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THE CEREAL PATHOGEN FUSARIUM PSEUDOGRAMINEARUM PRODUCES A NEW CLASS OF ACTIVE CYTOKININS DURING INFECTION

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SUMMARY

The fungal pathogen *Fusarium pseudograminearum* causes important diseases of wheat and barley. During a survey of secondary metabolites produced by this fungus, a novel class of cytokinins, herein termed Fusarium cytokinins, was discovered. Cytokinins are known for their growth promoting and anti-senescence activities and the production of a cytokinin mimic by what was once considered a necrotrophic pathogen that promotes cell death and senescence challenges the simple view that this pathogen invades its hosts by employing a barrage of lytic enzymes and toxins. Through genome mining, a gene cluster in the *F. pseudograminearum* genome for the production of Fusarium cytokinins was identified and the biosynthetic pathway established using gene knockouts. The Fusarium cytokinins could activate plant cytokinin signalling, demonstrating their genuine hormone mimicry. *In planta* analysis of the transcriptional response to one Fusarium cytokinin suggests extensive reprogramming of the host environment by these molecules, possibly through crosstalk with defence hormone signalling pathways.

INTRODUCTION

Plant growth and development is coordinated by phytohormones, which are small signal molecules active at low (nanomolar) concentrations. Eight classes of phytohormones are recognised; abscisic acid, auxins, cytokinins, ethylene, gibberellins, brassinosteroids, jasmonates and salicylic acid (SA) (Kende & Zeevaart, 1997, Creelman & Mullet, 1997). Phytohormones not only regulate physiological functions and reproduction, but also are involved in complex interconnected immune responses upon pathogen and insect attack (Pieterse *et al.*, 2009). SA, jasmonic acid (JA) and ethylene are well established as central hormones regulating plant defences against pathogens (De Vos *et al.*, 2005). However, a number of recent reports have implicated other hormones in plant defence or susceptibility including the cytokinins (Hann *et al.*, 2014, Siemens *et al.*, 2006, Jiang *et al.*, 2013, Jameson, 2000, Novak *et al.*, 2013). Cytokinins are involved in diverse processes including cellular proliferation, differentiation, the control of the morphological balance between shoot and root tissue, modification of source sink relationships as well as delaying leaf senescence (Sakakibara, 2006, Choi *et al.*, 2011). They also appear to have anti-apoptotic properties (Othman *et al.*, 2016). Cytokinin signalling has also been well characterised in the model plant *Arabidopsis thaliana* reviewed by To & Kieber (2008), where three histidine kinase receptors potentiate cytokinin signalling through a phospho-relay system to response regulators which bind DNA and regulate transcription (positively or negatively). The receptors and response regulators are conserved in the model monocot *Brachypodium distachyon* (hereafter *Brachypodium*) (Kakei *et al.*, 2015).

Cytokinins are adenine derivatives, which differ at the side chain at the N^6 position that can be either an isoprenoid or aromatic group (Strnad, 1997, Tarkowska *et al.*, 2003). Biosynthesis of cytokinins occurs through prenylation of AMP, ADP or ATP, which is catalysed by adenylate isopentenyltransferase (IPT) using dimethylallyl diphosphate

(DMAPP) as a substrate (Takei *et al.*, 2001, Kakimoto, 2001). This enzymatic step leading to the formation of isopentenyladenine riboside monophosphate (iPRMP), was first identified in the amoeba *Dictyostelium discoideum* (Taya *et al.*, 1978) and in *Agrobacterium tumefaciens* (Barry *et al.*, 1984, Akiyoshi *et al.*, 1984). iPRMP is subsequently hydroxylated by a cytochrome P450 monooxygenase before the cytokinins are transformed in a two-step reaction by a nucleotidase and a nucleosidase (Chen & Kristopeit, 1981) to the active forms trans-zeatin (tZ) and isopentenyladenine (iP). The final activation can also be performed in a single step by an enzyme named Lonely Guy (LOG) due to the mutant phenotype where rice flowers often contained only one stamen but no pistil (Kurakawa *et al.*, 2007).

An alternative route for cytokinin biosynthesis is through a transfer RNA (tRNA) pathway. In this pathway an adenine residue in tRNA is N^6-prenylated by a tRNA isopentenyltransferase (tRNA-IPT) and subsequently released from the tRNA by hydrolysis (Miyawaki *et al.*, 2006, Gray *et al.*, 1996, Koenig *et al.*, 2002). tRNA-IPTs are found in all organisms except Archaea and the addition of dimethylallyl diphosphate (DMAPP) to a tRNA-bound adenine nucleotide followed by degradation in Arabidopsis results in synthesis of cis-zeatin (cZ) (Miyawaki *et al.*, 2006).

Plant pathogenic fungi have developed numerous strategies to prevail over their hosts including production of phytohormones interfering with plant immune responses. The maize pathogen *Colletotrichum graminicola* is thought to alter cytokinin responses at infection sites, either through fungal production of a cytokinin mimic or via an indirect route (Behr *et al.*, 2012). Cytokinin activity has also been reported in extracts from the members of the *Fusarium* genus including the cereal pathogen *F. culmorum* (Michniewicz *et al.*, 1986, Vanstaden & Nicholson, 1989). The tRNA pathway for cytokinin biosynthesis is present in all fungi including the plant pathogens *Magnaporthe oryzae* (Chanclud *et al.*, 2016), *Leptosphaeria maculans* (Trdá *et al.*, 2016) and *Claviceps purpurea* (Hinsch *et al.*, 2016)

resulting in production of iP and cZ. *C. purpurea* also has a second pathway similar to the AMP prenylation route that produces iP via an IPT-LOG fusion enzyme (Hinsch *et al.*, 2015). A cytochrome P450 monooxygenase, which catalyses the iP hydroxylation to tZ, is located next to the IPT-LOG gene forming a small cytokinin production gene cluster in this species. Two active copies of this gene cluster responsible for tZ production is also present in members of the *Fusarium fujikuroi* species complex (Niehaus *et al.*, 2016).

A role for plant produced cytokinins in defence processes is emerging in model systems. In Arabidopsis, both exogenous and endogenous cytokinins can lead to decreased susceptibility to bacterial and oomycete pathogens. Mechanistically, cytokinins are thought to act via potentiation of SA-mediated responses (reviewed in Albrecht and Argueso (2016)). In rice, cytokinins also potentiate SA responses (Jiang *et al.*, 2013) where expression of classical marker genes such as *PR1b* was induced only by SA-cytokinin co-treatment in a SA-signalling dependent manner. In contrast to the potentiation of SA responses, cytokinins can also induce susceptibility to pathogens. Cytokinin production by *M. oryzae*, via the tRNA pathway, attenuates host defence responses and alters the sugar and amino acid distribution to favour the pathogen (Chanclud *et al.*, 2016). Similarly a Pseudomonas effector protein activates pools of inactive plant cytokinin precursors which in turn reduce pattern triggered immunity responses (Hann *et al.*, 2014). In *C. purpurea,* cytokinin production contributes to pathogen virulence (Hinsch *et al.*, 2016) presumably by the induction of susceptibility, but the precise mechanism of action of cytokinins in this pathosystem is yet unclear.

Fusarium pseudograminearum causes both Fusarium crown rot and Fusarium head blight diseases of wheat and barley (Obanor *et al.*, 2013). In this study, we report the identification of a new class of cytokinins produced by *F. pseudograminearum*, describe the underlying fungal genes for their biosynthesis, propose their biosynthetic route and go on to verify their

genuine cytokinin activity through receptor activity assays and analysis of host gene expression in response to the most highly produced compound.

