening using mutants and transgenic lines pointed to cytokinins as major players in symptom development upon *Moniliophthora perniciosa* infection. The reduced disease incidence and stem diameter after inoculation of *35S::AtCKX2* demonstrated that cytokinins are associated with the development of symptoms. The differential detection of a cytokinin signal via *ARR5::uidA* in inoculated plants at the initial stage of infection (24 h) in comparison to noninoculated plants indicates a role of cytokinin during *Moniliophthora perniciosa* infection. Our RNA-seq analysis of infected stems at 20 and 30 dai, when the symptoms were prominent, revealed the upregulation of some cytokinin responsive-genes, corroborating the role of cytokinins in the development of symptoms. Furthermore, a tomato *CKX* gene was 30× more upregulated in inoculated MT than in *35S::AtCKX2*; *35S::AtCKX2* containing low levels of cytokinins, and cytokinins failed to accumulate in these plants during *Moniliophthora perniciosa* infection. In support of this hypothesis, BA treatment of MT plants mimicked stem enlargement, resembling *Moniliophthora perniciosa*-infected tissues, as well as increasing the number of fruit locules and decreasing root biomass. The application of cytokinin agonists to infected MT plants reduced stem swelling, reinforcing the primary role of cytokinins in the development of symptoms.

Tomato *tRNA-IPT* was downregulated in infected stems, similar to what was found in infected cacao (Teixeira *et al.*, 2014). Notably, we found an *Moniliophthora perniciosa tRNA-IPT* expressed by the S-biotype during MT infection and by the C-biotype during cacao infection. The presence and expression of this *MptRNA-IPT* suggests that the pathogen can produce cytokinins via this alternative pathway. Indeed, we detected iP in the mycelia of *Moniliophthora perniciosa* grown *in vitro*. It is possible that *Moniliophthora perniciosa* produces cytokinin via *tRNA-IPT* biosynthesis and, consequently, represses tomato *tRNA-IPT* during infection, although this pathway is thought to contribute mostly cZ (Sakakibara, 2006). After inoculation of *35S::AtCKX2*, tomato *tRNA-IPT* was not repressed, suggesting that the mechanism of repression is active only in fully symptomatic MT plants. Therefore, we considered that although we detected the expression of only *tRNA-IPT*, this pathway might contribute to the accumulation of iP in *Moniliophthora perniciosa* mycelia.

The nematode *Heterodera schachtii* relies on *tRNA-IPT* to produce iP (Siddique *et al.*, 2015); silencing *HstRNA-IPT* reduced cytokinins at the infection site and decreased virulence in Arabidopsis. Infection of Arabidopsis mutants deficient in cytokinins resulted in reduced susceptibility, suggesting that the cytokinin produced by the nematode is required to establish the infection, while host cytokinins play a major role in maintaining the feeding site (Siddique *et al.*, 2015). In Arabidopsis, there are two cytokinin biosynthetic pathways: the AMP/ATP/ADP-IPT pathway, which produces iP followed by t*Z*, and the *tRNA-IPT* pathway, which is thought to mainly contribute to cZ synthesis (Sakakibara, 2006). Conversely, *Physcomitrella patens* produces cytokinins exclusively via the tRNA-IPT pathway, including iP (von Schwartzenberg *et al.*, 2007).

We believe that the combination of cytokinins produced by *Moniliophthora perniciosa* and MT leads to hypertrophic growth during infection. We detected the accumulation of tZ and DHZ in infected MT but not of iP found in *Moniliophthora perniciosa* mycelia. *Moniliophthora perniciosa* produces iP and possibly releases it to the MT host, acting as a precursor for the synthesis of tZ by the plant (Fig. S13). The RNA-seq data suggest that the iP produced by *Moniliophthora perniciosa* might presumably be released to the host to be converted into inactive iPRMP, then converted into tZ-riboside-monophosphate by *CYTOCHROME-P450* (Solyc02g094860), and then converted further to tZ to promote symptoms. Conversely, a recent study of cytokinin biosynthesis in Arabidopsis revealed no detection of the conversion of exogenously applied tZ to DHZ and concluded that the origin of DHZ-riboside-5′-monophosphate remains unknown (Hošek *et al.*, 2020). In our study, we observed increased levels of DHZ in infected stems, but additional studies are needed to clarify the origin of DHZ accumulation.

