

New Phytologist Supporting Information

Article title: *Lolium perenne* apoplast metabolomics for identification of novel metabolites produced by the symbiotic fungus *Epichloë festucae*

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The following Supporting Information is available for this article:



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b



Fig. S1 Principle component analysis (PCA) of 203 metabolite features with a false discovery rate of < 0.003, obtained by metabolite fingerprinting using ultra-performance liquid chromatography-electrospray-ionisation-time-of-flight mass spectrometry (UPLC-ESI-TOF-MS) analysis of apoplastic wash fluids from mock-treated (blue), or FI1 (red)- or CT (green)-infected *L. perenne*. (a) PCA scores plot and (b) PCA eigen values are shown. Five biological replicates per condition were used for the analysis.





Fig. S2 Extracted ion chromatogram of peramine ([M+H]⁺ 248.1506) in Fl1 and CT (a) and confirmation of the peramine structure by ultra high-performance liquid chromatography-



quadrupole time-of-flight mass spectrometry/mass spectrometry (UHPLC-QTOF-MS/MS) analysis. (b) Data are representative for five biological replicates per condition.





Fig. S3 Extracted ion chromatogram of epichloëcyclins A-E, obtained by ultra highperformance liquid chromatography-quadrupole time-of-flight-mass spectrometry (UHPLC-QTOF-MS) analysis of Fl1 samples. Data are representative for five biological replicates per condition.



Fig. S4 Confirmation of the identity of epichloëcyclins A-E by ultra high-performance liquid chromatography-quadrupole time-of-flight-mass spectrometry/mass spectrometry (UHPLC-



QTOF-MS/MS) analysis of the ions $[2M+H]^{2+}$. The fragment spectra shown here for epichloëcyclins A-E match the spectra described by Johnson RD, Lane GA, Koulman A, Cao M, Fraser K, Fleetwood DJ, Voisey CR, Dyer JM, Pratt J, Christensen M, et al. 2015. A novel family of cyclic oligopeptides derived from ribosomal peptide synthesis of an *in planta*-induced gene, *gigA*, in *Epichloë* endophytes of grasses. *Fungal Genetics and Biology* **85**: 14-24.





Fig S5 *gigA* gene cluster and GigA protein sequences. (a) The *gigA* gene cluster annotated with gene expression level (ranked highest to lowest for all *E. festucae* genes expressed *in planta*) and fold change in expression *in planta* compared to axenic culture. The transcriptome data used here is available from the Sequence Read Archive (SRA) under Bioproject PRJNA447872. (b) GigA pro-peptide sequences from *E. festucae* strains Fl1 and CT annotated with the epichloëcyclin repeat sequences.



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Fig. S6 Extracted ion chromatogram of a putative peptide of $[2M+H]^{2+}$ 562.2786 (put. peptide 1), which is strongly enriched in CT. (a) Data are representative for five biological replicates per condition. (b) The fragment spectrum of this putative peptide 1 obtained by



ultra high-performance liquid chromatography-quadrupole time-of-flight-mass spectrometry/mass spectrometry (UHPLC-QTOF-MS/MS) analysis is shown.



