Cytokinin production by *Pseudomonas fluorescens* G20-18 determines biocontrol activity against *Pseudomonas syringae* in *Arabidopsis*

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Plant beneficial microbes mediate biocontrol of diseases by interfering with pathogens or via strengthening the host. Although phytohormones, including cytokinins, are known to regulate plant development and physiology as well as plant immunity, their production by microorganisms has not been considered as a biocontrol mechanism. Here we identify the ability of *Pseudomonas fluorescens* G20-18 to efficiently control *P. syringae* infection in *Arabidopsis*, allowing maintenance of tissue integrity and ultimately biomass yield. Microbial cytokinin production was identified as a key determinant for this biocontrol effect on the hemibiotrophic bacterial pathogen. While cytokinin-deficient loss-of-function mutants of G20-18 exhibit impaired biocontrol, functional complementation with cytokinin biosynthetic genes restores cytokinin-mediated biocontrol, which is correlated with differential cytokinin levels *in planta*. *Arabidopsis* mutant analyses revealed the necessity of functional plant cytokinin perception and salicylic acid-dependent defence signalling for this biocontrol mechanism. These results demonstrate microbial cytokinin production as a novel microbiome-based, hormone-mediated concept of biocontrol. This mechanism provides a basis to potentially develop novel, integrated plant protection strategies combining promotion of growth, a favourable physiological status and activation of fine-tuned direct defence and abiotic stress resilience.

Throughout their life cycle, plants interact with a multitude of environmental factors, including unfavourable abiotic stress conditions and threats from a wide range of insects and pathogenic microbes. Phytohormone signalling plays a crucial role in accurately regulating plant responses. Ethylene (ET), jasmonic (JA) and salicylic acid (SA) are essential phytohormonal regulators of plant immunity that form a central signalling backbone which specifically coordinates defence responses against biotrophic and necrotrophic pathogens1. Detailed analyses of phytohormone function in plant immunity have extended this network to other classic growth-regulating...
phytohormones such as abscisic acid (ABA), auxins and gibberellins. The classic growth-stimulating phytohormone family of cytokinins (CKs) comprises important regulators of many physiological and developmental plant processes such as cell division, leaf senescence, nutrient mobilization, apical dominance, and seed germination. In the interaction of plants with insects and microbes, CK alterations have been identified to cause green island formation, galls, growth abnormalities, and modulation of primary carbon metabolism. As they induce sink metabolism, CKs have been suggested to alter host physiology to facilitate maximum access of (hemi)biotrophic pathogens to nutrients during early interactions. However, recently, significant direct functions for CKs in plant immunity have been identified in different plant species such as Arabidopsis thaliana, tobacco, and rice via induction of resistance against primarily (hemi)biotrophic pathogens such as Pseudomonas syringae and Hyaloperonospora arabidopsidis or by activation of defence responses (independent of pathogen infection). The underlying mechanisms mediating CK-dependent resistance against P. syringae include induction of SA in Arabidopsis and tobacco, induction of phytoalexin accumulation and reduction of ABA levels in tobacco. Furthermore, CKs were demonstrated to induce defence gene expression synergistically with SA and to enhance Diterpenoid phytoalexin accumulation in rice.

In addition to pathogens, plants interact with a multitude of beneficial microbes, many of which belong to the genera Azospirillum, Bacillus or Pseudomonas and are characterized by their ability to promote plant growth, increase tolerance to environmental stress and/or enhance disease resistance. Agricultural food production faces many challenges due to increasing world population, climate change and restrictions on use of classic pesticides. Consequently, alternative plant protection strategies are urgently required. The biological control of plant diseases by beneficial microbes offers significant potential for integrated plant disease management. To facilitate the development of microbe-based biocontrol strategies, their underlying mechanisms have to be fully elucidated. Known biocontrol mechanisms include (i) direct interference with competition for nutrients and space, secretion of antibiotics or degradation of virulence factors, and (ii) the induction of host plant resistance, which is often related to induced systemic resistance (ISR) involving the phytohormones ET and JA. Interestingly, beneficial microbes are capable of producing different phytohormones, notably including CKs. Therefore, it is intriguing that CKs exhibit similar biological effects as described for beneficial microbes including the induction of plant growth promotion (PGP), environmental stress tolerance and disease resistance. Despite this correlative evidence, microbial phytohormones - and particularly CKs - have not been considered as a determinant for effective biocontrol of plant diseases. Microbial CK production has so far only been linked to PGP and suggested as a mechanism for increasing abiotic stress tolerance in plants. Considering the widespread CK production by beneficial microbes and recent advances in understanding CK function in plant resistance, we analysed the impact of microbial CK production on the microbe’s biocontrol ability. We established a causal relationship between the production of CKs by Pseudomonas fluorescens (Pfl) strain G20-18 and its ability to control the infection of Arabidopsis by P. syringae pv. tomato DC3000 (Pto) through comparisons with G20-18-derived loss-of-function and gain-of-function strains in a leaf infiltration assay. Analyses of Arabidopsis mutant lines impaired in defence or hormone signalling pathways revealed the necessity of functional CK perception in combination with SA defence signalling and a potential minor impact of ET, JA signalling as well as camalexin accumulation to fully establish microbial CK-mediated biocontrol. These data provide the basis for a novel microbe-based concept of biocontrol.