In many cases, the role of cytokinins during infection has been to support pathogenesis and to promote symptoms. Evidence that pathogens produce cytokinins has been accumulating (Chanclud & Morel, 2016; Sørensen *et al.*, 2018). Inoculation of Arabidopsis overexpressing CKX with *Plasmodiophora brassicae*

resulted in reduced symptoms, suggesting a role for cytokinins in pathogenesis (Siemens *et al.*, 2006). In smut disease, *Ustilago maydis* is known to produce cZ *in vitro* and during infection of *Zea mays*, triggering the biosynthesis of cytokinins by the host to form tumours (Morrison *et al.*, 2017). Furthermore, *Magnaporthe oryzae* contains a gene related to cytokinin biosynthesis (*CKS*) and produces cZ-riboside, isopentenyl-adenosine, cZ-nucleotide, and iP-nucleotide in its mycelia (Chanclud *et al.*, 2016). Infection by the *Magnaporthe oryzae cks1* mutant did not promote the development of sugar or amino acid sinks around the site of infection, as observed during wild-type infection (Chanclud *et al.*, 2016). *Claviceps purpurea* was shown to produce cytokinins *in vitro* by the iP and *tRNA-IPT* biosynthesis pathways (Hinsch *et al.*, 2015). Deletion of *CpIPT-LOG* or *CpP450* did not affect virulence, but the deletion of *CptRNA-IPT* suppressed cZ synthesis and reduced *C. purpurea* virulence, whereas the double mutant Δ*ipt-log*/*tRNA-ipt* severely impaired virulence, confirming the role of cytokinins in pathogenicity (Hinsch *et al,* 2016). Despite numerous efforts, *Moniliophthora perniciosa* has been recalcitrant to genetic manipulation so far (Teixeira *et al.*, 2015), restricting the use of genome editing to demonstrate the involvement of *tRNA-IPT* in pathogenesis.

In addition to the increase in cytokinins, we also observed an increase in IAA, SA, and JA in infected stems of MT, whereas the dikaryotic mycelium of *Moniliophthora perniciosa* produced IAA and SA *in vitro*. We did not verify any change in disease incidence after inoculation of the auxin mutants *diageotropica* or *entire*, but we detected a contrasting response of stem enlargement for both mutants. Since auxin is also a hormone associated with the development of symptoms from pathogen infections (Kazan & Manners, 2009), we suggest that the increase in auxin may contribute to hypertrophic and hyperplastic tissue growth, especially increasing the size of xylem and phloem cells. It is well established that auxins and cytokinins play a major role during xylogenesis, increasing the size of the cells and stimulating cell division, respectively (Aloni, 1995). Using the auxin-responsive reporter construct *DR5::GUS*, Marelli *et al.* (2009) showed increased auxin signalling in the xylem of tomato plants infected by *Moniliophthora perniciosa*. Inoculated *35S::AtCKX2* showed less IAA than infected MT; therefore, cytokinins and auxin affect each other during *Moniliophthora perniciosa* infection of MT, and auxins may participate in the development of symptoms.

Altogether, our findings suggest that *Moniliophthora perniciosa* produces cytokinins that might interfere with host cytokinin biosynthesis to establish pathogenesis and develop symptoms. To our knowledge, this is the first report of cytokinins in the mycelia of *Moniliophthora perniciosa*. Although studies in cacao have suggested a role for cytokinins and auxins during *Moniliophthora perniciosa* pathogenesis, this work demonstrates that cytokinins are major players inducing symptoms in hosts. This increase in host cytokinins induced by *Moniliophthora perniciosa* infection might be associated with altering source-sink relationships and increasing the supply of sugars to help the pathogen establish and thrive in the apoplast. How the changes in cytokinins favour the pathogen requires further investigation.