RESULTS

F. pseudograminearum produces novel cytokinin-like molecules in axenic culture and during host infection

During profiling of secondary metabolite production by *F. pseudograminearum* (CS3096) three pyrrol-substituted purine derivatives, hereafter collectively termed Fusarium cytokinins, were identified. The three compounds were structurally elucidated by NMR (for detailed spectroscopic data, see Data file S1) and named fusatin (**1**; $C_{10}H_9N_5$), 8-oxo fusatin (**2**; $C_{10}H_9N_5O_1$) and fusatinic acid (**3**; $C_{10}H_7N_5O_2$) (**Fig. 1A**). Fusatinic acid and 8-oxo fusatin are novel compounds, while fusatin has previously been obtained synthetically through oxidation of zeatin (Haidoune *et al.*, 1990). A fourth compound, 8-oxo-isopentenyladenine (**4;** $C_{10}H_7N_5O_2$) was also confirmed as being produced by *F. pseudograminearum* by high resolution mass spectroscopy and NMR (data not shown). Trace amounts of a mass ($C_{10}H_7N_5O_3$ ([M+H]$^+$: m/z 246.062) corresponding to a carboxylic acid form of 8-oxo fusatin (= 8-oxo fusatinic acid) were also observed (data not shown). Fusatin and fusatinic acid were detected in wheat samples 14 days post single floret infection (**Fig. 1B**). 8-oxo-fusatin could not be detected *in planta* although its lower limit of detection was considerably higher than the other compounds. Fusatinic acid was the more abundant of the two detected Fusarium cytokinins with levels between 0.1 and 1 μg g^{-1} (average 0.2 μg g^{-1}). The mycotoxin deoxynivalenol, which is upregulated by *Fusarium* spp. during infection (Mudge *et al.*, 2006), was present at concentrations approaching 5 μg g^{-1} in these samples. A concentration of 0.2 μg g^{-1} of fusatinic acid is equivalent to approximately 0.1 μM, a concentration at which native plant cytokinins can activate cytokinin receptors and are physiologically active (Miwa

et al., 2007). Since these extracts were from whole heads, localised concentrations of fusatinic acid are likely to be well in excess of the measured levels.

Biosynthesis of cytokinin molecules is encoded in a gene cluster expressed during infection

To identify the genes responsible for Fusarium cytokinin biosynthesis, the *F. pseudograminearum* genome was searched for homologues of the *Arabidopsis* Lonely Guy 1 (LOG1, AT2G28305.1). Two matches both with ~40% amino acid identity to LOG1 were identified (FPSE_07269 and FPSE_06372). FPSE_06372 is hereafter named FCK1. In addition to the LOG1 homology, FCK1 contained an isopentenyl-transferase at its N-terminus. *FCK1* was deleted from *F. pseudograminearum* by homologous gene replacement (Fig. S1), and in all four independent mutants generated, the production of the Fusarium cytokinins was abolished when analysed qualitatively by inspection of HPLC-MS traces (data not shown). Relative quantification analysis of Fusarium cytokinins of replicated cultures of a single *FCK1* mutant confirmed the qualitative observations (**Fig. S2**). Furthermore, during the course of this study a similar protein identified in *C. purpurea* (CPUR_04177) and two in *F. fujikuroi* (FFUJ_03536 and FFUJ_14354) were shown to contribute to a large proportion of the tZ pool produced by these species (Hinsch *et al.*, 2015, Niehaus *et al.*, 2016). This prompted an analysis of a broader suite of fungal genomes for sequence homologues of these genes. Twenty-six additional IPT-LOGs were identified in *Fusarium* spp.. Eight *F. oxysporum* strains as well as *F. fujikuroi* and *F. verticillioides* contained two IPT-LOG orthologues. Outside of the *Fusaria* and *Claviceps,* there was a match in *Zymoseptoria tritici* although this did not cover the full length of the protein. Phylogenetic analysis of the IPT-LOG suggests three separate isoforms exist in fungal genomes (**Fig. 2A**).

Comparative genome analysis suggested that the *F. pseudograminearum* cluster consists of three additional genes (*FCK2 (FPSE_20001)*, *FCK3 (FPSE_06371)* and *FCK4*

(FPSE_20002)) (**Fig. 2B**). FCK2 is a cytochrome P450 monooxygenase; FCK3 has a weak (*e*-value 6×10^{-8}) hit to capsule polysaccharide biosynthesis protein domain (pfam05704) which suggests this protein might have a role as a glycosyl transferase; FCK4 has a domain match (*e*-value 2×10^{-15}) to an alcohol acetyltransferase (pfam07247). The original annotation of the *F. pseudograminearum* genome (Gardiner *et al.*, 2012) failed to predict *FCK2* and *FCK4,* but RNAseq data clearly demonstrated their presence (**Fig. S3**). The Fusarium cytokinin cluster is located 0.5 Mbp from the end of chromosome 3 in a region of high inter-strain diversity (Gardiner *et al.*, 2017).

As observed in the *C. purpurea and F. fujikuroi* clusters (Hinsch *et al.*, 2015, Niehaus *et al.*, 2016), cytochrome P450 monooxygenase encoding genes adjacent to IPT-LOGs were observed for all analysed *Fusarium* strains. However, phylogenetic analysis of cytochrome P450 monooxygenases suggested that like the IPT-LOG, there was three sequence types (**Fig S4**). Taken together, the analyses of IPT-LOG and the cytochrome P450 monooxygenases are suggestive of three separate cluster types that, when considered with the very different cytokinin like molecules produced by *F. pseudograminearum* and *C. purpurea,* are unlikely to be orthologous.

Biosynthesis of Fusarium cytokinins occur through two parallel pathways

To determine the biosynthetic route (**Fig. 3A**) for Fusarium cytokinins a combination of biochemical characterisation of heterologously produced enzyme and analysis of culture extracts of mutant strains was employed. Strains of *F. pseudograminearum* carrying deletions of *FCK1* were unable to produce any Fusarium cytokinins nor iP, tZ or cZ (**Fig. 3B**) which were all detectable in the progenitor strain (CS3096). A role for FCK1 in production of cZ was unexpected as this compound is commonly produced via degradation of prenylated-adenine moieties from tRNA, but since the tZ was also absent in the FCK1 mutant, it is possible that cZ is isomerized from tZ (Bassil *et al.*, 1993). Heterologously expressed FCK1

was able to use AMP and DMAPP *in vitro* (**Fig. 4**) as accumulation of both iPRMP and iP were observed. After extended incubation (18 hours), almost no iPRMP and only iP was detectable. The mutant analysis places FCK1 as the first committed step (from primary metabolites) in the biosynthesis of Fusarium cytokinins and the biochemistry suggests that the reaction catalysed by the bifunctional FCK1 proceeds via the prenylation followed by the removal of the phosphoribose. Detection of iPRMP in these reactions is consistent with release of this intermediate to the bulk solvent prior to utilisation by the second active site.

Deletion of the cytochrome P450 monooxygenase encoding gene (*FCK2*) resulted in the production of only two cytokinins, iP and 8-oxo-isopentenyladenine (**4**). The accumulation of iP and (**4**) and the absence of all other cytokinins in the *ΔFCK2* strains suggest that FCK2 is responsible for the step immediately downstream of these compounds, which we are proposing is tZ based on the knowledge that cytochrome P450 monooxygenases are responsible for this reaction in plants and fungi. Protein structural modelling and docking also support tZ as a product of FCK2 (**Fig. S5**). However it is also possible that FCK2 (directly or indirectly) is responsible for the formation of the C-N bond in the formation of the pyrrol ring, which might explain why the phylogenetic analysis suggests the cytochrome P450 monooxygenase in the *C. purpurea* cluster and FCK2 are not orthologous. Carbon-nitrogen bond formation by cytochrome P450 monooxygenases is unusual but not unprecedented; a *Streptomyces* sp. cytochrome P450 monooxygenase is involved in the formation of a C-N bond in biosynthesis of staurosporine (Onaka *et al.*, 2005). The *ΔFCK2* strain also provided the primary evidence for the parallel nature of the proposed biosynthesis pathway. Based on the accumulation of **4** in this mutant it must arise from either the prenylation of 8-oxo-AMP (by FCK1) and/or oxygenation of iP. C-8 carbonyl derivatives of adenine or cytokinins are uncommon in nature although formation has been observed in DNA damaged by ϒ-irradiation (Bonicel *et al.*, 1980) and is also catalysed by a xanthine dehydrogenase in the

rhizobacterium *Serratia proteomaculans* (Taylor *et al.*, 2006). Several putative xanthine dehydrogenases are present in *Fusarium* spp.

Deletion of *FCK3* resulted in enhanced production of cytokinins, including of a compound with a *m/z* of 236.1141 [M+H]$^+$ (**Fig. 3A**) which could be 8-oxo tZ ($C_{10}H_{13}N_5O_2$, theoretical [M+H]$^+$: 236.1142), an obvious intermediate for the parallel biosynthesis of 8-oxo-fusatin, but repeated attempts to elucidate the structure failed most likely due to insufficient amounts of compound. Deletion of *FCK4* also resulted in enhanced production of cytokinins compared to the wild type, which was most noticeable for cZ (**Fig. 3A**; **Fig. S2**). The enhanced production in the *ΔFCK3* and *ΔFCK4* strains could suggest that both the enzymes act downstream of the identified Fusarium cytokinins to produce yet unidentified compounds. The mechanism for oxidation of fusatin to fusatinic acid remains unknown. Heterologous expression of the cytokinin cluster in *F. graminearum* resulted furthermore in production of **1**, **2**, **3** and of the possible 8-oxo tZ, which were not observed in the wild type (**Fig. S6**).

