Supplemental Figures and table

HpDTC1, a Stress-Inducible Bifunctional Diterpene Cyclase Involved in Momilactone Biosynthesis, Functions in Chemical Defence in the Moss Hypnum plumaeforme

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HpDTC1 PpCPS/KS JsCPS/KS OsCPSsyn	10 20 30 40 50 60 70 80 90 100 RRLDOGVWGGGALVCRWRECLARFLGALEGFRRGTICQAQFVGEASVLSDEVFDDTAAVLAYQNVGSVTES-APDLWNAEVUL MASSTLJQNRSCGVTSSMSSFQIFRGQFLEFPGTRTPAAVQCLKKRECLAFTESVLESSPGSGSYRLVTGPSGTUPSSNGHLGGSGJTHRLPIFMESID MAARGAASSCT-LLSRTSKIDGELQQMTVAGTVAARSCRFHMPCHPRPFQSSLTARLPSRGMVWRAGEPRRASLASSPSASVPPLQAVFQAITD HFVTTASFQCVTLFGQFASAADAQFLLGGGRFFHLHARKRECGPMLISKSPYTPASETRWBADG
OsKSL4 SmMDS PaIPS	MASPMEAVARSEUVLAPERREALG-LLPAAAAPFULDCREENNGGMERENUSFACBAELDTGRRQLEFJGTRAVMSSCPG
HpDTC1 PpCPS/KS JsCPS/KS OsCPSsyn OsKSL4 SmMDS PaIPS	110 120 110 140 150 160 170 180 120 200 S
HpDTC1 PpCPS/KS JsCPS/KS OsCPSsyn OsKSL4 SmMDS PaIPS	210 223 240 250 250 250 250 200 280 200 280 200 280 200 280 200 280 200 280 200 280 28
HpDTC1 PpCPS/KS JsCPS/KS OsCPSsyn OsKSL4 SmMDS PaIPS	210 220 310 340 350 360 370 380 380 400 IPUELIRENTIMESLBERGESL-UPUNRTLEFGORGEILCERSTAVULMENGEKKEIDVISELERFENDEN IPUELIRENTIMESLBERGELERFENDEN 100 <td< th=""></td<>
HpDTC1 PpCPS/KS JsCPS/KS OsCPSsyn OsKSL4 SmMDS PaIPS	410 420 410 440 450 450 450 470 488 490 500 FDEEVSETLEF YRHINSDAKGIGWTKINGLQDIDDTSMAFRLLRLHGPVSSERFQSF-KDGKFYCFAGQS-QAVTGHWILYKASQVAPNEV-LVG FREERDCLGVYNYKKS-CGIGWASKSSVQDVDDTAMAFRLLR.HGFDVKEDCFRQFF-KDGFFCFAGQS-QAVTGHWISRASQVAPNEV-LVG FEKEINGSLDYVKYKS-VGIGWASKSSVQDVDDTAMAFRLLR.HGFDVKEDCFRQFF-KDGFFCFAGQS-QAVTGHWISRASQVAPNEV-LVG FEKEINGSLDYVKYKS-VGIGWASKSSVQDVDDTAMAFRLLR.HGFDVKEDCFRQFF-KDGFFCFAGQS-QAVTGHWYKSSVGFFPCFAG FEKEINGSLDYVKYKS-VGIGWASKSSVQDVDDTAMAFRLLR.HGFDVKEDCFRQFF-KDGFFCFAGQS-QAVTGHWYKSKSVGSSUFFFFF FEKEINGSLDYVKYKS-VGIGWASKSSVQDVDDTAMAFRLLR.HGFDVKEDCFRQFFCFAGQS-QAVTGHWYKSKSUSSVED FSSDEKRILDKYXLWS-G-NRDEFVKLDLFTCAMAFRLLR.HGYVVSDVLKFFFKDENSGEFCFGGAGS-QAVTGHWYTKASVSSSKSSKED FREIKAVLGVYSFWTN-GGIGWSTCUNNDIDTAMAFRLLR.HGYVVSSDVLKTFFRDENGEFFCFGQ70-RGVTDMLNVRCSHVAFPGFF-IMQE