Α	ACP-like superi (IPR036736)	amily Condensation domain (IPR001242)	AMP-dependent synthetase/ligase (IPR000873)	
_	100 aa			
В				
Zt	1 – – M E F T S K V N	ET L L NR L CA L L H V E P DR I K I	DE S F V R NGGD S L LA I R F S N V I K K S EQ LQ V G A G V I L R E KQ LG T L L D K T Q L	RTLA81
Rc	1 – – M E Q E L N I D	K D L L E G L CA L L H L E P NR I K I	NE A F V R NGGD S L LA I R F S N L I Q T L E H V R V G A G A I L R A K C L G T L L D R R S L	RELT81
Zb	1 – – M DY T S N V D	ET L L D R L C G L L H V E P DR I K I	NE S F V R NGGD S L LA I R F S N V I Q K S EQ LQ V G A G V I L R E KQ LG T L L D K T Q L	RTLA81
Ef	1 M EQQME L H M D	ER L L E R L C S L L H L E S K H I K I	NE T F I R NGGD S L LA I K F S N L I Q D L E H V R V G V G A I L R A K N LG S L L D R T Q L	RKLA83
Zt Rc Zb Ef	82 AG S STQQACS 82 TG S TVQP AQA 82 TEP STQPACS 84 TGHS SEP SQS	ST I DP ST TVT A SQDNLDDK I G L L AK S SAQDD I DDK ST I DP S I TVT A SQDNL EDK DK LP LTV SNDDRDDK	T L DP I QR F P K A I P ST G L P I T F I Q A GT A HYTH L I P GAAVQ HVTQY C F T D A L F L DP I QR F P K V I P T I G L P T T F I Q AAT A HYTH L V P GAAVQ HVTQY CYTD A L F L DP I QR F P K V I P ST G L P I T F I Q A GT A HYAH L I P GAAVQ HVTQY CYTD A L F L DP I QR I P T V I P T A G L P L T F I Q A GV A VYTH NV P GAAVQ HVTQY CYTD V L	P H L K 164 P Q L K 161 P K L K 164 P K L K 164 P R L R 163
Zt Rc Zb Ef	165 SAFAKAMSSY 162 QAFAKVMSSY 165 AAFAKAMSRY 164 SAFGEAMSRH	Q	. K DD F C L NW E E SQ V D S L EWA D L E D S F R NW S I E A S T E P V F R I L T P S N ST S N . K E S F E L NWT E Q H V E SP E E A D L E E S L R VWS VX A S S E P V F R V T P R E T ST G . K DD F R L NW E E SQ V D S L E G P D L E D S F R NW S I E A ST E P V F R I L T P S N ST S D . K E T F K L NWT E Q Q A E S L G E A D L E E L F R VWS S E A S A E P V F R V T P C K A A G E	R S L S 247 R K L S 244 R S L S 244 R S L S 247 R E L S 246
Zt	248	MDGR S I D I L L K H V D A L MQ D S	S S I S P N V N D NA F D V V K G LA E Y H DQ H S S A AK A F WAT R E V T S A S R N H P L L HA	L G P R 330
Rc		MDGR S MD I L M SQ V D A L MR N F	S P D I K C N D C T F D V V K G LA D Y H TQQ E A A AK A F WAT R D V P S G S R N H P L L Q A	L G T R 327
Zb		MDGR S I D I L L K H V D T L MQ D I	P I L L K V N D N T F D V V K G LA D Y H DQ H A A A A R T F WN I R E V P S A S R N H P L L HA	L G T R 330
Ef		L DGR S L D I L L R Q V D A L L D N F	S L P V E W G D S I F D V V R G LE D Y H G P R E A A AK A F WA S R E V R G G A R G H P L L R I	L G S R 329
Zt	331 T P S V V R M R S T	DV FYP EDVKA FQARTG FT FI	V LARAALAL V LAK LQ DTTT V SLMS V SSRR SLP I T G I E EAVG SFASSMIL	S V D I 413
Rc	328 V S S R V R M K S I	E I HYP GHLRE FQ SRTG FT FI	I LVRAAMG L V L SK LQ SK ST V P LMS V SSRR SLAV P G I E EAMG SFASSMV L	S I D I 410
Zb	331 T P S V V R M R S V	DV FYP EDVKA FQARTG FT FI	V LARAALAL V LAK LQ DA I T V SLMS V SSRR SLP I T G I E EAVG SFASSMIL	S V E I 413
Ef	330 P S S R V R M K T I	DV FYP EDVKA FS SRTG FT FI	I LVRAALAL V LSK FQ STRT V SLMS V SSRR SLP I K G I E HAVG C I AT SMIL	T M D V 412
Zt	414 N NTNTAHELL	EQ V FHK L L E L D D M S Y S D P S I	DG F S I GG L V V V S S D LQP H T P W Y S D NHR T E I M S P K E T L P T L Y V G S N G R V R F	C Y N S 496
Rc	411 N ENDTAHDLL	EQ V FNK V L E L D E M S Y S D P S I	DG F S L GG L V V V S S D L R DH C P W Y S G G K Q T E V M S P K E T L P T L Y V G S N G R L R F	C Y N S 493
Zb	414 N NTDTAHELL	EQ V FHK L L E L D D M S Y S D P S I	DG F S I G G L V V V S S D LQP H A P W Y S D NHR T E I M S P K E T L P T L Y V G S N G R V R F	C Y N S 496
Ef	413 N ENDAAQDLL	AQ V F EK V L E L E D M S Y S D P S I	DG F S L G G L V V V S S D LQP H R P W Y A G HQ T E V M A P K E T L P T L Y V S P T G R V R F	S Y N S 495
Zt	497 EWR TP A EMQ V	MADLFESALVGLGSGTLGVA	NECL S KML TAAAR E RTMT F GNC F SNKTTL K G I DDD I V S L F E KQ V G <mark>MR G</mark> E A	P A L T 579
