High cytokinin levels induce a hypersensitive-like response in tobacco

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INTRODUCTION

Cytokinins were first identified by their ability to promote cell division in tobacco tissue cultures (Miller et al., 1955). They have since been reported to promote shoot development, delay leaf senescence, contribute to stress and pathogen responses, and serve as important signals for co-ordinating growth rates throughout the plant (Argueso et al., 2009; Werner and Schmülling, 2009; Ha et al., 2012). The modulation of cytokinin metabolism or signalling could thus potentially be a powerful tool for improving crop yield. For example, rice plants that have elevated cytokinin levels due to a decrease in the expression of the cytokinin-degrading enzyme cytokinin oxidase/dehydrogenase can produce more grains per plant because of changes in their inflorescence architecture (Ashikari et al., 2005). Increasing leaf longevity via senescence-specific overexpression of a key cytokinin biosynthetic gene (ipt) was shown to increase biomass and seed production (Gan and Amasino, 1995), prolong the post-harvest shelf life of leaf crops (McCabe et al., 2001) and induce extreme drought tolerance (Rivero et al., 2007). Moreover, cytokinins play critical roles in plant–microbe interactions. In nitrogen-fixing plants, cytokinin receptors are important for symbiotic nitrogen fixation. Specifically, cytokinin receptor loss-of-function mutants are unable to form nodules (Gonzalez-Rizzo et al., 2006; Murray et al., 2007), while gain-of-function mutants form nodules spontaneously (Tirichine et al., 2007). Several lines of evidence indicate that cytokinins have a wide range of functions in plant–pathogen interactions. Pathogen infection has been reported to cause changes in cytokinin levels. For example, some pathogens seem to use their ability to produce cytokinins to facilitate disease development. Agrobacterium tumefaciens is the causal
agent of crown gall disease in a number of dicot species, including some important crops. The symptoms of the disease are caused by the insertion of T-DNA from a bacterial Ti plasmid into the plant genome. This T-DNA carries genes necessary for the biosynthesis of cytokinins (ipt), auxin and opines. While high cytokinin and auxin levels convert the transformed cells into rapidly growing tumours, opines secreted from the transformed cells serve as a source of carbon and nitrogen for the Agrobacterium (Chilton et al., 1977). Pathogen- (Plasmodiophora brassicae) derived cytokinins are also involved in the development of clubroot disease (Devos et al., 2006; Siemens et al., 2006), and transgenic plants that overexpress cytokinin oxidase/dehydrogenase show less severe disease symptoms (Siemens et al., 2006). On the other hand, beans inoculated with the viral pathogen White clover mosaic potexvirus reduced cytokinin levels (Clarke et al., 1999), and treatment of the inoculated bean plants with exogenous cytokinin resulted in reduced replication of the White clover mosaic potexvirus concomitant with upregulation of defence response genes such as those encoding pathogenesis-related (PR) proteins (Clarke et al., 1998).

Measures adopted by plants against disease-causing microorganisms include constitutive defences (in the form of antimicrobial secondary metabolites) and induced defence responses that require detection of the pathogen by the plant, which is followed by the rapid activation of defence-related genes. A plant–pathogen interaction that triggers the full defence syndrome is said to be incompatible. A common defence is the hypersensitive response, in which cells immediately surrounding the infection site die rapidly and thus deprive the pathogen of nutrients, preventing its spread. The hypersensitive response is preceded by the rapid accumulation of reactive oxygen species (ROS) (Dangl et al., 1996; Love et al., 2008; O’Brien et al., 2012). A characteristic feature of defence responses is the expression of PR proteins (Rushton and Somssich, 1998), which are a diverse group of enzymes, antifungal agents and secondary signalling components.

Despite their well documented function in delaying senescence, there are several lines of evidence linking cytokinins to the induction of cell death. It was shown that Arabidopsis and carrot cell cultures cultivated in a medium with high cytokinin content exhibit reduced growth and show hallmarks of programmed cell death (Carimi et al., 2003). The authors also showed that cytokinins promote premature senescence and the onset of apoptosis hallmarks in Arabidopsis plantlets cultivated in vitro. Similar results were obtained using tobacco BY-2 cells treated with the cytokinins isopentenyladenine and benzyladenine, as well as the corresponding ribosides (Mliejnek et al., 2002, 2003, 2005). Necrosis in old leaves was reported in transgenic tobacco and maize, in which targeted cytokinin accumulation in these leaves was achieved by placing ipt expression under the control of senescence-specific promoters (Robson et al., 2004; Wingler et al., 2005).

We have previously reported necrotic lesion formation in the leaves of transgenic tobacco plants within 3 d of ipt activation (Šámalová et al., 2005). Here, we employed this system to investigate in detail the diverse processes that lead from ipt activation to necrotic lesion formation. Based on the data obtained, we discuss the potential roles of cytokinins in the hypersensitive response to pathogen attacks.

### MATERIALS AND METHODS

#### Plant material and treatments

Transgenic plants CaMV35S > GR > ipt (pOp6-ipt/LhGR-N, lines 303 and 307; Šámalová et al., 2005) and the corresponding wild-type Nicotiana tabacum ‘SR1 Petit Havana’ were grown in an AR-36L growth chamber (Percival Scientific) with 16 h light at 24 °C/8 h dark at 21 °C under a photosynthetic photon flux density of 100 μmol photons m⁻² s⁻¹ provided by cool white fluorescent lamps (Philips). All experiments were performed using 5-week-old plants. A dexamethasone (DEX) (Sigma) solution with a final concentration of 20 μM was prepared by diluting a 20 mM stock solution in 96 % ethanol (Lachema, Czech Republic) with tap water. A 50 mL aliquot of the diluted solution was applied to the soil by watering 3 h before the start of the dark period. Control plants were watered with 0.096 % ethanol in tap water (mock). Samples were collected in the middle of the light period from non-damaged tissue at the indicated times after DEX application, frozen in liquid nitrogen and stored at −80 °C. For trans-zeatin (t-Z) feeding, leaves were detached from wild-type plants, immersed via petioles into t-Z solutions in tap water of concentrations ranging from 10 μM to 2.5 mM and incubated for 3 d under the light and temperature regime outlined above. Controls were fed with 1 % dimethylsulfoxide (DMSO; v/v) in tap water. To minimize gas exchange, lanolin was applied to the leaf surfaces according to Mateo et al. (2004).