Acknowledgements

This work was supported by the 'Fundação de Amparo à Pesquisa do Estado de São Paulo' (FAPESP) through a Thematic Research Grant (16/10498-4) and fellowships to JLC (13/04309-6; BEPE 16/10524-5), EMS (17/17000-4), JSS (15/00060-9), and RMC (19/12188-0). Additional support came from the 'Conselho Nacional de Desenvolvimento Científico e Tecnológico' CNPq through the research grant (471631/2013-2) and fellowships to DP (132376/2016-4) and scholarships to LF, LEPP and AF. The authors are grateful to Dr Gareth Griffith (Aberystwyth University, UK) for the generous gift of the APS1 and WMA5 isolates, and for Dr Sandra Pierre (*in memorium*) for preparing the pie-dishes. The authors wish to thank Dr Lukás Spíchal from Palacký University, Institute of Experimental Botany, Czech Republic for providing samples of PI55 and LGR-991. The support from Dr Beatriz Appezzato, Marli Soares, Cássia Figueiredo, and Aline Cruz were greatly appreciated. The authors are thankful for the technical help from Aline Pedroso, Leticia Angelini, Rodolfo Maniero, Juliana Deganello, Dr Gildemberg Leal Jr and Dr Danielle Scotton. The authors thank the University of North Carolina High Throughput Sequencing Facility for the support with the RNA-seq analysis and the Universitat Politècnica de València, Spain for the support with the hormonal analysis.

Author contributions

AF and JLC designed the research project. JLC, DP, JSS, RMC, EMS performed experiments. JLC and DP analysed and interpreted the data. PJPLT performed the bioinformatic analysis. EC, ILD, MLR, LF, PM, LEPP contributed reagents/materials/analysis tools. DP, AF and JLC wrote the manuscript. AF supervised the project and experiments. JLC and DP contributed equally to this work.

ORCID

Rafael M. do Carmo https://orcid.org/0000-0003-0527-4484
Esther Carrera https://orcid.org/0000-0002-3454-7552
Antonio Figueira https://orcid.org/0000-0001-8641-2556
Luciano Freschi https://orcid.org/0000-0002-0737-3438
Isabel López-Díaz https://orcid.org/0000-0003-4000-1633
Piotr Mieczkowski https://orcid.org/0000-0003-2418-0096
Daniele Paschoal https://orcid.org/0000-0001-6640-0451
Lazaro E. P. Peres https://orcid.org/0000-0002-8761-5934
Mônica L. Rossi https://orcid.org/0000-0003-2057-9470
Eder M. da Silva https://orcid.org/0000-0002-1450-511X
Paulo J. P. L. Teixeira https://orcid.org/0000-0002-0776-5044

Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

References

Agrios GN. 2005. *Plant pathology, 5th edn*. Burlington, MA, USA: Academic Press.

Aloni R. 1995. The induction of vascular tissues by auxin and cytokinin. In: Davies PJ, ed. *Plant hormones: physiology, biochemistry and molecular biology*. Dordrecht, the Netherlands: Kluwer Academic, 531–546.

Andrews S. 2010. *FastQC: a quality control tool for high throughput sequence data*. [WWW document] URL http://www.bioinformatics.babraham.ac.uk/projects/fastqc/ [accessed 5 January 2017].

Argueso CT, Ferreira FJ, Kieber JJ. 2009. Environmental perception avenues: the interaction of cytokinin and environmental response pathways. *Plant, Cell & Environment* **32**: 1147–1160.

Bassel GW, Mullen RT, Bewley JD. 2008. *Procera* is a putative DELLA mutant in tomato (*Solanum lycopersicum*): effects on the seed and vegetative plant. *Journal of Experimental Botany* **59**: 585–593.

Benjamini Y, Hochberg Y. 1995. Controlling the false discovery rate: a practical and powerful approach to multiple testing. *Journal of the Royal Statistical Society* **57**: 289–300.

Bolger AM, Lohse M, Usadel B. 2014. Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics* **30**: 2114–2120.

Brading PA, Hammond-Kosack KE, Parr A, Jones JD. 2000. Salicylic acid is not required for *Cf-2*- and *Cf-9*-dependent resistance of tomato to *Cladosporium fulvum*. *The Plant Journal* **23**: 305–318.

Calle H, Cook AA, Fernando SY. 1982. Histology of witches' broom disease caused in cacao by *Crinipellis perniciosa*. *Phytopathology* **72**: 1479–1481.