Rc	494 EWR TP M EMQ V	MVDLFKTALITIGMGTTGVG	GQ C L R NM L P V SQ R Q K V L EWG NC F ATTTX L E G I DDD I V S L F Q KQ V Q D R G E E	V A V Q 576
Zb	497 EWR TP A EMQ V	MADLFESALFGLGSGTLGVA	NECL S KM L TAAA <u>R</u> E R I MT F G N C L S NKTTQQ G I DDD I V S L F E K Q V E L R G A	P A L T 579
Ef	496 EWR SP S EMR V	MTDLFKGALVSLASGTLRVG	G C L R S M L P N SQ K T R I L R WG NC F S AQ T G L E S I DDD I T S L F Q KQ V L D R O V E	A A L Q 578
Zt	580 F R R E N I S Y N D	LARRIVIIGSRIKELLOPG	S V VM L HADG S T NWV I VM FA T LWA EC I F S PQ STNL P H S L R SQHYA I A NAQA	F L V P 662
Rc	577 F R E Q L I S Y T D	LAEXIRTVGRRIKELLKPG	S V V L L HADG S V NWV V AM FA V LWA EC V F S PQ GAA I P HQ L R SQYYA I ADAQA	F L V P 659
Zb	580 F C G E S I S Y N D	LARRIVIIGSRVKELLOPG	S V VM L HADG S I NWV V AM FA I LWADC I E S PQ STNL P HAL R SQHYA I ADAQA	F L V P 662
Ef	579 L G D Q E I S Y D G	VANMVRAVGQRIAELLEPR	S V V L I HADA S VYWV V AM FA V LWADC I L S PQ GVDL P HQ L R S HHYAVA EAQA	F L V P 661
Zt	663 QY ST SIPTPD	GCH L R L C V D T L L N E A T Q S P S	S S S T L D P R T P N P H S P AY I C F S S G T T G T P K A I L C T H S G V T S L	L R S P 736
Rc	660 QK DTAALTPD	G C N L S L C V E T I L R D A K E E K	S S R D E L H R P T K P L A G AY I C S S S G T T G K P K A I R C V H S G V G V	L S D P 735
Zb	663 QD SP SIPTPD	G C H Q R L C V E T L L S E A S Q T P S	S S T L T P R S P N P H C S AY I C F S S G T T G T P K A I L C T H S G V T S L	L C S P 736
Ef	662 QQDTDVLAPD	G C R L E L C V E T I L R E A E E R R C	S G H H H H R H P E P P A T R R Q P K P L A A AY I C F S S G S T G Q P K A I Q C T H S G AV S V	L R D P 744
Zt	737 A R L H V T P SH	T V SQT LAPQ FDGALLEV FAT	L C H G G T L L L K D P L N P FAH L Y G V G S L M L T P S L A L E L D P E D F I H D V R Y I Y F	A G E V 819
Rc	736 A R L N V V P G H	R V AQT LAP S FDGALLET F S	A L C F G G T L V L K D P L E P FAHMR T V D S L F V T P S L A A E L N P E D Y H H – L K N I Y I	G A E V 817
Zb	737 T A R L H V T P SH	T V SQT LAPQ FDGALLEV FAT	T L C H G G K L H L K D P L N P FAH L R G Y G T MML T P S L A L E L D P E D F I N N V R Y I Y F	A G E V 819
Ef	745 V A R L H V G P G H	R V AQT LAPA FDAALLEV FS	A L C Y G G T L I L K S P A E P F G H L R A A D S L L T T P S L A A E L N P D D Y N – L K Y V Y F	G A E V 826
Zt Rc Zb Ef	820 LPQATADRWT 818 LPQTTADRWS 820 LPQATADRWA 827 LPQHTADRWS	K G K A E V Y N V Y G P T E T H I L A A G K A I C R N I Y G P T E C H L V S K G K A E V C N V Y G P T E T H I L A A G K A S A Y N I Y G P T E C HMAC	AQ R VQ N C K P V T I G R P LM S T R VY I L D K NG V L L P P L V AG E I F I G C I S V S R C AQ T I K P C E P V T I G K P F S S T R I Y I L D EQ G Q L V P L V AG E I Y V S G VQ V S L C AQ R VQ T C K P V T I G R P L L S T R VY I L D K K G E L L P P L V AG E I Y I G C V S V S R C AQ R L R P C Q A V T I G T P F S S AR I Y I L D G Q G A L L P P L V AG E I H V A G V Q V A R C	Y L G L 902 Y I G L 900 Y L G L 902 Y I G L 902 Y I G L 909
Zt	903 E E E NA L R F V P	DT	VGYWT L E G E VA F L R R T DR Q V K V G G F R I DL ND VQ AR I Q S V V G G K V R VA V V	V G D V 985
Rc	901 E E E T K L R F V P		VGYWT L DCQ I A F I G R ND RQ I K L G G F R I DL ND VQ AR L Q NA I S S D AR V V V V	V G D V 980
Zb	903 K E E NA L R F V P		VG F WT L E G E V A F L G R T D R Q V K V G G F R V DL ND VQ AR I Q G V V G G K V K V AV V	V D D V 985
Ef	910 D Q E T R H R F I P		VG YWT L D Q V A F I S R T D R Q V K L F G F R I DL ND VQ AR L E K A I S P N V R V AV V V	V G D A 989
Zt	986 LGCAVEET	G N G I T E E S L R Q K I G Q V L P P C	IMVPRILRVVGKIPVSEVGKVDLRGVKAMLES	1044
Rc	981 LGCAISG	G H N L S P E Q V K A V A E D S L P P C	IQVPKVIRCLDAIPVSSFGKVDYRAVTKLLS-	1037
Zb	986 LGCAIEDT	G T G T T E E W L R Q K I E G V L P P C	MVPKILRIVERMPVSEVGKVDLRGVKAMLES	1044
Ef	990 LACAISPMSN	G H N L S E D Q V R A A A E T V L Q P C	ISVPKRIRLLEAMPVSPFGKIDYRAVTKLLE-	1049