#### Quantification of phytohormones

For endogenous cytokinin analysis, plant material with a fresh weight of approx. 0.2 g was extracted and purified according to the method described by Novák et al. (2003). A cocktail of isotopomeric deuterium and ¹³C₆-labelled standards (Olchemim) was added (each at 1 pmol per sample) to check the recovery during purification and to validate the determination. The cocktail consisted of [¹³C₆]c-Z, [²H₆]c-Z, [²H₆]i-ZR, [²H₆]i-Z7G, [²H₆]i-ZROG, [²H₆]i-ZROG, [²H₆]i-ZMP, [²H₃]DHZ, [²H₅]DHZR, [²H₅]DHZH9G, [²H₅]DHZH9G, [²H₅]DHZH9G, [²H₅]DP, [²H₅]i-P, [²H₅]i-P7G, [²H₅]i-P7G, and [²H₅]i-PMP (for a list of abbreviations, see Supplementary Data Table S2). The samples were purified using two ion-exchange chromatography steps (SCX followed by a DEAE-Sephadex column in conjunction with SPE C18 cartridges) and immunoaffinity purification. The cytokinin levels in each sample were quantified by ultra-performance liquid chromatography–electrospray tandem mass spectrometry (UPLC-MS/MS) (Novák et al., 2008). Endogenous salicylic acid (SA), jasmonic acid (JA) and abscisic acid (ABA) were determined in samples with fresh weights of 0·3 g using a combination of an octadecylsilica column (500 mg; Agilent Technologies) and a DEAE-Sephadex (Sigma Aldrich) column with a Sep-Pak C18 cartridge (360 mg; Waters). Plant material was extracted overnight in 80 % methanol, and 100 pmol of [²H₆]SA (Cambridge Isotope Laboratories), 50 pmol of [²H₅]ABA and [²H₆]JA (Olchemim) were added as internal standards. Quantification was performed as described by Bergougnoux et al. (2009). Separation was performed on an Acquity UPLC System (Waters) equipped with a Luna phenyl–hexyl column (250 × 2·0 mm, 5 μm; Phenomenex, Torrance, CA, USA), and the effluent was introduced into the...
electrospray ion source of a Xevo™ TQ MS (Waters) triple quadrupole mass spectrometer (Waters).

**Determination of leaf dry matter content and chlorophyll**

Leaf dry matter content (LDMC) was measured by drying 1–2 g of fresh plant material for 5 d in a drying oven (Memmert) at 70 °C and then for 2 d at 105 °C when constant weight of samples was reached.

Samples for chlorophyll determination were cut with a cork borer and analysed according to Wellburn (1994). Specifically, the samples were transferred into tubes containing 2 mL of 99.5 % DMSO (Sigma-Aldrich) and incubated for 2 h at 50 °C with occasional shaking. Their absorbance was then read at 649 and 665 nm (Helios B, Chromspec) and these measurements were used to calculate the sample’s chlorophyll content (Wellburn, 1994).

**Determination of hydrogen peroxide and lipid peroxidation**

Hydrogen peroxide was quantified using a slightly modified variant of the method reported by Alexieva et al. (2001). Leaves (300 mg f. wt) were homogenized to powder in liquid nitrogen, extracted in 3 mL of 0.1 % trichloroacetic acid (TCA) and centrifuged at 10 000 g at 4 °C for 10 min. The supernatant was separated and mixed with 0.1 mL potassium phosphate buffer (pH 7.0) and 0.5 mL potassium iodide (Fluka) solution at a ratio of 1:1:2 (v/v/v). The resulting mixture was allowed to react for 30 min in the dark, after which its absorbance at 390 nm was measured. The hydrogen peroxide content of the sample was then calculated using a standard curve.

The subcellular distribution of hydrogen peroxide was visualized by endogenous peroxidase-dependent histochemical staining using 3,3′-diaminobenzidine (DAB) (Sigma) according to Pogány et al. (2004) with slight modifications. Leaves were incubated in 2.5 mM Tris-phosphate buffer (pH 7.8) supplemented with 2.5 mM DAB for 2 h. The incubated leaves were then decolorized by incubation in a clearing solution comprising 79.85 % ethanol, 20 % chloroform (Penta, Czech Republic) and 0.15 % TCA (Sigma). The presence of hydrogen peroxide is indicated by the formation of a brown precipitate.

The extent of lipid peroxidation within samples was determined by estimating their malondialdehyde (MDA) content according to a modification of the method reported by Heath and Packer (1968). Samples were extracted with 0.1 % TCA using the procedure that was employed when determining hydrogen peroxide levels. A reaction mixture comprising 0.5 mL of supernatant, 0.5 mL of a 0.1 M potassium phosphate buffer (pH 7-0) and 1 mL of 0.5 % thiobarbituric acid (Sigma) was then prepared and heated on a water bath at 95 °C for 30 min, after which it was rapidly cooled in an ice bath. The cooled mixture was centrifuged at 10 000 g at 4 °C for 5 min and the absorbance of supernatant at 532 and 600 nm was determined. The non-specific absorbance at 600 nm was subtracted from the absorbance at 532 nm, and the concentration of MDA was calculated based on an extinction coefficient of 155 mmol L⁻¹ cm⁻¹.