Carvalho RF, Campos ML, Pino LE, Crestana SL, Zsögön A, Lima JE, Benedito VA, Peres LE. 2011. Convergence of developmental mutants into a single tomato model system: "Micro-Tom" as an effective toolkit for plant development research. *Plant Methods* **7**: 18.

Chanclud E, Kisiala A, Emery NR, Chalvon V, Ducasse A, Romiti-Michel C, Gravot A, Kroj T, Morel JB. 2016. Cytokinin production by the rice blast fungus is a pivotal requirement for full virulence. *PLoS Pathogens* **12**: e1005457.

Chanclud E, Morel JB. 2016. Plant hormones: a fungal point of view. *Molecular Plant Pathology* **17**: 1289–1297.

Chaves FC, Gianfagna TJ. 2006. Necrotrophic phase of *Moniliophthora perniciosa* causes salicylic acid accumulation in infected stems of cacao. *Physiological and Molecular Plant Pathology* **69**: 104–108.

Cortleven A, Leuendorf JE, Frank M, Pezzetta D, Bolt S, Schmülling T. 2019. Cytokinin action in response to abiotic and biotic stresses in plants. *Plant, Cell & Environment* **42**: 998–1018.

D'Agostino IB, Deruere J, Kieber JJ. 2000. Characterization of the response of the Arabidopsis response regulator gene family to cytokinin. *Plant Physiology* **124**: 1706–1717.

De Silva NI, Lumyong S, Hyde KD, Bulgakov T, Phillips AJL, Yan JY. 2016. Mycosphere Essays 9: defining biotrophs and hemibiotrophs. *Mycosphere* **7**: 545–559.

Deganello J, Leal GA Jr, Rossi ML, Peres LEP, Figueira A. 2014. Interaction of *Moniliophthora perniciosa* biotypes with Micro-Tom tomato: a model system to investigate the witches' broom disease of *Theobroma cacao*. *Plant Pathology* **63**: 1251–1263.

Dudman WF, Nichols R. 1959. Absence of gibberellin-like substances in filtrates of *Marasmius perniciosus* Stahel (witch broom disease of cacao). *Nature* **183**: 899.

Frias GA, Purdy LH, Schmidt RA. 1995. An inoculation method for evaluating resistance of cacao to *Crinipellis perniciosa*. *Plant Disease* **79**: 787–791.

Fujino DW, Burger DW, Yang SF, Bradford KJ. 1988. Characterization of an ethylene overproducing mutant of tomato (*Lycopersicon esculentum* Mill. Cultivar VFN8). *Plant Physiology* **88**: 774–779.

Gordon SP, Chickarmane VS, Ohno C, Meyerowitz EM. 2009. Multiple feedback loops through cytokinin signaling control stem cell number within the *Arabidopsis* shoot meristem. *Proceedings of the National Academy of Sciences, USA* **106**: 16529–16534.

Han X, Kahmann R. 2019. Manipulation of phytohormone pathways by effectors of filamentous plant pathogens. *Frontiers in Plant Science* **10**: 822.

Hinsch J, Galuszka P, Tudzynski P. 2016. Functional characterization of the first filamentous fungal tRNA-isopentenyltransferase and its role in the virulence of *Claviceps purpurea*. *New Phytologist* **211**: 980–992.

Hinsch J, Vrabka J, Oeser B, Novák O, Galuszka P, Tudzynski P. 2015. De novo biosynthesis of cytokinins in the biotrophic fungus *Claviceps purpurea*. *Environmental Microbiology* **17**: 2935–2951.

Hošek P, Hoyerová K, Kiran NS, Dobrev PI, Zahajská L, Filepová R, Motyka V, Kamínek M. 2020. Distinct metabolism of *N*-glucosides of isopentenyladenine and *trans*-zeatin determines cytokinin metabolic spectrum in Arabidopsis. *New Phytologist* **225**: 2423–2438.

Ioio RD, Nakamura K, Moubayidin L, Perilli S, Taniguchi M, Morita MT, Aoyama T, Costantino P, Sabatini S. 2008. A genetic framework for the control of cell division and differentiation in the root meristem. *Science* **322**: 1380–1384.

Jefferson RA, Kavanagh TA, Bevan MW. 1987. GUS fusions: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *EMBO Journal* **6**: 3901–3907.