HpDTC1 PpCPS/KS JsCPS/KS OsCPSsyn OsKSL4 SmMDS PaIPS	S10 S20 S10 S40 S50 S60 S70 S80 S90 600 GGL RELEFLOGEL BEINGY ARKCYLKKYL DG XVX X I KINPEROS PLLEV BY Y DIVG - TDDAWIGKTI YN Y UNINKYLDI AR AD YN OG LE FOD TO REF FERNEL HELL BENE GYDWI I TKYL DG XVX Y I KINPEROS PLLEV BY Y DIVG - TDDIWIGKTI YN Y UNINKYLDI AR AD YN OG LE FOD TO REF FERNEL BENE GYDWI I TKYL DG XVYXI AT YWY SI JPLLET BY LIDIYG - TDDIWIGKJYKK AV Y NGYVLLA AN AD YN UG LE KYL GENFARGY J YEHL - BYN BC YYNL I TKYL DG XVYXI AT YWY SI JPLLET BY LIDIYG - TDDIWIGKJYKK AV THOYAN AD YN DG AF YG XI SHOF YN DDIWI AT YND G XVYXI AT YWY SI JPLLET BY LIDIYG - TDDIWIGKJYKK AV THOYAN DDIWI AN AD YN UG AF YN YN D MGWRGALLERELCSDDINGY AT ALCH DG XVYXIAF YWY AN DYNWAR SI JPI I ET BY LIDIYG GODWIG XV LIRWIT FY DLALA AN AD FNO GYCG Y GYNT MGWRGALLERELCSDDING YN ALCH YN MGWN AF LINNF HOL DF AT YNN Y THWA Y DAN YN YN D YN D YN AF YN YN DYNH AN AD YN D YN ALCH YWY LEL AN YN YN DYNH AN AD YN D YN AN YN YN DYNH AN AD YN D YN ALCH YN HWY LEL AN YN D YN AN YN YN DYNH AN AD YN D YN ALCH YWY LEL AN YN YN DYNH AN AD YN D YN ALCH YN YN AN YN AD YN AN YN AD YN AD YN ALCH YN HYN D YN ALCH YN HYN AD YN D YN ALCH YN HYN AC YN AD YN AD YN AD YN AD YN AD YN AD YN ALCH YN HYN AN ALCH YN YN AN YN AD YN AD YN AN YN AD
11-0-001	
PpCPS/KS JsCPS/KS OsCPSsyn OsKSL4 SmMDS PaIPS	QILGMIKANSUPSESVA
HpDTC1 PpCPS/KS JsCPS/KS OsCPSsyn OsKSL4 SmMDS PaIPS	710 720 730 740 750 750 750 770 780 790 800 ALPDZVNVCRASALQGRNSTYIROWVRLVESTITAALWITORKIPPPDECKEVQIISAVEPTVMTAAFCOMPVNSTLSQLDSKGAMKI GLXKTINTIAEBAFMAQK RDVGHLQKIMLRWESCLTEALWITORKIPPPDEYKVAEISVLEPICGIFFACHWIDSLDSVD-HUVHL GLXNTLNAMTQEGTLAQG RDIGQHLQKIMLRWESCLTEALWITARKISGYPPPDEYKNALPSIALEPIVLCTLFFLGPLEPLSDEVGDSQK-LRLMEL SNLEELISUPPDDÀYSG SNLEELISUPPDDÀYSG ALVSTVNQLGANSASVQNRDVGVNUDFLESLATDAEWQRKVVPTMEEYKNISIVFFALGPILIALYFMQNLWBUJVKNAEY-DELFRL EFYNTULMTRAAKTIKHLNEPPFICKTISGUTVKLISELATDAEWGRKVVPTMEEYKNISIVFFALGPILIALYFMQNLWBUJVKNAEY-DELFRL EFYNTULMTAAKTIKHLSAFKIHMLASKITVKINSIVTEALWIJISITAVENSISITUMULUSLLAAVESQSFLGUTALLUTIAYFMQNLWSJLVGGNEFLGUTVLJSLFT VLYNTVNEIAVESGKRQGRDVLQYIRNVLBILLAAHTKEAEWSARYVPSFDEYIENASVSISISUSISIVUJSISVETGELLTDDVLSKIGROSRFLQL
HpDTC1 PpCPS/KS JsCPS/KS OSCPSsyn OsKSL4 SmMDS PaIPS	810 820 840 850 860 870 880 900 ISYSSELINDIATEY EROSGENENT-ISCYLDEROGUG EKERVALTETEKNEROINLELAROVPACHEVLENTARNYENSGEDTYSA NURVERILINGUKERASOKISI-VOITMAN NURVERILINGUKERASOKISI-VOITMAN NURVERILINGUKERASOKISI-VOITMAN NURVERILINGUKERASOKISI-VOITMAN NURVERILINGUKERASOKISI-VOITMAN NURVERILINGUKERASOKISI-VOITMAN NURVERILINGUKERASOKISI-VOITMAN NURVERILINGUERIAN
HpDTC1 PpCPS/KS JsCPS/KS OsCPSsyn OsKSL4 SmMDS PaIPS	910 920 -NTEVQHDVQKALFEPVA -TAKTGFVKKVLFEPVPZ -SARAKIKKVLFQEVVF HIDKVLFQDVI