**RT–qPCR analysis**

Steady-state transcript levels of genes coding for bacterial isopentenyl transferase (ipt), chlorophyll alb-binding protein (CAB), ferredoxin:NADP oxidoreductase (FNR1), violaxanthin de-epoxidase (VDE), pathogenesis-related protein 1b (PR-1b) and acidic phosphatase (PR-Q) were quantified by reverse transcription followed by quantitative PCR (RT–qPCR) using the fluorescent dye SYBR Green I. Leaves of 5-week-old tobacco were frozen in liquid nitrogen and total RNA was extracted using the TRIZol reagent (Invitrogen). The resulting total RNA extract was treated with TurboDNase (Ambion) to remove residual DNA. Reverse transcription was performed using the SuperScriptII reverse transcriptase (Invitrogen) and an oligo(dT) primer. qPCR was performed using the Rotor-Gene 6000 real-time analyzer (Corbett Research, Australia) with the primers and conditions listed in Supplementary Data Table S1. The amplification of a single specific product was verified by melting curve analysis and agarose gel electrophoresis. The measured expression levels were normalized by geometric averaging using the GeNorm VBA applet for Microsoft Excel (Vandesompele et al., 2002), with actin (Tob66) and EF-1α as the reference genes.

**Leaf gas exchange**

Leaf gas exchange was measured using an LI-6400 open gas-exchange system (LI-COR, Lincoln, NE, USA) on the third or fourth attached leaf (counted from the plant base) after 30 min of dark adaptation. Light induction of photosynthesis and transpiration was measured over 45 min of exposure to actinic light (330 μmol photons m⁻² s⁻¹) that was switched on after a 5 min period for the leaves to acclimate to the chamber conditions. The chamber conditions were as follows: mass flow of air 200 μmol s⁻¹, relative air humidity 50 %, block temperature 24.5 °C, CO₂ concentration 390 μmol mol⁻¹. The CO₂ response of photosynthesis was measured under an air mass flow of 300 μmol s⁻¹ at nine different ambient CO₂ concentrations (Cₐ); the starting Cₐ was 400 μmol mol⁻¹, followed by 300, 200, 100, 50, 400, 600, 800, 1000 and 1200 μmol mol⁻¹. The intercellular CO₂ concentration (Cᵢ) was calculated using the function described by von Caemmerer and Farquhar (1981). The carboxylation efficiency of Rubisco was indicated by the slope of the initial linear part of the A/Cᵢ curve.

**Chlorophyll fluorescence parameters**

Slow induction kinetic parameters were measured on the adaxial sides of the third or fourth attached leaves using a Dual-PAM-100 Chlorophyll Fluorescence & P700 Photosynthesis Analyzer (Heinz Walz GmbH, Effeltrich, Germany), which can be used to monitor chlorophyll fluorescence and changes in P700 absorbance simultaneously. Plants were pre-darkened for 30 min and then exposed to measuring light (approx. 1 μmol photons m⁻² s⁻¹) and red actinic light (approx. 100 μmol photons m⁻² s⁻¹) for 10 min together with a series of 0.5 s saturation pulses (6000 μmol photons m⁻² s⁻¹, red light) every 45 s. The non-photochemical chlorophyll fluorescence quenching (NPQ), the relative rates of electron transport via photosystem I (PSI) and PSII – ETR (I) and ETR (II), respectively – and the quantum yield of non-photochemical energy dissipation due to donor side limitations of PSI (Y(ND)) at steady-state were estimated (Klughammer and Schreiber, 2008). Images of steady-state NPQ were recorded using a
FluorCam 700MF imaging system (Photon Systems Instruments, Czech Republic). The adaxial side of the third or fourth attached leaf was irradiated for 10 min with actinic light (100 μmol photons m⁻² s⁻¹) and the measurement protocol described by Prokopová et al. (2010) was applied.

Statistical analysis

Three biological replicates were used in each experiment. In each biological replicate, three random samples were collected from each of three plants. The resulting nine samples were analysed in a single technical replicate in all experiments except those involving RT–qPCR, in which case two technical replicates were used. Student’s t-test was used to evaluate the statistical significance of the results obtained. The standard deviation of the measured ratios was calculated using the equation:

$$SD = \frac{X}{X_1} \sqrt{\left(\frac{SD_1}{X_1}\right)^2 + \left(\frac{SD_2}{X_2}\right)^2}$$

RESULTS

ipt activation results in a hypersensitive-like response in the leaves of tobacco plants

The binary pOp-ipt/LhGR system for DEX-inducible ipt expression (Šamalová et al., 2005) was used to increase endogenous cytokinin levels in transgenic tobacco plants, and the resulting morphological, histological, biochemical and molecular changes were investigated. Two independent CaMV35S > GR > ipt lines (303 and 307) were cultivated for 5 weeks under standard long-day photoperiod conditions and exhibited no detectable phenotypic differences relative to wild-type plants during this period. However, when the transgenic plants were watered once with 20 μM DEX in tap water, slightly translucent zones appeared on expanded horizontal leaves around 50 h after exposure to DEX. The translucent zones developed into lesions over the following 10 h (Fig. 1). No marks of chlorosis were apparent on these leaves and no statistically significant decrease in chlorophyll content occurred (Supplementary Data Fig. S1). Massive lesion formation continued until the fourth day of DEX application and then ceased. Lesion formation was associated with wilting, and both these processes resulted in the complete collapse of the expanded horizontal leaves in plants of line 303, which gradually died over the following 3 weeks. The extent of lesion formation and wilting was less severe in line 307 (Fig. 1), and plants of this line recovered from the effects of the single dose of 20 μM DEX, developed normally afterwards and even produced viable seeds. Lesion formation was dose dependent. In the more responsive line 303, a single treatment with 1 μM DEX was sufficient to provoke some lesion formation, but lower DEX concentrations were non-effective (Fig. 2).