Kazan K, Manners JM. 2009. Linking development to defense: auxin in plant–pathogen interactions. *Trends in Plant Science* **14**: 373–382.

Kiba T, Takei K, Kojima M, Sakakibara H. 2013. Side-chain modification of cytokinins controls shoot growth in *Arabidopsis*. *Developmental Cell* **27**: 452–461.

Kilaru A, Bailey BA, Hasenstein KH. 2007. *Moniliophthora perniciosa* produces hormones and alters endogenous auxin and salicylic acid in infected cocoa leaves. *FEMS Microbiology Letters* **274**: 238–244.

Kim D, Langmead B, Salzberg SL. 2015. HISAT: a fast spliced aligner with low memory requirements. *Nature Methods* **12**: 357–360.

Krupasagar V, Sequeira L. 1969. Auxin destruction by *Marasmius perniciosus*. *American Journal of Botany* **56**: 390–397.

Liao Y, Smyth GK, Shi W. 2013. The Subread aligner: fast, accurate and scalable read mapping by seed-and- vote. *Nucleic Acids Research* **41**: e108.

Lindsay DL, Sawhney VK, Bonham-Smith PC. 2006. Cytokinin-induced changes in *CLAVATA1* and *WUSCHEL* expression temporally coincide with altered floral development in *Arabidopsis*. *Plant Science* **170**: 1111–1117.

Lisboa DO, Evans HC, Araújo JP, Elias SG, Barreto RW. 2020. *Moniliophthora perniciosa*, the mushroom causing witches' broom disease of cacao: insights into its taxonomy, ecology and host range in Brazil. *Fungal Biology* **124**: 983–1003.

Logan BA, Reblin JS, Zonana DM, Dunlavey RF, Hricko CR, Hall AW, Schmiege SC, Butschek RA, Duran KL, Emery RJN *et al.* 2013. Impact of eastern dwarf mistletoe (*Arceuthobium pusillum*) on host white spruce (*Picea glauca*) development, growth and performance across multiple scales. *Physiologia Plantarum* **147**: 502–513.

Marelli JP, Guest DI, Bayley BA, Evans HC, Brown JK, Junaid M, Barreto RW, Lisboa DO, Puig AS. 2019. Chocolate under threat from old and new diseases. *Phytopathology* **109**: 1331–1343.

Marelli JP, Maximova SN, Gramacho KP, Kang S, Guiltinan MJ. 2009. Infection biology of *Moniliophthora perniciosa* on *Theobroma cacao* and alternate solanaceous hosts. *Tropical Plant Biology* **2**: 149–160.

Martin RC, Mok MC, Shaw G, Mok DW. 1989. An enzyme mediating the conversion of zeatin to dihydrozeatin in *Phaseolus* embryos. *Plant Physiology* **90**: 1630–1635.

Martínez-Bello L, Moritz T, López-Díaz I. 2015. Silencing C19-GA 2-oxidases induces parthenocarpic development and inhibits lateral branching in tomato plants. *Journal of Experimental Botany* **66**: 5897–5910.

McGurl B, Orozco-Cardenas M, Pearce G, Ryan CA. 1994. Overexpression of the prosystemin gene in transgenic tomato plants generates a systemic signal that constitutively induces proteinase inhibitor synthesis. *Proceedings of the National Academy of Sciences, USA* **91**: 9799–9802.

Melnick RL, Marelli JP, Sicher RC, Strem MD, Bailey BA. 2012. The interaction of *Theobroma cacao* and *Moniliophthora perniciosa*, the causal agent of witches' broom disease, during parthenocarpy. *Tree Genetics & Genomes* **8**: 1261–1279.

Mondego JMC, Carazzolle MF, Costa GGL, Formighieri EF, Parizzi LP, Rincones J, Cotomacci C, Carraro DM, Cunha AF, Carrer H *et al.* 2008. A genome survey of *Moniliophthora perniciosa* gives new insights into witches' broom disease of cacao. *BMC Genomics* **9**: 548.

Morrison EN, Emery RJN, Saville BJ. 2017. Fungal derived cytokinins are necessary for normal *Ustilago maydis* infection of maize. *Plant Pathology* **66**: 726–742.