FA has weak senescence delaying activity in planta and can activate cytokinin signalling

Analysis of RNAseq data demonstrated all four genes of the *F. pseudograminearum* cytokinin cluster are expressed during infection of barley and *Brachypodium*. Expression levels for *FCK1*, *FCK2* and *FCK3* approached that of the first step (*TRI5*) of deoxynivalenol biosynthesis (**Fig. S3**), which is important for stem-base infection of cereals by *Fusarium* spp. (Desmond *et al.*, 2008, Scherm *et al.*, 2011). Expression of *FCK4* was at least an order of magnitude lower than other genes in the cluster in barley and below quantification limits in *Brachypodium*. In contrast, the only available dataset of *F. verticillioides* infecting plants (Lanubile *et al.*, 2014) showed that only *FCK4* was expressed during ear rot (data not shown). Fusarium cytokinins were also not detected in metabolite extracts of *F. verticillioides* or *F. fujikuroi*, suggesting the cluster, with the exception of *FCK4*, may be inactive in culture,

consistent with the analysis of transcriptional data for five different strains from this lineage by Niehaus *et al.* (2016).

Despite the structural similarities of Fusarium cytokinin molecules to compounds with cytokinin activity, small structural differences can convert cytokinin agonists into antagonists (Spichal *et al.*, 2014). To test if fusatinic acid had cytokinin activity, its ability to prevent senescence of detached leaves was assessed and compared to the synthetic cytokinin 6-benzyl aminopurine (BAP), which elicits plant growth and developmental responses (D'Aloia *et al.*, 2011, Chen & Yang, 2013). The results showed that BAP prevented senescence in *Brachypodium* (**Fig. 5A**) with leaves appearing greener than DMSO controls. In contrast, fusatinic acid did not obviously reduce senescence based on visual inspection of the leaves (**Fig. 5A**). However using a quantitative measure leaf colour, the samples treated with fusatinic acid were slightly (~7%) darker than the solvent control (*p*-value 0.007) (**Fig. 5B**). BAP treatment caused leaves to be considerably darker (32%, *p*-value 2×10^{-7}). Thus fusatinic acid may have very weak senescence inhibiting activities in *Brachypodium*.

To further investigate the potential for cytokinin agonistic activities of Fusarium cytokinins, their ability to activate a cytokinin receptor was assayed in a bacterial system. In this system the Arabidopsis histidine kinase 3 (AHK3) cytokinin receptor controls the expression of β-galactosidase (Mizuno & Yamashino, 2010) which has been previously used to demonstrate the signalling activity of a range of cytokinins and functionally characterise the suite of cytokinin receptors in Arabidopsis (Inoue *et al.*, 2001, Miwa *et al.*, 2007, Spíchal *et al.*, 2004, Suzuki *et al.*, 2001). All Fusarium cytokinins activated AHK3 signalling (**Fig. 5C**). Taken together, the *in vitro* experiments suggest that Fusarium cytokinins are genuine cytokinin agonists as fusatinic acid has the ability to weakly delay senescence of detached leaves and to activate the AHK3 receptor.

F. pseudograminearum cytokinin molecules are cytokinin-signalling agonists in the model monocot, Brachypodium.

To further elucidate the effect of fusatinic acid on plants, a global analysis of *Brachypodium* gene expression in response to treatment with fusatinic acid or BAP was conducted. Fusatinic acid was chosen based on its terminal position in the proposed biosynthetic pathway and its measurement as the most abundant Fusarium cytokinin *in planta* (**Fig. 1** and **3**). Treatment with fusatinic acid, when compared to the solvent control resulted in statistically significant differential regulation of 1996 loci in *Brachypodium* (1485 up- and 511 down-regulated). Of these fusatinic acid regulated genes, 81% were also differentially regulated under BAP treatment and in 98.5% of these genes the direction of regulation was the same under both fusatinic acid and BAP treatment (**Fig. 5D**). In the cytokinin signalling pathway, fusatinic acid upregulated the expression of four type A response regulators (Bradi2g6100, Bradi3g45930, Bradi4g43090 and Bradi5g25960; *p*-value for all $5x10^{-5}$) between two and four-fold. Type A response regulators in Arabidopsis act as feedback regulators of not only cytokinin signalling but also SA signalling (To & Kieber, 2008, Argueso *et al.*, 2012). Two of the three cytokinin receptors were also significantly (*p*-value < 0.003) upregulated by fusatinic acid (Bradi1g10660 and Bradi2g59137) albeit by small fold changes of ~1.3 fold. The substantial overlap in the fusatinic acid and BAP regulon further supports the view that fusatinic acid has cytokinin agonistic activity in *Brachypodium*.

The role of cytokinins in plant resistance or susceptibility is poorly understood (Robert-Seilaniantz *et al.*, 2011). It was recently proposed that pathogen produced cytokinins in the *Magnaporthe*-rice interaction act to supress defence pathways along with altering or maintaining nutrient availability for the pathogen (Chanclud *et al.*, 2016). However, in Arabidopsis, current evidence suggests the opposite is true where cytokinins contribute to resistance against biotrophic pathogens via increasing SA responses including the expression

of the classical SA responsive gene *PR1* (Choi *et al.*, 2010). Following fusatinic acid treatment, *PR1* (Bradi1g57590) was down-regulated 2.1-fold (p-value = 0.006). However, this was not observed under BAP treatment and under pathogen inoculation at a later time point where this gene was in fact upregulated ~20 fold (Powell *et al.*, manuscript in preparation). This observation led to the querying of whether other defence pathways were partially regulated by fusatinic acid. To this end, genes responding to fusatinic acid or BAP were compared to those responsive to other hormones presented by Kakei *et al*. (2015). Apart from the substantial overlap with genes in the response to the cytokinin tZ, more than 30% of all genes upregulated by methyl jasmonate were also upregulated by fusatinic acid and BAP (**Fig. 5E**). This included up-regulation of JA biosynthesis (lipoxygenase 3 [Bradi5g11590, 1.8-fold p-value 5×10^{-5}]; three allene oxide synthases [Bradi1g69330, 1.5 fold p-value 5×10^{-5}; Bradi3g08160, 1.9 fold *p*-value 5×10^{-5}; Bradi3g23190 5-fold *p*-value 5×10^{-5}]) and response genes (nine different JAZ genes and Myc2 [Bradi3g34200, 3-fold p-value 1.3×10^{-3}]). The overlaps between responses to SA and fusatinic acid or BAP were lower at 18% of genes.

The observation of overlaps between the response to fusatinic acid and other plant hormones was consistent with a global analysis of gene ontologies conducted on the positively fusatinic acid regulated gene set (Data file S2). In this analysis, terms corresponding to the response to jasmonic acid, salicylate, auxin, gibberellin, abscisic acid and ethylene but surprisingly not cytokinin were all enriched (FDR $\leq 4\times10^{-3}$). When the $\log_2$ fold expression data comparing fusatinic acid with DMSO was mapped on to the *Brachypodium* metabolic pathways, 472 genes that were statistically significant (up or down) at the 0.05 level were assigned to known reactions. However, the majority of enzymes that could be assigned to reactions in the BrachyCyc database (Tello-Ruiz *et al.*, 2016) are yet to be assembled into larger pathways. Nonetheless, some trends were evident in the dataset. In this analysis, proteins related to cytokinin homeostasis were not observed. However, there appeared to be specific changes

that suggest SA and ethylene pathways might be modified by fusatinic acid. Ethylene is synthesised from ACC, which is a methionine derivative. Methionine adenosyl transferase (Bradi2g12150, 2-fold p-value 5×10^{-5}), three ACC synthases (Bradi1g10030, 6-fold p-value 5×10^{-5}; Bradi2g05790, 1.9-fold p-value 3×10^{-3}; Bradi5g19100 2.1-fold p-value 5×10^{-5}) and one ACC oxidase (Bradi2g35860 1.5-fold p-value 5×10^{-5}), are upregulated by fusatinic acid. This suggests methionine is preferentially being converted to ACC and in turn this may be converted to ethylene. A methyl-salicylate esterase which releases SA (Bradi2g52110, 2.4-fold p-value 2×10^{-4}) important for converting biologically inactive MeSA into active SA during systemic acquired resistance (Park $et\ al.$, 2007) is also upregulated, suggesting a possible activation of SA responses, in contrast to the observation of the down regulation of *PR1*. Taken together, the host gene expression response, at the level of hormone regulatory pathways, to fusatinic acid is complex but suggestive of substantive pathogen induced reprogramming of the plant environment. Presumably, this is to the benefit of the pathogen.