Results

Microbial CKs mediate G20-18 biocontrol. Since the CK-producing PGP Pfl strain G20-18 had not been tested for its biocontrol abilities, we first examined its biocontrol potential in the Arabidopsis–Pto pathosystem in comparison to its CK-deficient transposon mutants CNT1 and CNT2. As CKs have been demonstrated to induce defence responses or resistance against (hemi)biotrophic foliar pathogens when applied to leaves of Arabidopsis, rice, and tobacco, we decided to analyse the biocontrol potential of the Pfl strains when directly applied to Arabidopsis leaves by infiltration of cell suspensions 48 h prior to Pto infection. The leaf infiltration assay widely used in model pathosystems was chosen to allow us to relate the findings to the well-established immunity-relevant CK functions in leaf tissues. Although approaches such as spray inoculation or application to the root system would address more natural scenarios of interaction, they would contribute additional sources of interference with CK-mediated immunity responses, and thus, further complicate the analyses of a potential role of CK in biocontrol.

Pre-treatment with Pfl heavily suppressed Pto symptom development at 4 days post infection (dpi), resulting in maintenance of tissue integrity, an important beneficial aspect of biocontrol applications in sustaining biomass yield. Mock pre-treatment had no effect on Pto symptoms compared to control infections without pre-treatment (Fig. 1a). Thus, G20-18 is considered an efficient strain for biocontrol of Pto in Arabidopsis in the leaf infiltration assays. In comparison to G20-18, both CNT transposon mutants had only a slight suppressive effect on Pto symptom development (Fig. 1a). The quantification of the average symptom scores over all experiments further demonstrates this biocontrol effect: G20-18 pre-treatment efficiently suppressed Pto symptoms by approximately 75%, CNT pre-treatments suppressed Pto symptoms only by 15 to 20% compared to untreated and mock controls, indicating that the CK-deficient mutants were significantly less effective than G20-18 (Fig. 1b). This highly reduced effect of the CK-deficient CNT transposon mutants on Pto symptom development strongly supports a role for microbial CK production in the biocontrol ability of G20-18.

As the CNT transposon mutants were generated by undirected mutagenesis via the introduction of the TnphoA transposon into G20-18 and were selected based on CK deficiency without detailed genetic characterization, we analysed the only known CK biosynthetic gene in Pfl strains, tRNA delta(2)-isopentenylpyrophosphate transferase (miaA). Using primers based on known Pfl miaA sequences the gene was amplified from G20-18 and sequenced (Supplementary Fig. 1). Size comparison of full-length miaA amplicons of G20-18 and the CNT transposon mutants as well as sequence analysis ruled out miaA as the direct target of TnphoA. Subsequent
semi-quantitative RT-PCR analysis revealed that miaA transcript levels in the CNT transposon mutants were strongly reduced by approximately 50% compared to G20-18 (Supplementary Fig. 2). This suggests that regulatory components in the CNT mutants were affected by the transposon mutagenesis, potentially interfering with miaA transcription or the processing and stability of miaA transcripts. Since the mechanism of transcriptional regulation of miaA is not elucidated, we used directed functional approaches to further substantiate the link between miaA as a determinant of microbial CK production and subsequent biocontrol activity against Pto.