Muños S, Ranc N, Botton E, Berard A, Rolland S, Duffe P, Carretero Y, Le Paslier MC, Delalande C, Bouzayen M *et al.* 2011. Increase in tomato locule number is controlled by two single nucleotide polymorphisms located near WUSCHEL. *Plant Physiology* **156**: 2244–2254.

Nisler J, Zatloukal M, Popa I, Doležal K, Strnad M, Spíchal L. 2010. Cytokinin receptor antagonists derived from 6-benzylaminopurine. *Phytochemistry* **71**: 823–830.

Oh K, Ivanchenko MG, White TJ, Lomax TL. 2006. The *diageotropica* gene of tomato encodes a cyclophilin: a novel player in auxin signaling. *Planta* **224**: 133–144.

Orchard J, Collin HA, Hardwick K, Isaac S. 1994. Changes in morphology and measurement of cytokinin levels during the development of witches' brooms on cocoa. *Plant Pathology* **43**: 65–72.

Pertry I, Václavíková K, Depuydt S, Galuszka P, Spíchal L, Temmerman W, Stes E, Schmülling T, Kakimoto T, Van Montagu MC *et al.* 2009. Identification of *Rhodococcus fascians* cytokinins and their modus operandi to reshape the plant. *Proceedings of the National Academy of Sciences, USA* **106**: 929–934.

Pierre S, Griffith GW, Morphew RM, Mur LA, Scott IM. 2017. Saprotrophic proteomes of biotypes of the witches' broom pathogen *Moniliophthora perniciosa*. *Fungal Biology* **121**: 743–753.

Pino LE, Lombardi-Crestana S, Azevedo MA, Scotton DC, Borgo L, Quecini V, Figueira A, Peres LEP. 2010. The Rg1 allele as a valuable tool for genetic transformation of the tomato 'Micro-Tom' model system. *Plant Methods* **6**: 23.

Purdy LH, Schmidt RA. 1996. Status of cacao witches' broom: biology, epidemiology, and management. *Annual Review of Phytopathology* **34**: 573–594.

Robinson MD, McCarthy DJ, Smyth GK. 2010. edgeR: a bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics* **26**: 139–140.

Roman H, Girault T, Barbier F, Péron T, Brouard N, Pěnčík A, Novák O, Vian A, Sakr S, Lothier J *et al.* 2016. Cytokinins are initial targets of light in the control of bud outgrowth. *Plant Physiology* **172**: 489–509.

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

SAS. 1999. *SAS user's guide statistics.* Cary, NC, USA: Statistical Analysis Systems Institute Inc.

Sato S, Tabata S, Hirakawa H, Asamizu E, Shirasawa K, Isobe S, Kaneko T, Nakamura Y, Shibata D, Aoki K *et al.* 2012. The tomato genome sequence provides insights into fleshy fruit evolution. *Nature* **485**: 635–641.

Scarpari LM, Meinhardt LW, Mazzafera P, Pomella AWV, Schiavinato MA, Cascardo JCM, Pereira GAG. 2005. Biochemical changes during the development of witches' broom: the most important disease of cocoa in Brazil caused by *Crinipellis perniciosa*. *Journal of Experimental Botany* **56**: 865–877.

Schneider CA, Rasband WS, Eliceiri KW. 2012. NIH Image to ImageJ: 25 years of image analysis. *Nature Methods* **9**: 671–675.

von Schwartzenberg K, Nunez MF, Blaschke H, Dobrev PI, Novák O, Motyka V, Strnad M. 2007. Cytokinins in the bryophyte *Physcomitrella patens*: analyses of activity, distribution, and cytokinin oxidase/dehydrogenase overexpression reveal the role of extracellular cytokinins. *Plant Physiology* **145**: 786–800.

Sena K, Alemanno L, Gramacho KP. 2014. The infection process of *Moniliophthora perniciosa* in cacao. *Plant Pathology* **63**: 1272–1281.

Seo M, Jikumaru Y, Kamiya Y. 2011. Profiling of hormones and related metabolites in seed dormancy and germination studies. *Methods in Molecular Biology* **773**: 99–111.

Shi X, Gupta S, Lindquist IE, Cameron CT, Mudge J, Rashotte AM. 2013. Transcriptome analysis of cytokinin response in tomato leaves. *PLoS ONE* **8**: e55090.