Young, recently opened upward-facing leaves underwent some distinctive processes immediately after exposure to DEX. The leaves initially curled and then (on the fourth day after being treated with DEX) developed chlorosis (Fig. 3). These leaves did not develop substantial necrotic lesions. Interestingly, a slight but significant transient increase in chlorophyll content was seen in line 307 following activation.

Root growth was also negatively affected in the transgenic plants exposed to DEX for 4 d. The fresh weight of the root system harvested from DEX-treated line 303 was lower by 25 % (P < 0.05) compared with the mock-treated line 303, while DEX treatment did not result in any statistically significant difference in the root system of wild-type plants.

Dynamics of the cytokinin pool upon ipt activation in the tobacco plants

To investigate the dynamics of the cytokinin pool following ipt activation, the cytokinin bases and their metabolites were analysed in samples of expanded horizontal leaves that did not

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**Fig. 1.** Phenotype of CaMV35S > GR > ipt plants after treatment with dexamethasone. Five-week-old CaMV35S > GR > ipt lines 303 (A, C), 307 (B) and the wild type (D) after 4 d of treatment with 50 mL of 20 μM dexamethasone (A, B, D) or 0.096 % ethanol (C).
contain lesion areas (Fig. 4) by UPLC-MS/MS. Dramatic increases in the levels of both cytokinin bases and their ribosides and ribotides were observed within 19 h of exposure to a single dose of 20 μM DEX (first day after DEX treatment; 1 DAT). These increases levelled off over the following 24 h (2 DAT), reaching values about four orders of magnitude higher than those observed in non-activated samples. In absolute terms, the levels of cytokinin free bases in the activated leaves reached 30 nmol g⁻¹ f. wt, while the levels of the ribosides and ribotides rose to 64 nmol g⁻¹ f. wt. O-Glucosylation represented a significant part of conjugative inactivation of free cytokinins. While there was a very significant increase in the levels of cytokinin-O-glucosides and cytokinin-N9-glucosides by 1 DAT, it remained much less pronounced than the increase in the levels of the corresponding free bases until 2 DAT. From 3 DAT onwards, the levels of cytokinin-O-glucosides were of the same order of magnitude as those of free bases, but those of the cytokinin-N9-glucosides were around three orders of magnitude lower. The cytokinin pool was dominated by t-Z and its metabolites (Supplementary Data Table S2). In general, following ipt activation, the cytokinin pool was consistently somewhat higher in line 303 than in line 307, which is consistent with the stronger phenotypic response observed in line 303: as discussed above, a DEX solution of only 1 μM was sufficient to induce lesion formation in tobacco leaves. Analysis of the cytokinin pool (Supplementary Data Fig. S2) revealed that in line 303, on the third day after a single application of 1 μM DEX, the levels of free cytokinin bases, their riboside and ribotides, and O- and N9-glucosides were only 2-, 9-, 10- and 3-fold lower, respectively, than those observed following treatment with 20 μM DEX. This implies that the levels of free biologically active cytokinins, i.e. free bases, must increase by about four orders of magnitude to reach the threshold level required to induce the processes that lead to lesion formation in tobacco leaves. To prove that the main biologically active cytokinin increased upon ipt activation (t-Z) is sufficient to induce lesion formation per se, detached tobacco leaves were fed with t-Z by dipping their petioles into t-Z solution in tap water. The effective concentrations of t-Z start from 1 mM (Supplementary Data Fig. S3).

The increase in cytokinin levels is followed by oxidative stress and dehydration in expanded leaves

Cytokinins were shown to induce oxidative stress related to cell death in tobacco BY-2 cells (Mlejnek et al., 2003) and

FIG. 2. Effects of different dexamethasone concentrations on lesion formation in CaMV35S > GR > ipt plants. Five-week-old plants of CaMV35S > GR > ipt line 303 were induced with 50 mL of a dexamethasone solution at the indicated concentrations. The images show plants photographed 91 h after dexamethasone treatment (4 DAT). Scale bars = 3 cm.

FIG. 3. Time course of chlorophyll content in young leaves of CaMV35S > GR > ipt plants following ipt activation. Five-week-old CaMV35S > GR > ipt lines 303, 307 and the wild type (SR1) were induced with 50 mL of 20 μM dexamethasone. Samples for chlorophyll analysis were collected at 19 h (1 DAT), 43 h (2DAT), 67 h (3 DAT) and 91 h (4 DAT) after treatment with dexamethasone. (A) The chlorophyll content of plants treated with dexamethasone (+DEX) is expressed as a percentage of that observed in plants treated with 0.096 % ethanol (–DEX) and all reported values represent the means of nine measurements. Error bars indicate the s.d. One and two asterisks indicate statistically significant differences between +DEX and –DEX plants at P ≤ 0.05 and P ≤ 0.01, respectively, based on the t-test. (B) Typical leaves of DEX-treated plants.
hydrogen peroxide was identified as a major orchestrator of molecular responses to both biotic and abiotic stresses (Vandenabeele et al., 2003). Therefore, we examined the hydrogen peroxide levels and membrane damage in leaves that developed lesions following the activation of the ipt gene. The potassium iodide assay (Pogány et al., 2004) was used to monitor the levels of hydrogen peroxide in samples of expanded horizontal leaves. To avoid potential interference arising from oxidative stresses associated with the degradation processes taking place in dying cells, only lesion-free plant material was used in these experiments. Significantly elevated hydrogen peroxide levels were detected in samples harvested 43 h after DEX application (2 DAT), peaking at 3 DAT (Fig. 5A). The rates and extent of hydrogen peroxide accumulation in lines 303 and 307...
were similar. It thus seems that hydrogen peroxide accumulation preceded the initiation of lesion formation by at least 7 h. Histochemical localization of hydrogen peroxide using DAB staining (Liu et al., 2007) in leaves harvested 3 DAT indicated that most of the hydrogen peroxide was present in the chloroplasts (Fig. 6). This suggests that the chloroplasts are primarily responsible for the hydrogen peroxide produced following increases in cytokinin levels.