Considering the reduced miaA transcript levels in the CNT transposon mutants, compared to G20-18, as the cause for the difference in biocontrol efficacy, functional complementation for CK production by the CNT transposon mutants (gain-of-function) was performed to assess the possible restoration of their biocontrol ability. Therefore, the CK biosynthetic genes isopentenyltransferase from Agrobacterium tumefaciens (ipt) for heterologous expression and the endogenous PflG20-18 miaA for homologous expression were fused to a lac-promoter in the expression vector pBBR1MCS-5 (EV) and analysed for their biocontrol activities. The presence of the EV did not affect biocontrol activity of G20-18 as this strain efficiently restricted Pto symptom development (Fig. 2a) comparable to G20-18 wild-type (Fig. 2b). In contrast, the ipt- or miaA-complemented CNT transposon mutants, exhibited restored biocontrol activities as evidenced by a strong suppression of Pto symptom development (Fig. 2a), comparable to G20-18 biocontrol activity (Fig. 2b). This wild-type-like biocontrol activity in the two CNT transposon mutants functionally complemented via restored CK production by two different CK biosynthetic genes supports the role of microbial CKs as a key determinant for efficient biocontrol of Pto.

To substantiate the gain-of-function data, a complementary loss-of-function approach was followed, addressing the function of miaA and subsequent CK production in G20-18-mediated biocontrol of Pto. To this end, the impact of directed knockout of the G20-18 miaA gene by insertion of a kanamycin resistance cassette into
Figure 3. Distinct ΔmiaA knockout in Pfl G20-18 exhibits a reduced biocontrol activity. (a) ΔmiaA loss-of-function mutant is impaired in its biocontrol ability indicated by stronger Pto symptom development (right leaf halves) 4 days post infection (dpi) with 10^6 cfu ml^-1 compared to Pfl G20-18 pre-treatment. (b) Average Pto symptom score in Arabidopsis 4 dpi with 10^6 cfu ml^-1 after indicated pre-treatments. Data are means ± s.e. n ≥ 79, letters indicate different significance groups (P < 0.05).

the miaA coding region on the biocontrol ability was assessed. This resulted in the Pfl knockout mutant ΔmiaA, which tested PCR-positive for the integration of the disrupted miaA gene sequence in its genome. RT-PCR confirmed the lack of miaA transcripts and thus the functional knockout in this strain (Supplementary Fig. 2). Assays with this ΔmiaA knockout mutant revealed a significant reduction in biocontrol compared to G20-18 wild-type as illustrated by stronger Pto symptom development (Fig. 3). Together, the gain-of-function and directed loss-of-function approaches prove the importance of microbial CK production for their biocontrol ability in the leaf infiltration assays. Interestingly, the distinct functional miaA knockout in ΔmiaA (Supplementary Fig. 2) did not further reduce the biocontrol ability compared to the transposon mutants CNT1 and 2 (Fig. 3) in which low levels of miaA transcripts were still detectable (Supplementary Fig. 2). This suggests that the described biocontrol effect depends on minimum threshold levels of miaA transcripts which subsequently determine CK levels that suffice to induce resistance under particular conditions.

G20-18 biocontrol affects CKs in planta. Based on the established link between Pfl G20-18 CK production and its biocontrol abilities described above, the in planta CK levels were analysed as these should ultimately reflect their contribution to the induction of resistance or defence responses^{16-18}. Therefore, we analysed the accumulation of 25 individual CK species comprising the free nucleobases as well as conjugates^{29} in pooled samples of whole Arabidopsis leaves 48 h post infiltration with the different Pfl strains, which corresponds to the time-point of Pto infection. Thus, these samples integrate all processes related to each individual pre-treatment and determine the plant tissue status at the critical time-point of infection that defines the outcome of the plant-pathogen interaction. CKs were analysed in two sample sets, one comparing the pre-treatments with G20-18, the miaA- or ipt-complemented CNT transposon mutants, and mock control (Table 1 and Supplementary Table 1), and the second comparing pre-treatments with G20-18, the CNT transposon mutants, the ΔmiaA knockout mutant, and mock control (Table 1 and Supplementary Table 2). Eight of ten CK levels that increased after G20-18 treatment in the first set (Supplementary Table 1) also increased in the second set (Supplementary Table 2). A clear trend of lower CK levels in plant tissue pre-treated with loss-of-function CNT transposon or ΔmiaA knockout mutants was observed compared to G20-18 (ratios of 0.82 to 0.89). In contrast, this effect was reversed in tissue treated with the functionally complemented CNT transposon mutants that showed even higher CK levels compared to G20-18 (ratios of 1.08 to 1.11, Table 1). Since CK types differ in their biological activity and signalling function, the individual consideration of specific CK species is important. Total tZ-, cZ-, DHZ- and iP-type CK levels showed similar trends as total CK levels with lower levels after treatments with CK-deficient mutants (Supplementary Table 2) and reversion in the functionally complemented CNT transposon mutants (Supplementary Table 1), which correlates with their differential effect on Pto symptom development (Fig. 1–3). Similarly, levels of the free nucleobases as the most active CK species were lower in tissue treated with CK-deficient mutants (ratios of 0.63 to 0.97) and higher in tissue treated with the functionally complemented CNT transposon mutants (ratios of 1.34 to 2.38) compared to G20-18 (Table 1). In particular, the individual nucleobases tZ, cZ, and iP accumulated to higher levels after treatment with the functionally complemented CNT transposon mutants compared to G20-18 (ratios of 1.30 to 2.50), while these nucleobases in general showed lower accumulation after mutant treatments (Table 1). The most prominent differences were detected in the accumulation of the highly active tZ. Treatment with the three CK-deficient mutants caused significantly lower tZ.
levels (ratios of 0.47 to 0.60) than G20-18 treatment (Table 1), which can directly be related to the defects in miaA expression as miaA has been identified to be responsible for the specific formation of tZ and derivatives from tRNA in different bacteria. Intriguingly, exogenously supplied tZ efficiently restricted infections of Magnaporthe oryzae and tobacco with P. syringae pv. tabaci, while cZ had a much weaker effect on the resistance against Pst and iP treatment did not increase the resistance of rice against Magnaporthe oryzae. This could explain why increased cZ or iP levels in some samples after treatment with the CK-deficient mutants had no effect on resistance against Pto and emphasizes the role of tZ levels as a key parameter in G20-18-mediated biocontrol.