DISCUSSION

The identification of novel cytokinins produced by *F. pseudograminearum* adds to the growing collection of plant pathogens that produce or directly manipulate plant hormones during infection. Indeed based on its close relationship to *F. graminearum*, *F. pseudograminearum* is likely also able to produce auxin and interfere with ethylene synthesis (Adam $et\ al.$, 2015). Cytokinin production by pathogens during infection has in many cases been shown to contribute to virulence or symptom development (Hinsch $et\ al.$, 2016, Chanclud $et\ al.$, 2016, Siddique $et\ al.$, 2015). However, in initial experiments with a *FCK1* mutant on wheat, a clear role for Fusarium cytokinins in pathogen virulence could not be ascertained (data not shown), although a comprehensive study has yet to be undertaken. Furthermore, the tRNA pathway for cytokinin (cZ) production is also active in *F. pseudograminearum* and it is possible that the abolishment of both pathways is required

before a virulence-related effect can be observed. Indeed, in *C. purpurea* which also has two cytokinin production pathways, no role in virulence was observed until both pathways were mutated simultaneously (Hinsch *et al.*, 2016).

The production of an active cytokinin during infection simultaneously with the cell death inducing toxin deoxynivalenol *prima facie* appears counterproductive. However, the production of Fusarium cytokinins in such high quantity during infection presumably imparts some selective advantage to the pathogen. The analysis of host gene expression following fusatinic acid treatment suggested the possibility that this may be via phytohormone crosstalk. However the direct effect of fusatinic acid on cytokinin responses of the host is an equally plausible evolutionary reason for the maintenance and activity of this cluster, and would suggest *F. pseudograminearum* behaves like a hemi-biotroph during infection. This has been proposed previously based on histopathological studies and biomass monitoring time course experiments where crown infection by *F. pseudograminearum* and *F. graminearum* of wheat and barley has distinct phases of fungal growth and symptom development (Stephens *et al.*, 2008, Knight & Sutherland, 2016). Experiments to simultaneously monitor the spatial and temporal production of both deoxynivalenol and the Fusarium cytokinins will be necessary to further dissect the apparent co-production of deoxynivalenol and Fusarium cytokinins during plant infection. *F. pseudograminearum* infection is exacerbated in plants undergoing drought stress and given cytokinins can ameliorate drought symptoms in grasses, possibly via increasing sink strength at the site of production (Chang *et al.*, 2016, Reguera *et al.*, 2013), the Fusarium cytokinins may be promoting plant survival by directly activating cytokinin responses. Promoting host survival may be beneficial to the pathogen to ensure sufficient plant biomass is available to the pathogen post host senescence or crop harvest. This strategy would be akin to "anti-virulence" loci in bacteria that while they reduce the measured virulence of a pathogen in

laboratory assays, they nonetheless contribute to overall pathogen fitness by, for example, contributing positively to pathogen transmissibility or ecological survival between hosts (Foreman-Wykert & Miller, 2003). This is a component of pathogen virulence underexplored in molecular studies of plant pathogenic fungi largely due to the difficulty in establishing suitable experimental systems (Preston, 2017). Such systems, that also consider the host-pathogen interaction over evolutionary timescales, are likely to be important for us to fully understand the intricacies of interactions between plant pathogens and their hosts. Cytokinin production by *F. pseudograminearum*, and secondary metabolism by fungi in general, represent one of these areas of exquisite intricacy that maybe difficult to understand without probing the entire life cycle of the producing organisms in the context of their own pathogen population and environment.

EXPERIMENTAL PROCEDURES

Fungal strains

Fusarium pseudograminearum isolate CS3096 was used for metabolite analyses and isolate RBG5266 (Bentley *et al.*, 2008) was used for wheat head blight infections. *F. verticillioides* strains were BRIP14953a, BRIP53263a, BRIP53273b, BRIP53590a and BRIP54043a, (provided by the Queensland Department of Primary Industries and originally isolated from Maize or Sorghum in New South Wales or Queensland, Australia), as well as the genome sequenced *F. verticillioides* (FGSC7600), *F. fujikuroi* (IMI58289) and *F. oxysporum* (FGSC9935) (Wiemann *et al.*, 2013, Ma *et al.*, 2010).

Identification of cytokinin biosynthetic genes

FCK1 was identified in the *F. pseudograminearum* genome based on its homology to the Arabidopsis Lonely Guy 1 (LOG1, AT2G28305.1). To conduct phylogenetic analyses a set of IPT sequences from bacterial and eukaryotic sources was assembled from diverse sources using the Arabidopsis LOG1 as a query in a blastP analyses in GenBank. The set also

included tRNA IPTs and is available in Data file S3. The amino acid sequences were aligned with by multiple alignment using fast fourier transform (MAFFT) using T-REX web server (Boc *et al.*, 2012). The alignments were analysed with MetaPIGA v2.0 (Helaers & Milinkovitch, 2010) using maximum likelihood using 100 bootstraps and visualized with EvolView (http://evolgenius.info/evolview) (Zhang *et al.*, 2012). Protein sequences of fungal cytochrome P450 monooxygenases located adjacent to IPT-LOGs were included in a phylogenetic analysis using the same procedure described above.

Biochemical characterisation of FCK1

FCK1 was expressed as an N-terminal His tag fusion protein in *E. coli*. The coding sequence was synthesised and cloned into pET-28a by GenScript (USA) using *Nhe*I and *Hin*dIII cloning sites. Expression and purification of the protein was performed as previously described (Kettle *et al.*, 2015) except that the His tag was not removed from the protein prior to biochemical characterisation. The final protein storage conditions were 50 mM sodium phosphate buffer, 300 mM NaCl and 20% glycerol, pH 7.5. Purification of the protein was assessed using sodium dodecyl sulphate-polyacrylamide gel electrophoresis using a BOLT 4-12% Bis-Tris gel stained with SimplyBlue^{TM} SafeStain (Thermo Fisher Scientific). A SeeBlue® Plus2 prestained marker was used (Thermo Fisher Scientific). The enzyme assay was modified from those previously used to characterise an Arabidopsis IPT (Takei *et al.*, 2001). The reaction mixture contained a final concentration of 880 mM betaine, 17.5 mM triethanolamine, 44 mM KCl, 8.8 mM MgCl2, 880 μM DTT, 880 μg mL^{-1} bovine serum albumin, 1 mM adenosine monophosphate, 286 μM dimethylallyl pyrophosphate, pH 8.0. The reaction was initiated by the addition of purified protein or buffer control. The reaction was incubated at room temperature and sampled at 2 and 18 hours and stopped with an equal volume of 100% ethanol and heated to 90°C for 10 minutes. Ten micro litres of the reactions were analysed via LC-MS using system 1 as described in the supplementary methods file.

In silico FCK2 model building and docking analysis

A similar approach to model building and docking analysis was carried out as previously done for another Fusarium cytochrome P450 monooxygenase (Droce *et al.*, 2016). An initial homology model of FCK2 was generated with SWISS MODEL (Bordoli *et al.*, 2008) (Bazel, Switzerland) using PDB ID 4D6Z as the template structure. Following coordination of the deoxy-heme group, subsequent energy minimization and a 500 ns molecular dynamics model refinement with the md_refine macro was carried out in YASARA/WHAT IF Twinset (Vienna, Austria; version 16.7.22) using the Yasara2 force-field in explicit water (TIP3P water model) (Krieger *et al.*, 2004, Krieger & Vriend, 2014). Following energy minimizations of trans-zeatin, cis-zeatin, isopentenyl adenenine, and fusatinic acid in YASARA (Yasara2 forcefield, TIP3P water model), each compound was globally docked in AutoDock VINA (Trott & Olson, 2010) to the best Z-scoring, low-energy, conformer of FCK2 (dock_run macro with 100 docking runs). Results were based on a comparison of the calculated dissociation constant of the high-scoring clusters.