To assess the degree of membrane damage caused by increased hydrogen peroxide levels, we determined the MDA content of leaf material, which reflects the extent of lipid peroxidation. The time course of MDA accumulation matched that of hydrogen peroxide in all cases except that at 2 DAT, when the increase in MDA levels was statistically significant only in line 303 (Fig. 5C).

Water losses associated with membrane damage are probably responsible for the wilting of affected leaves (and in the case of line 303, their eventual collapse). To quantify this loss, the LDMC for the two lines was determined (Fig. 5C). Significant increases in LDMC were observed 3 DAT, i.e. 24 h after the increase in hydrogen peroxide levels. This coincided with a strong increase in membrane peroxidation. The LDMC in line 303 was consistently higher than that for line 307, which is consistent with the much stronger leaf phenotype found in line 303.

The increase in cytokinin levels is followed by downregulation of photosynthesis-related genes and upregulation of defence-related genes

Pathogen attacks trigger changes in photosynthetic activity, while the hypersensitive response involves the induction of pathogenesis-related proteins. To investigate further the similarities between lesion formation resulting from a massive increase in cytokinin levels and the hypersensitive response caused by pathogen attack, we examined the expression of photosynthesis- and defence-related genes in leaves that developed lesions following ipt activation (Fig. 7). RT-qPCR analysis confirmed a dramatic increase in ipt transcript abundance 1 DAT. The transcript’s abundance then decreased slightly at 2 DAT and remained at this slightly lower level 3 DAT. The CAB, FNR1 and VDE genes code for chlorophyll a/b-binding protein, ferredoxin:NADP oxidoreductase and violaxanthin de-epoxidase, respectively, and were selected as representative photosynthesis-related genes. In all cases except that of VDE in line 307, the abundance of the transcripts of these genes fell at 2 DAT. A further decrease (in many cases, to below the limit of detection) occurred 3 DAT. Once again, VDE in line 307 was exceptional, exhibiting only a mild decrease in transcript levels 3 DAT relative to the situation 1 DAT. The PR-1b and PR-Q genes code for pathogenesis-related protein 1b and an acidic phosphatase, respectively, and were selected as representative pathogenesis-related genes. The abundance of their transcripts began to increase 2 DAT, and rose dramatically over the following 24 h. In keeping with the relative strength of the leaf phenotypes in the two lines, the changes in the transcript profiles were more pronounced in line 303 than in line 307.

Effect of increased cytokinin level on photosynthesis

The gene expression analysis indicated that high cytokinin levels have inhibitory effects on photosynthesis. To examine this issue on a physiological level, we analysed a number of photosynthetic parameters in line 303 following the activation of the ipt gene. First, the degree of stomatal opening and carboxylation efficiency were determined 67 h after DEX application (3 DAT; Fig. 8). Light-induced stomatal opening was reduced significantly in the leaves of plants in which ipt expression was activated. Microscopic examination revealed that this decreased stomatal conductance was accompanied by stomatal closure (data not shown). Similarly, a light-induced increase in the rate of CO₂ assimilation was inhibited, which indicated downregulation of Rubisco activation. The carboxylation efficiency of Rubisco was also inhibited, as demonstrated by a decrease in the slope of the linear region of a plot of A against Cₜ (from 0.025 to 0.015; Fig. 9). We then monitored the changes in chlorophyll fluorescence over time following ipt activation (Figs 10 and 11). A very significant increase in the NPQ occurred within 43 h of ipt activation (2 DAT) and became slightly more pronounced over the following 24 h (3 DAT), reflecting an increase in the dissipation of excitation energy. The values of the ETR (II) and ETR (I) parameters decreased 3 DAT, indicating that electron transport through PSII and PSI was inhibited. There was also an increase in Y(ND), which indicates increased energy dissipation at the donor side of PSI due to the reduced rate of electron donation to PSI (Klughammer and Schreiber, 2008).

Fig. 6. Hydrogen peroxide accumulation in chloroplasts of CaMV35S > GR > ipt plants following ipt activation. Five-week-old plants of CaMV35S > GR > ipt line 303 were induced with 50 mL of 20 μM dexamethasone (B, D) or 0.096 % ethanol (A, C) as a control. Leaf samples for histochemical staining using 3,3’-diaminobenzidine were collected 64 h (3 DAT) after treatment with dexamethasone.

Dynamics of stress hormones following ipt activation in tobacco leaves

Ipt activation in lines 303 and 307 triggered a number of processes similar to those observed in the hypersensitive response to...
pathogen attack. Therefore, we examined the dynamics of pathogenesis- and stress-related hormones following *ipt* activation (Fig. 12). Pronounced increases in the levels of SA and JA were observed within 43 h of *ipt* activation (2 DAT) and these peaked over the following 24 h at levels that were at least two orders of magnitude higher than those observed in non-activated controls. Levels of abscisic acid increased to a lesser degree but were still between eight and ten times higher than those in the controls.