The analyses of a large set of individual CK levels revealed subtle though distinct changes in the host plant. In both datasets specific differences between G20-18 and its derivatives were successfully monitored and appeared to be robust even against variable background levels indicated by the variable CK levels in the control samples (Supplementary Table 1 and Supplementary Table 2). The complex regulation of CK levels in Arabidopsis depends on nine biosynthesis and seven catabolism genes that are potentially affected by biocontrol. The number of viable Pfl mutants could cause lower CK production. Therefore, the number of viable Pfl cells may determine biocontrol by competition with

Table 1. Cytokinin levels in Arabidopsis Col-0 48 h post infiltration with Pfl strains. CK-levels in pmol g\(^{-1}\) fresh weight. Data are represented as mean ± s.e. n = 3. Ratios to G20-18 treatment are given in bold. * and ** indicate significant differences at the 0.05 and 0.01 levels of confidence, respectively. cZ, cis-zeatin; DHZ, dihydrozeatin; iP, isopentenyladenine; ND, not detected; tZ, trans-zeatin.

| G20-18 vs. transposon mutants CNT1 and CNT2 functionally complemented with G20-18 miaA or Atipt | Mock | G20-18 | CNT1 + miaA | CNT1 + ipt | CNT2 + miaA | CNT2 + ipt |
|---|---|---|---|---|---|---|
| tZ | 0.41 ± 0.04 | 0.35 ± 0.04 | 0.46 ± 0.03 | 0.46 ± 0.02 | 0.53 ± 0.05 | 0.61 ± 0.06 |
| 1.15 | 1.00 | 1.30 | 1.31** | 1.51* | 1.73* |
| cZ | 0.048 ± 0.004 | 0.064 ± 0.005 | 0.104 ± 0.010 | 0.159 ± 0.011 | 0.125 ± 0.013 | 0.136 ± 0.013 |
| 0.75* | 1.00 | 1.63* | 2.50** | 1.95 | 2.13** |
| DHZ | ND | ND | ND | ND | ND | ND |
| – | – | – | – | – | – | – |
| iP | 2.97 ± 0.26 | 3.16 ± 0.38 | 4.24 ± 0.54 | 7.92 ± 0.75 | 6.04 ± 0.52 | 5.65 ± 0.58 |
| 0.94 | 1.00 | 1.34 | 2.50** | 1.91* | 1.78* |
| Total CK-bases | 3.43 ± 0.28 | 3.58 ± 0.40 | 4.80 ± 0.56 | 8.54 ± 0.77 | 6.70 ± 0.54 | 6.40 ± 0.63 |
| 0.96 | 1.00 | 1.34 | 2.38** | 1.87** | 1.79* |
| Total CKs | 211.19 ± 16.89 | 185.12 ± 10.31 | 205.12 ± 4.99 | 201.14 ± 16.83 | 205.81 ± 6.51 | 200.42 ± 5.15 |