Figure S7 LgsA predicted protein structure and multiple sequence alignment. (a) *Epichloë festucae* LgsA protein structure with respective InterproScan domains as indicated. Bar = 100 amino acids (aa). (b) Multiple sequence alignment of *E. festucae* (Ef) EfM3.056230, *Zymoseptoria tritici* (Zt) ZT1E4_G1886, *Ramularia collo-cygni* (Rc) RCC_03904, *Zymoseptoria brevis* (Zb) TI39_contig403g00012-encoded proteins. Domains as per (a), are indicated.







Fig. S8 *lgsA* deletion and complementation construct design and strain screening. (a) Schematic of the WT (F11) *lgsA* genomic locus and linear insert of *lgsA* deletion construct pKG39 and complementation construct pKG40. Regions of recombination are indicated by grey shading. *Hin*dIII(H) restriction enzyme sites used for Southern analysis and PCR primers used for Gibson assembly (red), split marker transformations (green) and knock-out screening (blue) are as shown. (b) nitro-blue tetrazolium/5-bromo-4 chloro-3'-indolyphosphate (NBT/BCIP)-stained Southern blot of *Hin*dIII genomic DNA digests (1.5 µg) probed with digoxigenin (DIG)-11-dUTP-labeled linear pKG39 purified PCR fragment (primers 171/172). Fragments of the expected size are as shown.





Fig. S9 Culture morphology of WT and $\Delta lgsA$ strains. Culture morphology of WT and $\Delta lgsA$ strains grown on PD agar for seven days at 22°C (upper row). DIC images of hyphae undergoing cell-cell fusion on 1.5% water agar (highlighted by broken circles) in lower row. Bar = 20 µm.