**Effect of decreased stomatal conductance on cell death**

The *ipt*-expressing plants were shown to have strongly reduced stomatal conductance and thus very limited potential for gas exchange. Such conditions can be simulated by applying lanolin to the leaf surfaces of non-activated plants (Mateo et al., 2004). This was done to determine whether the reduced gas-exchange capabilities of the activated plants could be responsible for the observed lesion formation. While lanolin treatment did cause lesion formation, this was only observed when both sides of the leaves were treated. More importantly, lesion formation was preceded by chlorosis and it took 3–4 weeks of lanolin treatment to achieve a level of lesion formation comparable with that observed in line 303 only 4 d after *ipt* activation (Supplementary Data Fig. S4).

**DISCUSSION**

We report a detailed analysis of necrotic lesion formation triggered in tobacco leaves following a dramatic increase in cytokinin levels due to activation of the bacterial cytokinin biosynthesis gene *ipt*. Our findings indicate that this process is remarkably similar to the hypersensitive response in plant–pathogen interactions. We therefore discuss our findings in the context of the potential role of cytokinins in plant–pathogen interactions.
ipt activation triggers distinct phenotypes in leaves depending on their developmental stage

There is a large body of evidence suggesting that cytokinins are important positive regulators of shoot development (e.g. Werner et al., 2003; Higuchi et al., 2004; Nishimura et al., 2004; Riefler et al., 2006). However, we and others have reported cell death in the leaves of transgenic plants that ectopically express a gene encoding the key cytokinin biosynthetic enzyme isopentenyl transferase (Li et al., 2004; Robson et al., 2004; Šámalová et al., 2005; Wingler et al., 2005). While developing and characterizing a stringent and highly responsive DEX-inducible gene expression system (pOp6/LhGR) for tobacco, we observed the development of necrotic lesions, severe wilting and eventual cell death following ipt activation in CaMV35S > GR > ipt transgenic tobacco plants (Šámalová et al., 2005). Here, we present a detailed analysis of this phenomenon. A single dose of DEX at saturating concentration (20 µM) caused a dramatic increase in ipt transcription and the size of the cytokinin pool in 5-week-old transgenic tobacco plants. The best performing previously characterized systems of inducible ipt expression did not yield levels of free endogenous cytokinin bases above 0.6 nmol g⁻¹ f. wt (Faiss et al., 1997; McKenzie et al., 1998). In contrast, our system achieved levels of 20 nmol g⁻¹ f. wt at 2 DAT, at which point the cytokinin response was almost saturated. Whether such high cytokinin levels are produced under natural conditions in plant tissues remains a challenge for future work. One can anticipate that high increases in cytokinin levels would be limited to small regions of plant tissue. Currently, methods allowing reliable determination of plant hormone metabolites in very small plant samples are being developed (Novák et al., 2012). In this context, it is worth mentioning that some pathogen-derived cytokinins were shown to increase plant tissue sensitivity even to classical cytokinins (Pertry et al., 2009).

In shoots, within the first 4 d after ipt activation, clear phenotypic responses were only observed in the leaves, and depended on their stage of development. Older leaves that had already achieved a horizontal orientation but were still growing developed necrotic lesions without any prior or accompanying chlorosis, wilted, and eventually died in the more responsive line 303. Young, upward-oriented leaves developed chlorosis but only rarely formed lesions. These phenotype changes were observed only when free cytokinin levels increased by about four orders of magnitude over their baseline levels. Leaf necrosis associated with cytokinin overproduction has previously been reported in leaves of transgenic plants expressing ipt under the control of senescence-specific promoters. However, in these older studies, necrosis formation was only observed in old leaves that were already senescing. In transgenic SAG12-IPT tobacco plants, cell death was observed only in a fraction of old leaves and only under conditions of limited nutrient availability (Wingler et al., 2005). In the more strongly responsive transgenic maize line Sg3, which carries the PmaeXhbaIPTNOS transgene, old leaves progressed directly from fully green to bleached and dead without an intervening yellowing phase, even under conditions of standard nutrient supply (Robson et al., 2004).
analysis presented herein thus represents the first investigation of cytokinin-induced necrosis in expanding leaves that have not undergone any significant reduction in their chlorophyll content. The responses observed in this work are thus wholly uncoupled from senescence and are therefore more similar to the hypersensitive response – a type of cell death associated with pathogen infection.

In general, cytokinins are considered to be positive regulators of chloroplast biogenesis and function based on physiological and anatomical evidence (Chory et al., 1995), as well as transcriptomic (Brenner et al., 2005) and proteomic analyses (Lochmanová et al., 2008; Černý et al., 2011). Interestingly, the predominant cytokinin biosynthesis pathway and some metabolism pathways have been reported to be localized in the chloroplasts (Brzobohaty et al., 1993; Kristoffersen et al., 2000; Takei et al., 2004; Kiran et al., 2006), and the chloroplast cytokinin pool has been found to be dynamic (Benková et al., 1999). Nevertheless, chlorosis has been observed in some transgenic plants that overproduce cytokinins. Decreases in the chlorophyll content of young leaves similar to those presented in this work were reported in \textit{SAG12-IPT} tobacco under conditions of nitrogen remobilization (Jordi et al., 2000), and significant chlorosis in emerging young leaves was found in \textit{Psee1Xba IPTNOS} transgenic maize grown under low nutrient conditions (Robson et al., 2004). This chlorosis was linked to defects in the standard remobilization of nutrients from old leaves, in which senescence is strongly delayed due to a targeted

![Fig. 10](image1.png)

**Fig. 10.** Time course of chlorophyll fluorescence parameters in \textit{CaMV35S > GR > ipt} plants following \textit{ipt} activation. Plants of line 303 were treated as described in Fig. 3. The fluorescence parameters (A) NPQ, (B) ETR (II), (C) ETR (I) and (D) Y (ND) were determined under steady-state conditions (10 min of actinic PAR, 100 μmol photons m$^{-2}$ s$^{-1}$) 1, 2 and 3 DAT. Means and s.d. are shown ($n=3–5$). One and two asterisks indicate statistically significant differences between +DEX and −DEX plants at $P \leq 0.05$ and $P \leq 0.01$, respectively, based on the $t$-test.