| G20-18 vs. CK-deficient mutants CNT1, CNT2, and ΔmiaA | Mock | G20-18 | CNT1 | CNT2 | ΔmiaA |
|---|---|---|---|---|---|
| tZ | 0.60 ± 0.07 | 0.79 ± 0.06 | 0.47 ± 0.05 | 0.46 ± 0.06 | 0.37 ± 0.05 |
| 0.77 | 1.00 | 0.60* | 0.59* | 0.47** |
| cZ | 0.036 ± 0.005 | 0.087 ± 0.011 | 0.093 ± 0.005 | 0.102 ± 0.010 | 0.096 ± 0.009 |
| 0.41* | 1.00 | 1.10 | 1.17 | 1.11 |
| DHZ | ND | ND | ND | ND | ND |
| – | – | – | – | – | – |
| iP | 3.21 ± 0.40 | 1.33 ± 0.06 | 1.56 ± 0.16 | 1.28 ± 0.10 | 0.93 ± 0.08 |
| 2.42** | 1.00 | 1.17 | 0.96 | 0.70* |
| Total CK-bases | 3.85 ± 0.43 | 2.20 ± 0.13 | 2.12 ± 0.21 | 1.84 ± 0.16 | 1.39 ± 0.09 |
| 1.75* | 1.00 | 0.97 | 0.84 | 0.63* |
| Total CKs | 181.52 ± 15.75 | 197.10 ± 11.80 | 160.69 ± 11.52 | 174.59 ± 6.03 | 167.54 ± 16.70 |
| 0.92 | 1.00 | 0.82 | 0.89 | 0.85 |
time-point of Pto infection - 48 hours post infiltration (hpi) of Pfl-, was determined for G20-18 and the different mutant strains (Fig. 4a). Similar numbers of viable cells were determined directly after the infiltration (0 hpi) and at 48 hpi for all strains except CNT2 for which viable cells decreased (significantly compared to CNT1). Based on these data, growth differences between G20-18 and the analysed mutant strains can be excluded as the cause of the variations in their biocontrol abilities.

As CKs can directly contribute to a favourable physiological status by modulating primary metabolism\(^8,9\) and thus potentially affect tissue integrity, suppression of symptom development during CK-mediated resistance does not necessarily correlate with restriction of pathogen growth\(^{28}\), which is a direct result of increased resistance. To discriminate between increased resistance induced by G20-18-derived CKs and general impact on tissue integrity, we determined Pto proliferation in planta after pre-treatment with G20-18 and its CK-deficient mutants. Pto proliferation was significantly reduced after G20-18 pre-treatment compared to the mutant and mock pre-treatments at 72 hpi (Fig. 4b) and thus restricted Pto proliferation can be considered as the cause for reduced symptom development in the leaf infiltration assays. Further, Pto proliferation was strongly negatively correlated with the tZ levels determined at the time-point of infection (Table 1) following pre-treatments with the different Pfl strains (ranked data, Spearman’s correlation coefficient of \(-0.8\)). G20-18 pre-treatment resulted in the lowest Pto proliferation and the highest tZ levels, followed by pre-treatments with the CNT transposon mutants which similarly caused lower tZ levels and higher Pto proliferation comparable to mock treatment, while ΔmiaA pre-treatment resulted in the lowest tZ levels and the highest Pto proliferation. This correlation supports the role of specific active CKs in determining biocontrol activities by inducing defence responses that act directly on the pathogen in a dose-dependent manner, similar to resistance effects induced by exogenously applied CKs\(^{11,12}\), which in a certain range can act in a dose-dependent manner and require specific threshold levels to be active.