Heterologous expression in *Fusarium graminearum*

The fungal cytokinin gene cluster was PCR-amplified from *F. pseudograminearum* CS3096 genomic DNA in three overlapping fragments with primers provided in Table S1. Primers used for the two outermost PCR reactions were added a 30 bp tail with homology to the plasmid multiple cloning site for integration through homologous recombination. A multi-purpose shuttle vector was prepared for capture of gene cluster and introduction *F. graminearum* PH-1 (**Fig. S7**). Vector construction was performed by transforming *S. cerevisiae* strain BY4743 cells with *Bam*HI/*Xho*I linearized plasmid DNA and three PCR products (Gietz & Schiestl, 2007). Transformants were plated on synthetic yeast drop-out medium without uracil (Sigma Cat. No. Y1501) and incubated at 28 C for 3 days. Plasmid DNA was purified from yeast colonies using the Zymoprep Yeast Plasmid II (Zymoresearch

Cat No. D2004). The cytokinin cluster was transformed into *F. graminearum* through *Agrobacterium tumefaciens*-Mediated Transformation (Malz *et al.*, 2005). A transformant carrying the cytokinin gene cluster was identified by PCR with primers hybridizing outside the tubulin locus in combination with primers hybridizing inside the inserted gene cluster.

Targeted gene deletion

Vectors were synthesized by GenScript (USA) and consisted of; 1000 bp of sequence immediately upstream of the target gene's start codon, a 20 bp sequence (gatgtccacgaggtctct), a unique 20-bp sequence (see later), another 20 bp sequence (cgtacgctgcaggtcgac), the *Aspergillus nidulans TrpC* promoter and nourseothricin resistance cassette corresponding to nucleotide positions 437–1387 of GenBank accession AY631958.2 (Gardiner *et al.*, 2005), and 993 bp of sequence immediately following the stop codon of each gene. The 20-bp unique sequences were agctcaatattgcgtgcgca (*FCK2*), tcgctgatccacaggtagat (*FCK1*), agtggacgtatcacatctcg (*FCK4*) and actagacgctacgatcttgg (*FCK3*). The synthesised fragments were amplified using Phusion DNA polymerase with M13 forward and reverse primers. Eight 50 µL PCR reactions were pooled, polyethylene glycol precipitated and transformed into CS3096 protoplasts as previously described (Gardiner *et al.*, 2012). Transformants were screened using a triplex PCR assay with primers as listed in Table S1. Absence of the wild type band was used as confirmation of successful deletion of the target gene (**Fig. S1**).

Production and analyses of cytokinins and deoxynivalenol

Fusarium pseudograminearum (CS3096) was cultivated for two weeks at 25 °C in the dark on yeast extract sucrose agar (YES) medium (yeast extract from Scharlau, Barcelona, Spain) (Sørensen & Sondergaard, 2014). Secondary metabolites were extracted using the micro-scale extraction procedure (Smedsgaard, 1997) modified as previously described (Sørensen *et al.*, 2014). The extracts were initially examined on LC system 2 and then LC system 3 (supplementary methods file).

In planta quantification of Fusarium cytokinins and deoxynivalenol and analysis of Australian isolates of *F. verticillioides* were performed on LC system 4 (supplementary methods). For plant samples extracts were made from individual freeze dried heads that had been point inoculated with *F. pseudograminearum* isolate RBG5266 (Bentley *et al.*, 2008) 14 days prior to harvest. These were ground with a mortar and pestle and resuspended in ten millilitres of extraction solution as per the modified micro-scale extraction procedure, placed in a sonicator bath for 45 minutes, dried under nitrogen and resuspended in 400 μL of methanol. Standard curves for all compounds were constructed from pure compounds (verified by NMR) for the Fusarium cytokinins or deoxynivalenol purchased from Sigma. *In planta* molar concentration equivalents were calculated based on 1 gram of fresh material being equivalent to 1 mL of solution and the water content of samples being about 90%.

Isolation and structural elucidation of cytokinins

F. pseudograminearum CS3096 was each grown on 50 petri dishes (90 mm) with YES medium for two weeks at 25 °C in the dark and cytokinins were isolated from the *F. pseudograminearum* extract on a semi-preparative HPLC system (LC system 5 in supplementary methods). The structures of the isolated cytokinins were elucidated by nuclear magnetic resonance (NMR) spectroscopy. Fusatinic acid (3), fusatin (1), 8-oxofusatin (2) and N^6-(3-methylbut-2-en-1-yl),8-oxo-adenine (4) were dissolved in methanol-d_4. ^{1}H-NMR, $[^1H,^{13}C]$-HSQC, $[^1H,^{13}C]$-HMBC (not molecule 4) and DQF-COSY spectra were recorded on a BRUKER AVIII-600 MHz NMR spectrometer for all compounds. For fusatinic acid, additional $[^1H,^{15}N]$-HMBC spectra optimized for J_{HN}=4, 8 and 16 Hz were recorded on a BRUKER AVIII-800 MHz spectrometer. Spectra were recorded and processed in TopSpin 3.2. For spectroscopic data and the argumentation for the structure elucidation see the supplementary information. Structures were checked against the CSEARCH Robot Referee (Haider & Robien, 2016) for consistency.

Detached leaf cytokinin activity assay

Stocks of fusatinic acid or 6-benzylaminopurine (BAP) were dissolved in DMSO at 5 mM. 10-13 individual whole first leaves were treated in 15 mL of 0.1% agarose in 50 mL tubes to which 30 μL of test compounds were added. Leaves were maintained in the treatment solution on a slowly rotating tube roller in a laboratory maintained at 22°C for 10 days prior to photographing using a 10 MegaPixel digital camera on a black velvet background. The black background in the image was selected and deleted using the colour range selection feature set to shadows in Adobe Photoshop CS2. This area was replaced with a true black (RBG, 000) background and the image saved and opened in ImageJ version 1.50i (Schneider *et al.*, 2012). Each leaf was individually selected using the wand tool with default settings and the average red, green and blue pixel content across each leaf measured using the RBG Measure plugin with data exported to Microsoft excel for statistical analysis.

An identical experimental system was used for gene expression analysis. Treatment occurred for two hours prior to sampling by removing the leaves from the treatment solution and snap freezing. Each replicate consisted of a pool of 12 detached first leaves (treated in a single tube) and four biological replicates were used per treatment for RNA extraction and RNAseq analysis.

RNAseq analysis

RNA was extracted from freeze dried leaves using a QIAgen RNeasy spin plant RNA extraction kit as per the manufacturer's instructions. RNA was sent to the Australian Genome Research Facility (Melbourne, Australia) for Illumina TruSeq stranded mRNA library construction and sequencing using two lanes of a HiSeq2000 with 125 bp paired end sequencing. Four biological replicates were used per treatment. All twelve libraries were sequenced in both lanes as technical replicates to eliminate lane effects and output files for these technical replicates were combined during the analysis.

SolexaQA++ was used to trim reads to a minimum length of 50 nucleotides and trimmed to the longest contiguous segments with quality scores above Phred 30 (Cox *et al.*, 2010). The Cufflinks package version 2.2.1 was used for RNAseq analysis (Trapnell *et al.*, 2012). Trimmed reads were aligned using tophat 2.1.0 (Kim *et al.*, 2013) to the *Brachypodium distachyon* genome v3.0 downloaded from phytozome. Alignment was guided by a genome feature file (v3.1) and default parameters were used. Transcripts were assembled using cufflinks for individual samples and then merged into a final transcriptome assembly using cuffmerge both run with default parameters. Differential expression was identified using cuffdiff run with default parameters.

GO analysis was performed at AgriGO (Du *et al.*, 2010). Whilst an annotation of *Brachypodium* is available, the *Arabidopsis* GO term annotation is considerably richer in the AgriGO database. Therefore, orthologus of the *Brachypodium* primary protein set of annotation version 3.1 were identified in the Arabidopsis (TAIR10) primary protein set. Blastp version 2.2.25 was run on cluster computing resources available in CSIRO with default blastp parameters and output limited to the best hit in tabular format. Reciprocal best hits were extracted using custom perl scripts.

Pathway analysis was conducted at Gramene (http://pathway.gramene.org/) in the BrachyCyc version 2 database (Tello-Ruiz *et al.*, 2016). The enzyme database used at BrachyCyc was downloaded and the corresponding entries in this database to that in *Brachypodium* annotation version 3.1 identified using reciprocal best blast hits. Locus ids and their corresponding expression values for genes that were significantly up or down regulated by fusatinic acid treatment were uploaded to the BrachyCyc query form and the resulting metabolic pathways inspected manually.