![Fig. 11](image2.png)

**Fig. 11.** False colour images of NPQ in leaves of \textit{CaMV35S > GR > ipt} plants following \textit{ipt} activation. Plants of line 303 were treated as described in Fig. 3. NPQ was determined under steady-state conditions (10 min of actinic PAR, 100 μmol photons m$^{-2}$ s$^{-1}$) on 1, 2 and 3 DAT.
increase in cytokinin levels. Thus, chlorosis reported previously in ipt-expressing plants is unlikely to result directly from the effects of cytokinins in young leaves. Similarly, the chlorosis observed in young leaves in this work might result from decreased assimilate availability due to extensive damage in older leaves and/or reductions in the uptake and transport of mineral nutrients from the soil. In addition to lesion formation, photosynthesis in older leaves was dramatically inhibited by increased cytokinin levels by 3 DAT. Growth of the root system decreased by 25% in plants with an increased cytokinin level 4 DAT. In roots, cytokinins were reportedly shown to affect negatively the uptake of mineral nutrients including nitrate, ammonium, phosphate and iron (reviewed in Rubio et al., 2009). This is consistent with the observation that chlorosis only became apparent 4 DAT. As a matter of fact, there was a slight increase in chlorophyll levels 2 DAT in the young leaves of the less responsive line 307, although they declined later on. Nevertheless, our data cannot exclude that increased cytokinin levels per se caused the chlorosis in young leaves directly.

The hypersensitive-like response triggered by increased cytokinin levels

Cytokinins reportedly play distinct roles in plant–pathogen interactions (reviewed by Argueso et al., 2009; Choi et al., 2011) which are dependent, at least in part, on the nature of the pathogen. While necrotrophs kill the cells of the host plant, biotrophs cause minimal cellular damage and require living host tissues. They can produce cytokinins and auxins to modulate the physiology of host plants to support biotroph proliferation. On the other hand, plant derived-cytokinins are reportedly involved in plant resistance to viral infection and other pathogens that do not secrete cytokinins. For example, cytokinins were shown to modulate SA signalling to increase resistance against Pseudomonas syringae in Arabidopsis (Choi et al., 2010). Infected plants can activate localized programmed cell death related to the hypersensitive response which impairs pathogen spread. The hypersensitive response is induced by ROS and reactive nitrogen species. Cytokinins were shown to induce programmed cell death (Mlejnek and Procházka, 2002; Carimi et al., 2003); however, their involvement in the hypersensitive response has not been reported.

Here we show that the dramatic increase in cytokinin levels triggered by ipt activation resulted in extensive cell death and lesion formation in older leaves. Since this was not accompanied or preceded by chlorosis, the overall process closely resembled the hypersensitive response associated with pathogen attack. The hypersensitive-like nature of the cytokinin response is further demonstrated by the observed changes in various biochemical and molecular parameters prior to the appearance of the first islets of dead cells in affected leaves. One of the early consequences of increased cytokinin levels identified in this work was an increase in the levels of chloroplastic-associated hydrogen peroxide. This was accompanied by oxidative membrane damage, increases in stress hormone levels and PR transcripts, and decreases in the abundance of transcripts related to photosynthesis. High doses of exogenous cytokinin have also been reported to increase the levels of ROS in tobacco BY2 cells (Mlejnek et al., 2003). Our data provide the first evidence for a chloroplast-associated burst of hydrogen peroxide production in response to endogenously produced Z-type cytokinins in planta. Since milder increases in cytokinin levels have been reported to stimulate enzymes involved in ROS detoxification (Zavaleta-Mancera et al., 2007; Barna et al., 2008), our data suggest that cytokinins have two separate and concentration-
dependent effects on the cellular ROS pool. Hydrogen peroxide has been shown to orchestrate molecular responses to both biotic and abiotic stresses, and transcriptomic analysis demonstrated that hydrogen peroxide production is closely linked to the levels of stress hormones such as SA, JA and ethylene (Vandenabeele et al., 2003). Salicylic acid is important in plants’ defences against pathogens: it can prevent local infections and also mediate systemic acquired resistance, promoting resistance at sites remote from the point of infection. Jasmonic acid mediates local and systemic defence responses to wounding by herbivores. Cytokinins were reported to modulate SA signalling to enhance resistance against P. syringae (Choi et al., 2010), and a systems analysis revealed a synergism between cytokinin and SA in plant disease networks (Naseem et al., 2012). However, direct stimulation of either SA or JA accumulation in response to cytokinin action has not been documented. On the contrary, SA levels decreased relative to those in an untransformed control in transgenic tobacco explants that were propagated in vitro and overproduced cytokinins due to ipt expression driven by a promoter of the Rubisco small subunit (Schnablóva et al., 2006). In poplars, treatment with exogenous cytokinins did not affect JA levels in undamaged leaves. However, cytokinin priming increased the wound-inducible accumulation of JA during insect attacks on the poplar leaves (Dervinis et al., 2010). It thus seems that hydrogen peroxide mediated the increase in the levels of SA and JA that was observed in this work following increases in cytokinin abundance. The induction of PR protein expression is a key component of local and systemic defence responses. The transcriptional activation of many PR genes is regulated by an SA-mediated signal transduction cascade (reviewed by Van Loon, 1997), and a similar system may be responsible for the transcriptional activation observed in this work. On the other hand, a number of PR proteins are encoded by late cytokinin response genes (Rashotte et al., 2003). It is therefore possible that the activation of the PR-1b and PR-Q genes observed in this work could be a direct consequence of cytokinin signalling.