**G20-18 biocontrol requires plant pathways.** Pfl G20-18 showed suppressive effects on Pto symptom development and multiplication in Arabidopsis indicating direct activation of plant defences, which were lacking after pre-treatment with CK-deficient Pfl mutants. To dissect the underlying plant mechanisms, the efficiency of G20-18-mediated biocontrol was determined in several Arabidopsis lines impaired in phytohormone and/or defence-related mechanisms (Fig. 5a). Since we identified microbial CK production as a determinant of Pfl G20-18-mediated biocontrol against Pto, we assumed functional CK perception as the initial step of CK signalling in the plant to be essential. In Arabidopsis CK perception depends on the three membrane-bound histidine kinases AHK2, AHK3 and AHK4/CRE1/WOL\(^6\). The function of these Arabidopsis CK receptors in G20-18 biocontrol was assessed in the double mutant lines ahk2-2/ahk3-3 and cre1-12/ahk3-3, and the triple mutant cre1-12/ahk2-2/ahk3-3\(^{\text{38}}\) (homozygous for cre1-12 and ahk2-2, heterozygous for ahk3-3)\(^{\text{38}}\). G20-18-mediated biocontrol was reduced in all three mutant lines (Fig. 5a), illustrated by significantly elevated Pto symptom development compared to the wild-type Col-0 (Fig. 5b). This indicates that all three receptors function as signalling components...
of CK-dependent biocontrol by G20-18, which is supported by the finding that the triple mutant exhibited the strongest defect (Fig. 5b). However, a rudimentary G20-18 biocontrol effect is still observed in these mutant plant lines, which is either due to residual CK perception or is unrelated to G20-18 CK production and/or plant CK signalling.

SA was demonstrated as a key central defence signalling component of CK-mediated resistance, mainly depending on NPR1 signalling, against Pto in Arabidopsis10, but also as a parameter of CK-induced resistance or defence responses in other plant species12,13,28. The role of SA in G20-18-mediated biocontrol was assessed in Arabidopsis lines either overexpressing nahG (35S::nahG), a SA-degrading enzyme from Pseudomonas putida39, or defective in SA biosynthesis (sid2)40 or SA signalling (npr1)41. In agreement with the known SA-dependent tZ-mediated resistance effect in Arabidopsis10, G20-18 pre-treatment was almost completely ineffective in these lines as Pto symptoms were not suppressed (Fig. 5a). 35S::nahG, sid2, and npr1 (Fig. 5b) showed Pto symptoms after G20-18 treatment comparable to the mock treatment in the plant mutants and Col-0 wild-type, hence SA accumulation as well as functional SA signalling have to be considered as major parameters in CK-mediated biocontrol.

To examine involvement of the defence-related phytohormones JA and ET, which are important for inducing ISR as part of biocontrol and for priming effects mediated by beneficial microbes4,20, G20-18 biocontrol assays were performed in the mutant lines myc2 (jin1)42 which is partially insensitive to JA43, and ein244, which is insensitive to ET. In both Arabidopsis lines, the suppressive effect of G20-18 on Pto symptoms was reduced (Fig. 5a). Although this reduction was significant compared to Arabidopsis wild-type Col-gl (myc2) and Col-0

Figure 5. Pfl G20-18 biocontrol depends on functional hormonal and defence pathways of the host. (a) Pto symptom development (right leaf halves) 4 days post infection (dpi) with 106 cfu ml−1 in indicated Arabidopsis lines after Pfl G20-18 pre-treatment. (b) Average Pto symptom scores 4 dpi with 106 cfu ml−1 in indicated Arabidopsis mutant or transgenic lines (red bars) compared to Col-0 (Col-gl for myc2) wild-type (blue bars) pre-treated with Pfl G20-18 or the appropriate mock. Data are means ± s. e. n ≥ 28, letters indicate different significance groups (P < 0.05).
immunity, particularly considering the strong dependency on SA of CK-induced resistance against pathogen infection and signalling of the host plant (Fig. 5). This is in agreement with known CK-SA interactions in plant defence as competition or antibiotic production, and strongly depends on functional CK perception as well as SA accumulation and signalling of the host plant (Fig. 5). This is in agreement with known CK-SA interactions in plant immunity, particularly considering the strong dependency on SA of CK-induced resistance against Pto in Arabidopsis leaves by exogenously applied T2. In contrast, the CK-mediated biocontrol of G20-18 in Arabidopsis seems to depend not on or only to a limited extent on JA, ET and camalexin accumulation. The apparent minor effect of these defence signalling pathways could be due to the specific biocontrol assays performed in Arabidopsis leaves and particularly the contribution of JA and ET is probably more relevant in natural microbe-root interactions. Furthermore, these mechanisms do not necessarily depend on microbial CK production and could therefore be responsible for the rudimentary suppression of Pto symptoms caused by the CK-deficient P1 mutants. Generally, the contribution of all these underlying, networked mechanisms to CK-mediated biocontrol as well as the relevance of microbial CKs for biocontrol per se, can vary depending on the conditions of the interaction between the beneficial microbe and the plant. Based on the presented results, which strongly support the potential of microbial CKs for biocontrol effects, directed analyses of CK function in more complex biocontrol systems such as the natural interaction between beneficial microbes and plant roots or their relevance in practical applications such as spraying or seed coating can be conducted in the future.