Bacterial assay for cytokinin receptor activation

Arabidopsis cytokinin receptor 3 (AHK3) activation was assayed using a bacterial based assay that used β-galactosidase activity as a read out of activity as previously described (Mizuno & Yamashino, 2010) with the minor modifications as follows. The Arabidopsis histidine kinase 3 receptor was used in pSTV28 in *E. coli* KMI001. 400 μL of an overnight culture that had been grown at room temperature with 10 μM of the test compounds (dissolved in DMSO with the final DMSO concentration in the culture of 0.1%) was pelleted by centrifugation and resuspended in 700 μL Z-buffer. 200 uL of this was transferred to a 96 well culture plate for measurement of optical density at 600 nm using a Perkin Elmer Envision multimode plate reader fitted with monochromators. To the remaining 500 μL 10 μL of toluene was added and the mixture incubated at 37°C for 60 minutes. 100 μL of 4 mg mL^{-1} ONPG in Z-buffer was added to the tubes and allowed to incubate at 28°C for 50 minutes, after which 900 μL of 1 M NaCO$_3$ was added to stop the reaction. This was centrifuged for 2 minutes at 20,000×*g* in a microcentrifuge to pellet the cellular debris. 200 μL was transferred to a 96 well plate and the optical density at 420 nm of this cleared reaction measured. A simplified equation for calculating beta-galactosidase activity was also used removing the approximation of light scatter due to cellular debris since a cleared reaction was used. The equation was: $1000*OD_{420}/(time*volume*OD_{600})$.

Accessions

The raw reads for the RNAseq experiment assaying *Brachypodium* response to cytokinins can be found under BioProject PRJNA325847, SRA accession SRP076768. *F. pseudograminearum* gene expression was quantified from RNAseq data that can be found under BioProject PRJNA326033, SRA accession SRP076777.

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SUPPLEMENTARY FIGURES

Fig. S1: Generation and verification of mutant strains of *F. pseudograminearum*. The design of the targeting vectors and PCR screening assays are shown for all genes. Central to the image is the entire Fusarium cytokinin biosynthetic gene cluster and two flanking genes labelled as "wild type locus". Below or above this for each of the four genes in the cluster a schematic of the synthesised targeting vectors are shown. These consist of upstream and downstream homologous flanking sequences of each gene, with the regions of homology to the wild type locus indicated by grey shading. The black box and arrow in each targeting vector represents the TrpC promoter driven nourseothricin acetyl transferase used to select transformants. Transformants were screened with a triplex PCR assay in which a vector specific and/or parental gene specific product can be generated depending of the genotype of the transformant. In each case the vector specific product (depicted as blue lines) were designed to be larger than the parental gene specific (red lines) products. Agarose gels are shown of the products of the triplex PCR assays for each genes. Samples nomenclature are unique identifiers given to each transformant. In the case of the *FCK1*, *FCK2* and *FCK4* transformation screens, mutants at one of the other genes in the cluster were used as controls. The amplification of the wildtype band in these strains demonstrate that for these strains deletion of the target gene has not impacted other genes in the cluster. *FCK1* mutants were generated in both Aalborg University and CSIRO. A single mutant (AAU2.2) was identified in transformations conducted at Aalborg University with other transformants with ectopic integrations of the targeting cassette (lower gel) and three mutants were identified in transformations conducted at CSIRO with only these selected mutants included in the screen depicted in the upper gel for *FCK1*. For AAU2.2 the flanking genes, *FCK2* and *FCK4* showed amplification identical to the CS3096 wild type strain (data not shown).

Fig. S2: Quantification of aurofusarin, fusatin, 8-oxo fusarin, fusatinic acid and 8-oxo fusatinic acid using the protonated ion traces [M+H]$^+$ for each compound. The columns represent mean values of three replicates with standard error of the mean error bars. A single mutant in each gene was selected for this analysis but the loss of production of Fusarium cytokinins in independent mutants of *FCK1* and *FCK2* and enhanced production in *FPSE_20002* and *FPSE_06371* mutants, was consistently observed.

Fig. S3: Expression of the four genes of the Fusarium cytokinin cluster in *Fusarium pseudograminearum* during infection of barley and *Brachypodium* in comparison to the trichodiene synthase encoding gene (TRI5). FPKM, fragments per kilobase of transcript per million mapped reads. *FCK4* derived RNAseq reads were observed in *Brachypodium* derived samples but their abundance was lower than that required to calculate a FPKM value. Values represent the average of four biological replicates with error bars representing the standard error of the mean. 0.2% of all reads were of fungal origin from barley samples. In the Brachypodium set this was 2.2-3.6%.

Fig. S4: Phylogenetic analysis of cytochrome P450 monooxygenases encoded in fungal cytokinin production clusters. White, grey and black hexagons illustrate which clade the neighbouring IPT-LOG belongs to (Fig 2A)

Fig. S5: Active site modelling of the cytochrome P450 monooxygenase FCK2. **(a)** Of the tested compounds (trans-zeatin (tZ), cis-zeatin, fusatin, isopentenyl adeinine and fusatinic acid), docking analyses suggests that tZ is the most likely product of this enzyme. Outputs of the docking are represented as relative binding strengths (dissociation constant). The model cannot exclude that other untested compounds are better fits for the active site nor does it take into account the possibility of other conformational states. **(b)** Ribbons structural representation of the entire FCK2 protein. **(c)** Active site of FCK2 with tZ bound.

Fig. S6: Chromatograms of *F. graminearum* wild type (PH-1) and transformed with the cytokinin cluster (PH-1::*FCK*). A: UV (280 nm) chromatogram. B. Extracted chromatograms ([M-H]⁻) for **1**, **2**, **3** and 8-oxo tZ.

Fig. S7: Illustration of the procedure for heterologous expression of the cytokinin cluster in *F. graminearum*. Three overlapping PCR fragments were cloned into the Y-GOTL vector and transferred into the tubulin locus of *F. graminearum* (Josefsen *et al.*, 2012).

SUPPLEMENTARY DATA FILES

Data file S1: Structural elucidation of fusatin, 8-oxo-fusatin and fusatinic acid.

Data file S2: Gene ontology analysis of genes differentially regulated by fusatinic acid in *Brachypodium*.

Data file S3: Amino acid sequences used in the construction of the IPT phylogeny.

Supplementary methods: *F. pseudograminearum* RNAseq and the five different LC systems used are presented.

SUPPLEMENTARY TABLE LEGEND

Table S1: Primers for screening transformants for successful gene deletion and for heterologous expression of cytokinin cluster in *F. graminearum*.

FIGURE LEGENDS

Fig. 1: *Fusarium pseudograminearum* produces cytokinins in culture and *in planta*. (**A**) Structures of four Fusarium cytokinins isolated and elucidated from *F. pseudograminearum* wild type (CS3096) grown in yeast extract sucrose media. **1**, fusatin; **2**, 8-oxo-fusatin; **3**, fusatinic acid; **4**, 8-oxo-isopentenyladenine. (**B**) Quantification of Fusarium cytokinins and deoxynivalenol in infected wheat. 8-oxo-fusatin (**2**) was not detected *in planta* and 8-oxo-isopentenyladenine (**4**) was not searched for. Values represent the average of 12 individual infected heads with error bars representing the standard error of the mean.

Fig. 2: Fungal genomes encode three distinct cytokinin production clusters. (**A**) Phylogenetic analysis of IPT proteins. (**B**) Three distinct cytokinin production gene clusters are found in various *Fusarium* spp. and *Claviceps purpurea*.

Fig. 3: Biosynthesis of Fusarium cytokinins in *Fusarium pseudograminearum*. (**A**) Proposed biosynthetic pathway based on accumulation of cytokinins in deletion mutants and biochemical characterisation of FCK1. (**B**) Extracted ion chromatograms of Fusarium cytokinins and related molecules in *F. pseudograminearum* wild type and deletion mutants. Data are shown for one deletion mutant only in each gene, but are representative of traces for independent mutant generated for all genes.

Fig. 4: FCK1 catalyses the formation of isopentenyl adenine *in vitro* from adenosine monophosphate and dimethylallyl pyrophosphate. (**A**) The FCK1 protein was expressed in *E. coli* as an N-terminally His-tagged fusion protein. The expected size of the protein is ~55 kDa. (**B**) Extracted ion chromatograms of the final expected protonated product (isopentenyl

adenine, iP) and intermediate (isopentenyl adenosine monophosphate, iPMRP) of the two reactions catalysed by the bi-functional FCK1.