Inhibition of photosynthesis upon ipt activation in transgenic tobacco

Photosynthesis, ROS levels and the hypersensitive response are intertwined at several levels (for a recent review, see Kangasjärvi et al., 2012). It has been argued that chloroplastic ROS production contributes to plant immunity (Karpinski et al., 1999; Vandenabeele et al., 2004), and photosynthetic electron transfer reactions are known to be a significant source of ROS (Hideg et al., 2002). In addition, the photoactive nature of chlorophyll provides another mechanism for ROS formation in chloroplasts (Lorrain et al., 2003). However, photosynthesis is inhibited by high levels of ROS (Vandenabeele et al., 2003). We observed downregulation of CAB, FNRI and VDE transcripts related to photosynthesis within 43 h of ipt activation, which might indicate a reduction in photosynthetic activity. A similar downregulation of the corresponding genes was observed following the activation of the ipt gene in Arabidopsis (Hoth et al., 2003). Together with the fact that chlorophyll levels remained unchanged, the downregulation of CAB transcript levels might indicate the presence of free chlorophyll in the chloroplasts, which could contribute to ROS generation in the tobacco leaves that developed lesions following ipt activation. Analyses of chlorophyll fluorescence and CO2 assimilation revealed that photosynthetic activity declined following ipt activation. Moreover, electron transport through PSII and PSI was inhibited, there was an increase in the amount of energy dissipated at the donor side of PSI due to reduced electron donation and there was an increase in the dissipation of excitation energy by heat emission, as demonstrated by an increase in NPQ. The need to dissipate excess chlorophyll excitation energy via NPQ reflects a decrease in electron consumption by CO2 assimilation resulting from the downregulation of Rubisco activity, together with a decrease in CO2 availability due to reduced stomatal conductance. Decreases in CO2 assimilation reportedly lead to an excess of excitation energy, the formation of ROS and hypersensitive-like cell death (Mateo et al., 2004; Liu et al., 2007). However, in our system, the decrease in CO2 availability did not seem to be the main contributor to the hypersensitive-like response induced by cytokinins since reductions in CO2 availability following treatment with lanolin paste (Mateo et al., 2004) were not by themselves sufficient to trigger this response on a time scale of 4 d. The decrease in stomatal conductivity correlated with closure of the stomata. Cytokinins are generally considered to promote stomatal opening and decrease sensitivity to ABA. However, the responses to hormones are often dependent on their abundance (reviewed by Acharya and Assmann, 2009). We found a good correlation between stomatal closure and the kinetics of the increase in ABA levels. We have previously observed elevated ABA levels in tobacco plants overexpressing the Sho gene, which codes for a cytokinin biosynthetic enzyme from Petunia hybrida (Polanská et al., 2007). The occurrence of cross-talk between cytokinin and ABA on the metabolic level is further supported by findings such as those obtained in an analysis of transgenic tobacco overexpressing Arabidopsis cytokinin oxidase/dehydrogenase AtCKX3. In these plants, the decrease in cytokinin levels caused by AtCKX3 activity was accompanied by a decrease in ABA content (Polanská et al., 2007). It therefore seems that an increase in the levels of ABA and hydrogen peroxide is probably responsible for the observed stomatal closure following ipt activation in CaMV35S > GR > ipt tobacco plants. The stomatal closure probably represents an insufficient attempt to prevent the water losses that accompany elevated cytokinin levels. However, stomatal closure also protects the plant against pathogen invasion (Melotto et al., 2006) and so would be an expected response to pathogen-related hormones.

Conclusions

Dramatic increases in endogenous cytokinin levels triggered by activating the ipt gene from the bacterial soil plant pathogen A. tumefaciens cause a hypersensitive-like response in transgenic tobacco leaves. The data presented herein are consistent with the suggestion that the molecular processes underpinning this hypersensitive-like response are orchestrated by increases in cellular hydrogen peroxide levels. The hypersensitive-like response includes inhibition of photosynthesis, increases in stress...
hormone levels, oxidative damage of membranes and stomatal closure. Overall, the data indicate that cytokinins can act as mediators in plant–pathogen interactions. This conclusion is consistent with the results of recent dynamic modelling studies and systems analyses, which identified multiple cytokinin-mediated regulatory interactions in plant disease networks (Naseem et al., 2012).

**SUPPLEMENTARY DATA**

Supplementary data are available online at https://www.aob.oxfordjournals.org and consist of the following. Table S1: primers and amplification conditions used for gene expression analysis. Table S2: time course of cytokinin accumulation in CaMV35S > GR > ipt plants following ipt activation. Figure S1: time course of chlorophyll content in older leaves of CaMV35S > GR > ipt plants following ipt activation. Figure S2: cytokinin content of CaMV35S > GR > ipt plants following ipt activation. Figure S3: effects of different t-Z concentrations on lesion formation in detached leaves of wild-type plants. Figure S4: effects of lanolin on lesion formation in tobacco leaves.

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**LITERATURE CITED**


Cytokinins can trigger hypersensitive-like response