Fig. S10 Host interaction and cellular phenotypes of *L. perenne* infected with WT and $\Delta lgsA$ strains. (a) Host interaction phenotype of *L. perenne* plants infected with WT and $\Delta lgsA$ strains at 8 weeks post planting. (b) Confocal depth series images of *L. perenne* leaf sheaths infected with WT and $\Delta lgsA$ strains. Samples were stained with aniline blue, which detects β -glucans (red) and wheat germ agglutinin-alexa fluor 488 (WGA-AF488) which detects chitin (blue). Bar = 25 µm.





Fig. S11 Negative mode ESI-MS/MS spectra. (a) High-resolution accurate mass negative electro spray ionisation-mass spectrometry/mass spectrometry (ESI-MS/MS) spectrum of the 470 m/z *E. festucae* LGS precursor ion fragmented by higher-energy collisional dissociation (HCD) at 20% energy. (b) High-resolution accurate mass negative ESI-MS/MS spectrum of the 306 m/z putative *Z. tritici* LGS precursor ion fragmented by HCD at 20% energy.



Fig. S12 Observed (black) and simulated (red) isotope distributions for the 472 m/z (top) and 308 m/z (bottom) metabolites assuming molecular formulas of C₁₈H₃₃O₁₃N + H⁺ and C₁₂H₂₁O₈N + H⁺, respectively. Isotope distribution simulations were performed using Thermo FreeStyle 1.6.







Fig. S13 Strategy for deletion of *E. festucae lgsC*, Southern and PCR analysis. (a) Schematic of the *E. festucae* F11 (wild-type, WT) *lgsC* genomic locus, the linear *lgsC* deletion construct amplified from plasmid pCE57 using primers pRS426-cpsA-F and cpsA-pRS426-R, and the $\Delta lgsC$ genomic locus. WT and mutant loci are annotated with *Bcl*I cut sites and amplified regions to aid interpretation of the Southern blot and PCR screening results, respectively. Homologous flanking regions enabling targeted recombination are indicated by grey shading. (b) nitro-blue tetrazolium/5-bromo-4 chloro-3'-indolyphosphate (NBT/BCIP)-stained Southern blot of *Bcl*I-digested genomic DNA (1.5 µg per lane) probed with digoxigenin (DIG)-11-dUTP-labeled linear pCE57 purified PCR fragment (primers AR84/AR85). Fragments of the expected size are as shown. (c) Gel photo showing products of PCR amplification across the left (primers cps8/TC45) and right (TC44/cps7) flanking regions used to mediate integration at the target locus in four putative $\Delta lgsC$ strains. No amplification of the WT control is expected. (d) Gel photo showing products of PCR amplification (primers cps7/cps8) across the entire $\Delta lgsC$ integration site. Amplification of *lgsC* is expected for the WT control.





Fig. S14 Host interaction phenotype of *L. perenne* infected with WT and $\Delta lgsC$ strains. Host interaction phenotype of *L. perenne* plants infected with WT and $\Delta lgsC$ strains #6-6 and #12-6 several months post-planting.



Table S1 Biological material.

Table S2 Primers used in this study.

Table S3 Data matrix of raw data obtained by UPLC-ESI-TOF-MS-based metabolite

 fingerprinting analysis of the polar extraction phase, analysed in positive ESI-mode.

Table S4 Data matrix of raw data obtained by UPLC-ESI-TOF-MS-based metabolite

 fingerprinting analysis of the polar extraction phase, analysed in negative ESI-mode.

Table S5 Data matrix of raw data obtained by UPLC-ESI-TOF-MS-based metabolite

 fingerprinting analysis of the non-polar extraction phase, analysed in positive ESI-mode.

Table S6 Data matrix of raw data obtained by UPLC-ESI-TOF-MS-based metabolite

 fingerprinting analysis of the non-polar extraction phase, analysed in negative ESI-mode.

Table S7 Data matrix of 203 high quality metabolite features (false discovery rate < 0.003)</th>obtained by metabolite fingerprinting (UPLC-ESI-TOF-MS analysis) of apoplastic washfluids from mock-treated, FI1- or CT-infected *L. perenne*.

Table S8 Infection markers identified by metabolite fingerprinting (UPLC-ESI-TOF-MS analysis) and verified by UHPLC-ESI-QTOF-MS/MS analysis or coelution.

Table S9 Epichloë metabolite database



 Table S10 Differences in expression of lgs cluster genes in planta compared to axenic culture.