In the model biocontrol assays used here G20-18 seems to cause only subtle and fine-tuned, but highly efficient changes in the host plant CK levels. Induction of the subsequent resistance seems to be specifically regulated, probably in concert with additional mechanisms such as direct modulation of the metabolic plant-pathogen interface or interference with the niche establishment of Pto. Since CKs are critically involved in various aspects of plant growth, development and physiology, such as spraying or seed coating can be conducted in the future.

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Methods

Plant lines and growth conditions. Arabidopsis plants were grown in soil under controlled short day (8/16 h, 22/20 °C day/night) conditions at 60% relative humidity in growth chambers (APT.line™ KBW 720,
Bacterial strains. The virulent hemibiotrophic bacterial pathogen Pseudomonas syringae pv. tomato DC3000 (Pto) was used for all infections as described before\textsuperscript{58}. For determination of Pto proliferation in planta following pre-treatments with Pseudomonas fluorescens (Pf) strains, Pto was transformed with pMP4662\textsuperscript{2} to facilitate additional selection against tetracycline which is necessary to avoid unspecific background (co-cultivation) by spontaneous rifampicin resistance of Pf cells\textsuperscript{2}. Freshly grown (28 °C, 200 rpm) Pto cells from liquid cultures in 50 mL LB medium containing 50 mg L\textsuperscript{-1} rifampicin (and 20 mg L\textsuperscript{-1} tetracycline for the pMP4662 transformed strain) were pelleted, re-suspended in 10 mM MgCl\textsubscript{2} and adjusted to the desired concentration for the experiments using the BioPhotometer plus (Eppendorf AG).

Pf G20-18 was tested for its biocontrol ability and subsequently used for biocontrol assays. It was tested against its transposon (TphoA) mediated CK-deficient mutants CNT1 and CNT2\textsuperscript{24}, CNT transposon mutants functionally complemented with functional CK biosynthetic genes (homologous expression of G20-18miaA or heterologous expression of Agrobacterium tumefaciens ipt [Atip]) in pBBR MCS-5 and a loss-of-function mutant of G20-18 with a distinct disruption of its CK biosynthetic gene miaA (ΔmiaA). A detailed description of the cloning procedure and generation of Pf derivatives are available as Supplementary Methods. The different Pf strains were cultivated in 50 mL LB medium (28 °C, 200 rpm) containing 10 μM adenine\textsuperscript{24} and appropriate antibiotics: 50 mg L\textsuperscript{-1} ampicillin for G20-18; 50 mg L\textsuperscript{-1} ampicillin and 20 mg L\textsuperscript{-1} gentamycin for G20-18 transformed with pBBR MCS-5; 50 mg L\textsuperscript{-1} ampicillin and kanamycin for CNT1, CNT2, and ΔmiaA; 50 mg L\textsuperscript{-1} ampicillin, kanamycin, and 20 mg L\textsuperscript{-1} gentamycin for CNT transformed with pB BrCS-5 derivatives. Pfi cells were processed as described for Pto including a washing step in 30 mL 10 mM MgCl\textsubscript{2} before the final resuspension.

Biocontrol experiments. For biocontrol assays, whole Arabidopsis leaves were infiltrated with Pf cell suspensions (OD\textsubscript{600} = 0.02) or 10 mM MgCl\textsubscript{2} as a mock control using a needleless syringe two days prior to infection with Pto. Pto infection was performed as described before\textsuperscript{58} by infiltration of Arabidopsis leaf halves with 10\textsuperscript{5} cfu mL\textsuperscript{-1} for Pto proliferation determination or 10\textsuperscript{6} cfu mL\textsuperscript{-1} for analysis of symptom development, respectively.

Pto symptom development (infiltrated leaf halves) was evaluated 4 days post infection (dpi) based on an adapted scale\textsuperscript{12} consisting of 7 categories (Supplementary Fig. 3). Viable Pf cells and Pto proliferation in planta were determined similar to published procedures\textsuperscript{60}. Discs of infiltrated leaves were excised at indicated hours post infiltration (hpi) using a cork borer of 0.4 cm diameter, ground and re-suspended in 1 mL 10 mM MgCl\textsubscript{2} 100 μl of serial 1:10 dilutions were plated in triplicate on LB medium containing appropriate antibiotics for selection and colony formation was determined after 36 h incubation at 28 °C.