Fig. 5: Fusarium cytokinin molecules have cytokinin activity. (**A**) Examples of gross effects of fusatinic acid and BAP (6-benzyl aminopurine) treatment on detached *Brachypodium* leaves. Leaves were treated in 15 mL of 0.1% agarose with 10 μM of the hormone for 10 days on a laboratory bench. DMSO (0.2% final concentration) was used as a control. (**B**) Relative leaf colour following treatment with fusatinic acid or BAP in *Brachypodium* measured as the average red, green and blue pixel content per unit leaf area. Lower numbers indicate darker leaves. The data consists of a minimum of 10 individual leaves per treatment with DMSO, fusatinic acid or BAP. (**C**) Fusarium cytokinins can activate the Arabidopsis histidine kinase 3 cytokinin receptor in a bacterial assay system. (**D**) RNAseq analysis of gene expression changes in *Brachypodium* in response to 6–benzylaminopurine (BAP) and fusatinic acid. Overlap between global gene expression changes in each treatment. All included genes were statistically significantly differentially regulated genes at *p*-value 0.05 after Benjamini-Hochberg false discovery correction. (**E**) Overlap between the positively fusatinic acid or BAP regulated genes and the high stringency hormone regulated gene sets defined by Kakei *et al*. (2015). The positively hormone regulated high stringency gene sets contained for ABA contained 423 genes, IAA 112 genes, brassinolide 4 genes, trans-Zeatin 23 genes, salicylic acid 52 genes, methyl jasmonate 362 genes. The overlap presented for ethylene was based on the low stringency set from Kakei *et al*. as these authors found no gene differentially regulated by ethylene when high stringency filtering was applied (Kakei *et al.*, 2015).
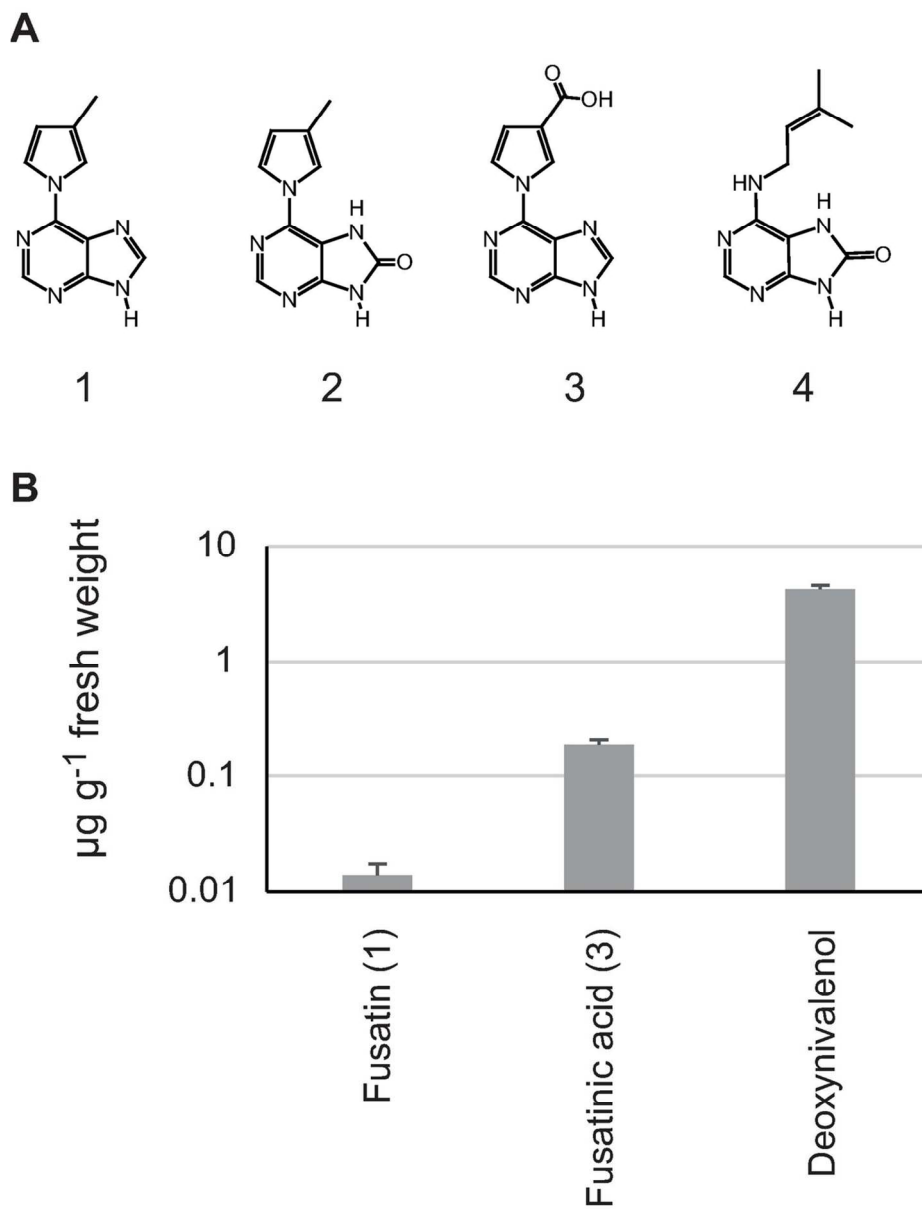
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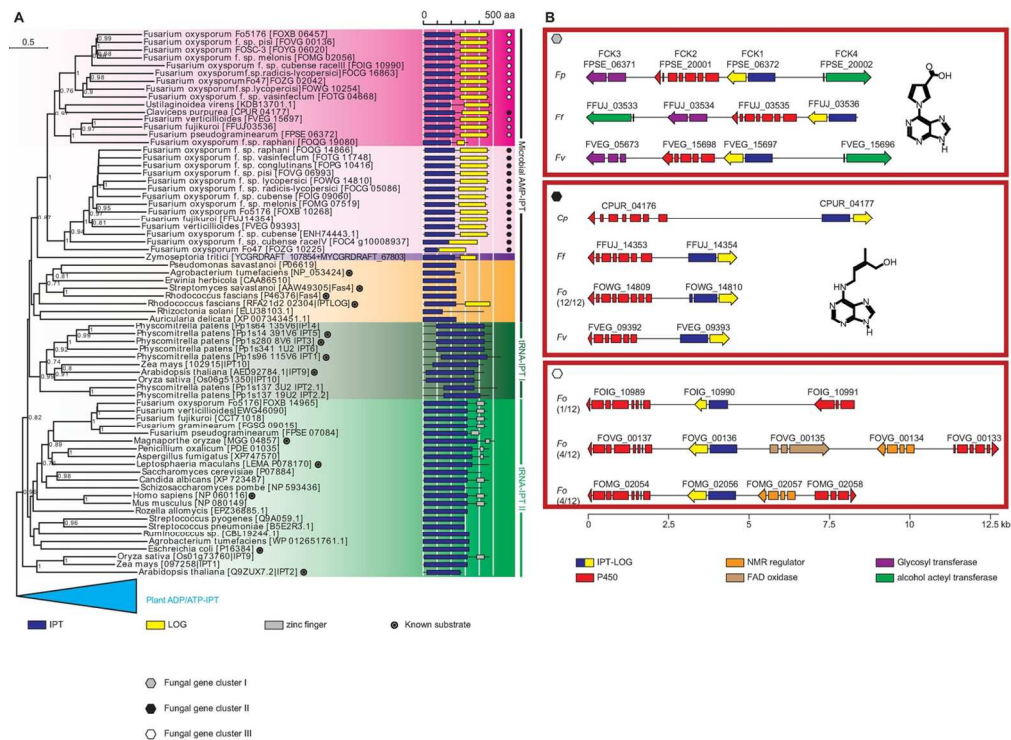
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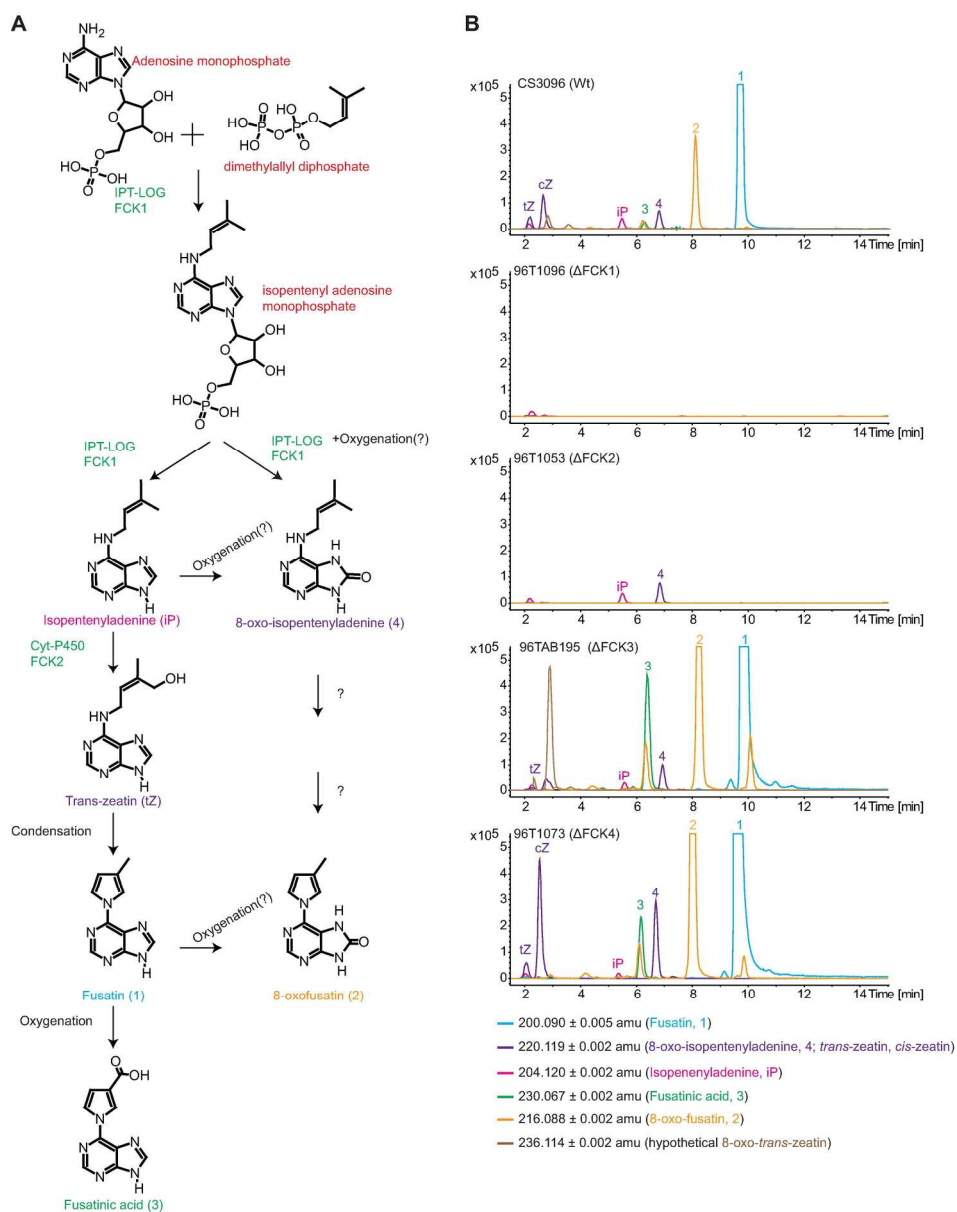
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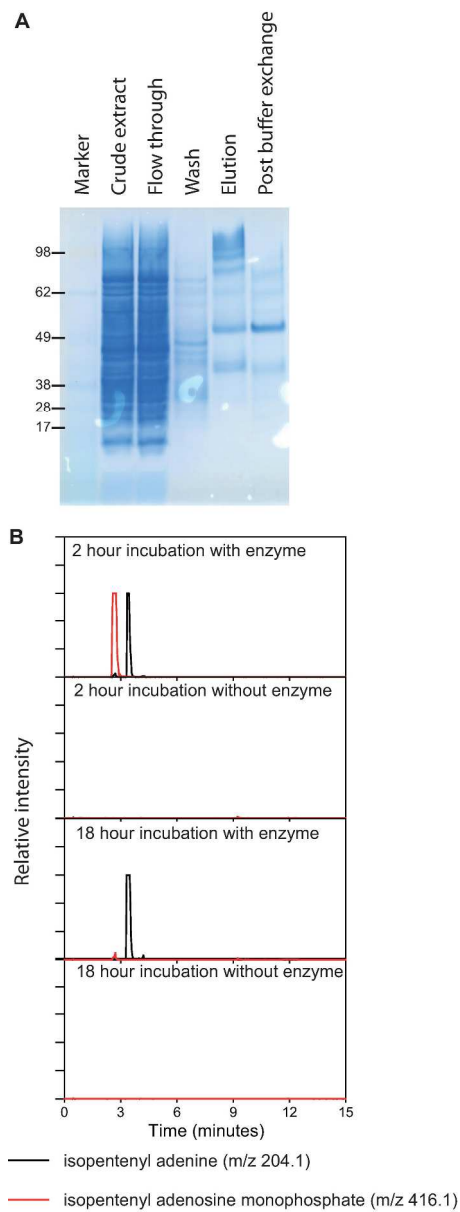
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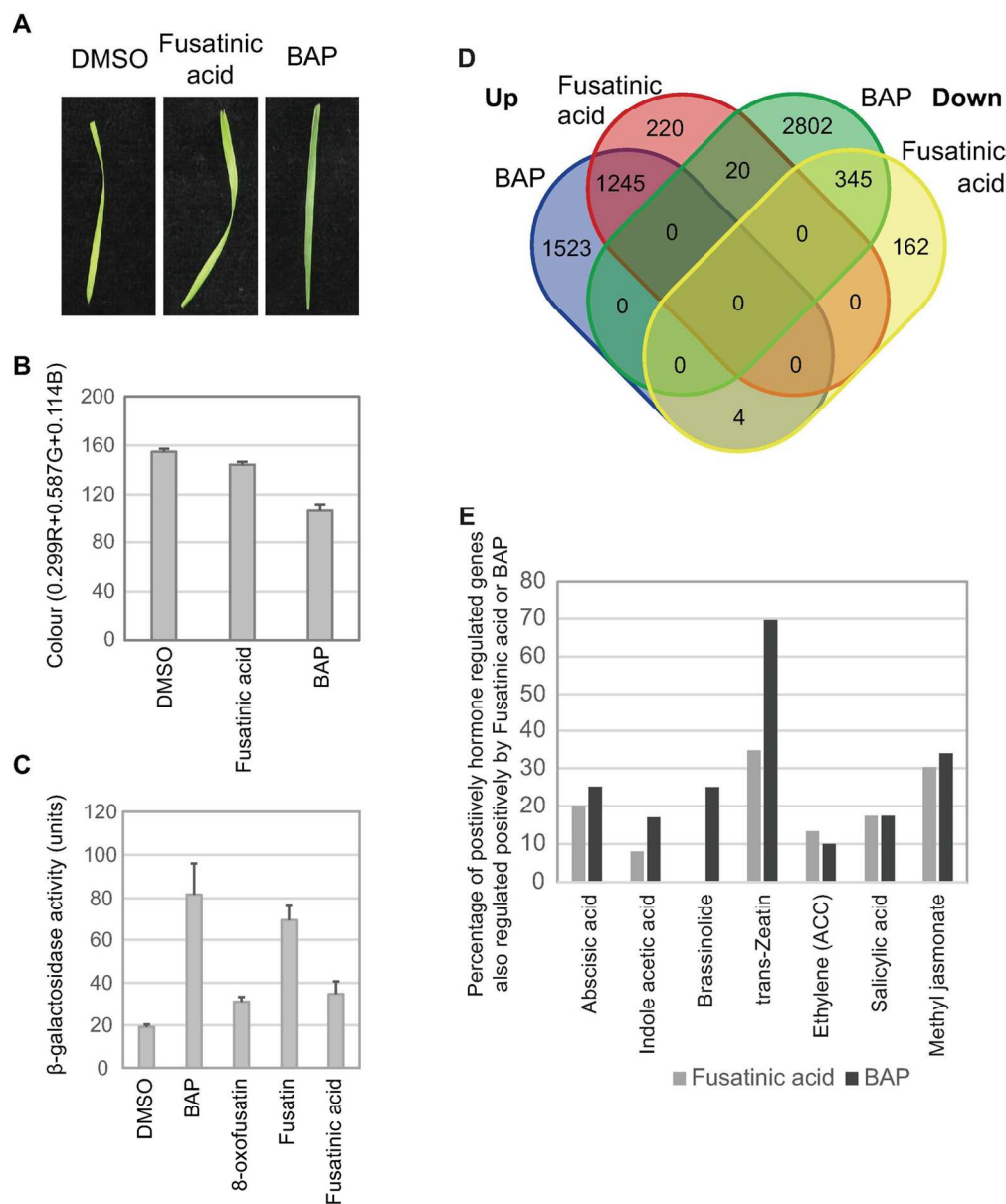
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