Siddique S, Radakovic ZS, De La Torre CM, Chronis D, Novak O, Ramireddy E, Holbein J, Matera C, Hutten M, Gutbrod P *et al.* 2015. A parasitic nematode releases cytokinin that controls cell division and orchestrates feeding site formation in host plants. *Proceedings of the National Academy of Sciences, USA* **112**: 12669–12674.

Siemens J, Keller I, Sarx J, Kunz S, Schuller A, Nagel W, Schmülling T, Parniske M, Ludwig-Müller J. 2006. Transcriptome analysis of Arabidopsis clubroots indicate a key role for cytokinins in disease development. *Molecular Plant–Microbe Interactions* **19**: 480–494.

Somssich M, Je BI, Simon R, Jackson D. 2016. CLAVATA-WUSCHEL signaling in the shoot meristem. *Development* **143**: 3238–3248.

Sørensen JL, Benfield AH, Wollenberg RD, Westphal K, Wimmer R, Nielsen MR, Nielsen KF, Carere J, Covarelli L, Beccari G *et al.* 2018. The cereal pathogen *Fusarium pseudograminearum* produces a new class of active cytokinins during infection. *Molecular Plant Pathology* **19**: 1140–1154.

Spallek T, Gan P, Kadota Y, Shirasu K. 2018. Same tune, different song — cytokinins as virulence factors in plant–pathogen interactions? *Current Opinion in Plant Biology* **44**: 82–87.

Spíchal L, Werner T, Popa I, Riefler M, Schmülling T, Strnad M. 2009. The purine derivative PI-55 blocks cytokinin action via receptor inhibition. *The FEBS Journal* **276**: 244–253.

Takei K, Yamaya T, Sakakibara H. 2004. *Arabidopsis CYP735A1* and *CYP735A2* encode cytokinin hydroxylases that catalyze the biosynthesis of *trans*-zeatin. *Journal of Biological Chemistry* **279**: 41866–41872.

Teixeira PJPL, Thomazella DPDT, Reis O, do Prado PFV, do Rio MCS, Fiorin GL, José J, Costa GGL, Negri VA, Mondego JMC *et al.* 2014. High-resolution transcript profiling of the atypical biotrophic interaction between *Theobroma cacao* and the fungal pathogen *Moniliophthora perniciosa*. *Plant Cell* **26**: 4245–4269.

Teixeira PJPL, de Toledo Thomazella DP, Pereira GAG. 2015. Time for chocolate: current understanding and new perspectives on cacao Witches' broom disease research. *PLoS Pathogens* **11**: e1005130.

Walters DR, McRoberts N. 2006. Plants and biotrophs: a pivotal role for cytokinins? *Trends in Plant Science* **11**: 581–586.

Wang J, Tian C, Zhang C, Shi B, Cao X, Zhang TQ, Zhao Z, Wang JW, Jiao Y. 2017. Cytokinin signaling activates *WUSCHEL* expression during axillary meristem initiation. *Plant Cell* **29**: 1373–1387.

Werner T, Motyka V, Laucou V, Smets R, Van Onckelen H, Schmülling T. 2003. Cytokinin-deficient transgenic Arabidopsis plants show multiple developmental alterations indicating opposite functions of cytokinins in the regulation of shoot and root meristem activity. *Plant Cell* **15**: 2532–2550.

Werner T, Schmülling T. 2009. Cytokinin action in plant development. *Current Opinion in Plant Biology* **12**: 527–538.

Wilkinson JQ, Lanahan MB, Yen HC, Giovannoni JJ, Klee HJ. 1995. An ethylene-inducible component of signal transduction encoded by Never-ripe. *Science* **270**: 1807–1809.

Xu C, Liberatore KL, MacAlister CA, Huang Z, Chu Y-H, Jiang Ke, Brooks C, Ogawa-Ohnishi M, Xiong G, Pauly M *et al.* 2015. A cascade of arabinosyltransferases controls shoot meristem size in tomato. *Nature Genetics* **47**: 784–792.