Cytokinin determination. For CK determination, a minimum of 10 Arabidopsis leaves per sample were harvested 48 hpi with Pf strains or the mock control, immediately frozen and ground in liquid nitrogen. Cks were extracted and determined by UHPLC-MS/MS as described before\textsuperscript{2}. The CK-types quantified in this study are cis-zeatin (cZ), cZ-O-glucoside (cZOG), cZ-riboside (cZR), cZR-O-glucoside (cZROG), cZ-9-glucoside (cZ9G), cZR-5′-monophosphate (cZR5′MP), dihydrozeatin (DHZ), DHZ-O-glucoside (DHZOG), DHZ-riboside (DHZR), DHZR-O-glucoside (DHZROG), DHZ-7-glucoside (DHZ7G), DHZ-9-glucoside (DHZ9G), DHZR-5′-monophosphate (DHZR5′MP), isopentenyladenine (iP), iP-riboside (iPR), iP-7-glucoside (iP7G), iP-9-glucoside (iP9G), iPR-5′-monophosphate (iPR5′MP), trans-zeatin (tZ), tZ-O-glucoside (tZOG), tZ-riboside (tZR), tZR-O-glucoside (tZROG), tZ-7-glucoside (tZ7G), tZ-9-glucoside (tZ9G), tZR-5′-monophosphate (tZR5′MP).

Statistical analysis. Statistical analyses were performed for datasets deriving from a minimum of three biological experiments. Unpaired Student’s t-test was used to compare group differences. P values < 0.05 were considered significant and letters in bar graphs indicate different significance groups. *, **, and *** indicate significant differences at the 0.05, 0.01, and 0.001 levels of confidence, respectively.

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pBBR1MCS-5, numbers LO1204 and 15-22322S, to M.S. and the Czech Ministry of Education, Youth and Sports of CR within UNCPBA to S.A.S., the Czech Ministry of Education grant from the National Program for Sustainability I, grant No. 4093-00255 of the Danish Council for Independent Research, Danish Ministry of Higher Education and the Society for the Advancement of Plant Sciences (Vienna, Austria) and by the Individual Postdoctoral Grant for critical reading and constructive comments on the manuscript. This work was supported by funding from Pto

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How to cite this article: Großkinsky, D. K. et al. Cytokinin production by Pseudomonas fluorescens G20–18 determines biocontrol activity against Pseudomonas syringae in Arabidopsis. Sci. Rep. 6, 23310; doi: 10.1038/srep23310 (2016).

Acknowledgements

We thank Helga Hammer for superb plant care, Peter Krbez for excellent technical assistance, Kerstin Edelsbrunner for supporting initial miaA identification, Thomas Engelke for supplying pMP4662 transformed Pto, Susanne Berger and Tatsuo Kakimoto for supplying genetically modified Arabidopsis lines and Matthias Ulrich for supplying the vectors pBBR1MCS-5, pK18mobGII and pRK2013. We are grateful to Claus Wasternack for critical reading and constructive comments on the manuscript. This work was supported by funding from the Society for the Advancement of Plant Sciences (Vienna, Austria) and by the Individual Postdoctoral Grant No. 4093-00255 of the Danish Council for Independent Research, Danish Ministry of Higher Education and Science to D.K.G., by an ERASMUS–EADIC II fellowship to M.V.M., funding from the University of Graz and UNCPBA to S.A.S., the Czech Ministry of Education grant from the National Program for Sustainability I, grant numbers LO1204 and 15-22322S, to M.S. and the Czech Ministry of Education, Youth and Sports of CR within the National Sustainability Program I (NPU I), grant number LO1415, to T.R.

Author Contributions

D.K.G. designed and performed the experiments, analysed and interpreted the data, and wrote the manuscript. R.T. performed the experiments, analysed and interpreted the data. M.V.M. and S.A.S. performed the experiments and analysed the data. I.E.G.d.S. and L.M.N. supplied material and interpreted the data. O.N. and M.S. performed cytokinin determination and analysed the data. E.v.d.G. analysed and interpreted the data, and wrote the manuscript. T.R. designed the experiments, interpreted the data, and wrote the manuscript. All authors discussed the results and commented on the manuscript.

Additional Information

Accession codes: The obtained sequence of the Pseudomonas fluorescens G20-18 miaA gene has been deposited in the GenBank database under the accession code KM593658.

Supplementary information accompanies this paper at http://www.nature.com/srep.

Competing financial interests: The authors declare no competing financial interests.

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