Yuste-Lisbona FJ, Fernández-Lozano A, Pineda B, Bretones S, Ortíz-Atienza A, García-Sogo B, Müller NA, Angosto T, Capel J, Moreno V *et al.* 2020. *ENO* regulates tomato fruit size through the floral meristem development network. *Proceedings of the National Academy of Sciences, USA* **117**: 8187–8195.

Zhang J, Chen R, Xiao J, Qian C, Wang T, Li H, Ouyang B, Ye Z. 2007. A single-base deletion mutation in *SlIAA9* gene causes tomato (*Solanum lycopersicum*) *entire* mutant. *Journal of Plant Research* **120**: 671–678.

Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Fig. S1 Histogram of classes of number of locules per fruit expressed in percentage from Micro-Tom (MT) plants, inoculated or not with three isolates of the S-biotype of *Moniliophthora perniciosa*.

Fig. S2 Histogram of classes of number of locules per fruit expressed in percentage from Micro-Tom (MT), mutants

(*diageotropica, entire, Never ripe, epinastic* and *procera*) and transgenic lines (*35S::PS*, *35S::nahG*, and *35S::AtCKX2*) introgressed into MT, inoculated or not with *Moniliophthora perniciosa*.

Fig. S3 Expression in FoldChange of the tomato *tRNA DIMETHYLALLYLTRANSFERASE* (*tRNA IPT*) (Solyc01g080150.3, Solyc09g064910.1) and *CYTOKININ OXIDASE* (Solyc10g017990.2) genes in inoculated Micro-Tom (MT) or *35S::AtCKX2* plants.

Fig. S4 Box-plots indicating the expression in RPKM of the *Moniliophthora perniciosa tRNA ISOPENTENYLTRANSFERASE-tRNA-IPT* gene in *Moniliophthora perniciosa* mycelium, and in Micro-Tom (MT) or cacao infected plants.

Fig. S5 Cladogram constructed with tRNA-isopentenyl transferase (tRNA-IPT) protein sequences from plant pathogens.

Fig. S6 Quantification of hormones in Micro-Tom (MT) or *35S::AtCKX2* plants inoculated with the Tiradentes isolate of the S-biotype of *Moniliophthora perniciosa*, or in *in vitro*-grown mycelium of the same isolate.

Fig. S7 Effect of topical application of 20 mM 6-benzyladenine (BA) in tomato cv. M82 shoot apex and axillary buds, inoculated or not with the S-biotype of *Moniliophthora perniciosa*.

Fig. S8 Histology of stem cross-section of Micro-Tom (MT) plants treated with 20 mM benzyladenine (BA) after inoculation or not with the S-biotype of *Moniliophthora perniciosa*.

Fig. S9 Pith, xylem, phloem, and cortex areas of stem cross-section of Micro-Tom (MT) plants treated or not with 20 mM benzyladenine (BA) after inoculation or not with the S-biotype of *Moniliophthora perniciosa*.

Fig. S10 Histogram of classes of number of locules per fruit in percentage from inoculated Micro-Tom (MT) plants, treated or not with 20 mM benzyladenine (BA), or noninoculated plants treated or not with 20 mM BA.

Fig. S11 Doses evaluation of the cytokinin-receptors inhibitors PI55 and LGR-991 in Micro-Tom (MT) shoot apices and axillary buds, inoculated or not with the S-biotype of *Moniliophthora perniciosa*.

Fig. S12 Application of the cytokinin-receptors inhibitor PI55 in tomato cultivar M82 shoot apices and axillary buds, inoculated or not with the S-biotype of *Moniliophthora perniciosa*.

Fig. S13 Proposed hypothetical model of infected Micro-Tom (MT) plants compared to inoculated *35S::AtCKX2* plants.

Table S1 Annotation of differentially expressed cytokinin marker genes of Micro-Tom tomato (*Solanum lycopersicum*) in response to inoculation with the S-biotype of *Moniliophthora perniciosa*.

Table S2 Annotation of all differentially expressed genes of Micro-Tom and *35S::AtCKX2* tomato (*Solanum lycopersicum*) in response to inoculation with the S-biotype of *Moniliophthora perniciosa* at 5, 10, 20 and 30 d after infection (dai).

Please note: Wiley Blackwell are not responsible for the content or functionality of any Supporting Information supplied by the authors. Any queries (other than missing material) should be directed to the *New Phytologist* Central Office.