Supplementary Figure S1. Amino acid sequence alignment of HpDTC1

Moss bifunctional *ent*-kaurene synthase (PpCPS/KS: AB302933.1), liverwort bifunctional *ent*-kaurene synthase (JSCPS/KS: BAJ39816), lycophyte bifunctional miltiradiene synthase (SmMDS:BAL41682), rice monofunctional *syn*-pimaradiene synthase (OsKSL4: Q0JEZ8) and gymnosperm bifunctional isopimaradiene synthase (PaIPS: ADZ45512).



Supplementary Figure S2. Phylogenetic tree of plant diterpene cyclases.

The tree was constructed using the bootstrap neighbour joining method from alignment with the ClustalW program. HpDTC1 belongs to the gymnosperm and lycophyte bifunctional diterpene cyclases for specialized diterpene biosynthesis.

	ACCESSION	Name
PpCPS/KS	AB302933.1	ent-kaurene synthase [Physcomitrella patens]
JsCPS/KS	BAJ39816	ent-kaurene synthase [Jungermannia subulata]
SmCPS1	J9QS23	Copalyl diphosphate synthase 1, SmTPS9
PgCPS	ADB55707	ent-copalyl diphosphate synthase [Picea glauca]
SICPS	NP_001234008	copalyl diphosphate synthase [Solanum lycopersicum]
LsCPS	BAB12440	copalyldiphosphate synthase No1 [Lactuca sativa]
AtCPS	NP_192187	Ent-copalyl diphosphate synthase [Arabidopsis thaliana]
OsCPS-syn	BAD42451	syn-CDP synthase [Oryza sativa Japonica Group]
ZmCPS	AAT70083	ent-copalyl diphosphate synthase [Zea mays]
HvCPS	AAT49065	copalyl diphosphate synthase-like protein [Hordeum vulgare]
OsCPS1	BAD42449	ent-copalyldiphosphate synthase [Oryza sativa]
PaLPS	ADZ45517	levopimaradiene/abietadiene synthase [Picea sitchensis]
PaIPS	ADZ45512	isopimaradiene synthase [Picea sitchensis]
AgAS	AAK83563	abietadiene synthase [Abies grandis]
PcmISO1	M4HYP3	Monofunctional isopimaradiene synthase [Pinus contorta]
PcmPIM1	M4HYC8	Monofunctional pimaradiene synthase [Pinus banksiana]
GbLPS	Q947C4	Levopimaradiene synthase [Ginkgo biloba]
SmCPSKSL1	AEK75338	labda-7,13E-dien-15-ol synthase [Selaginella moellendorffii]

SmCPSKSL2	BAP19109	sandaracopimaradiene synthase [Selaginella moellendorffii]
SmMDS	BAL41682	miltiradiene synthase [Selaginella moellendorffii]
OsKS	AAQ72559	ent-kaurene synthase 1A [Oryza sativa]
OsKSL7	Q00G37	Ent-cassa-12,15-diene synthase [Oryza sativa]
HvKS	AAT49066	ent-kaurene synthase-like protein 1 [Hordeum vulgare]
OsKSL4	Q0JEZ8	Syn-pimara-7,15-diene synthase [Oryza sativa]
OsKSL11	Q1AHB2	Stemod-13(17)-ene synthase [Oryza sativa]
OsKSL10	ABH10735	ent-sandaracopipmaradiene synthase [Oryza sativa]
AtKS	AAC39443	ent-kaurene synthase [Arabidopsis thaliana]
LsKS	BAB12441	ent-kaurene synthase No1 [Lactuca sativa]
PgKS	ADB55711	ent-kaurene synthase [Picea glauca]
SmKS	BAP19110	ent-kaurene synthase [Selaginella moellendorffii]



Supplementary Figure S3. GC-MS spectra of enzymatic products of recombinant HpDTC1 mutant enzymes.

(a) *syn*-copalol, product of OsCPS4. (b) The product of the HpDTC1-GGLFD mutant enzyme was identical to *syn*-copalol. (c) *syn*-pimara-7,15-diene, HpDTC1 product. (d) The reaction product from OsCPS4 and the HpDTC1-DIGG mutant enzyme was identical to *syn*-pimara-7,15-diene. The reaction product, *syn*-CDP, in (a and b) was dephosphorylated by calf intestinal alkaline phosphatase.



Supplementary Figure S4. Photograph of *Botrytis cinerea* **infected** *Hypnum plumaeforme Hypnum plumaeforme* was cultured at 24 °C for 5 days after inoculation with *Botrytis cinerea* mycelia. Scale bar: 10 mm.



Supplementary Figure S5. *HpDTC1::GUS* reporter gene expression in protonema cells of *P. patens*. Protonema colonies of *HpDTC1::GUS* and vector control lines were incubated with 500 μ M CuCl₂ and chitosan (500 μ g/ml) for 24 h and then histochemically stained with X-Gluc. Scale bar: 5 mm.

Supplementary Figure S6. The effects of jasmonate and 12-oxo-phytodienoic acid on *HpDTC1::GUS* expression.

After 24 h treatment with jasmonate (JA) and 12-oxo-phytodienoic acid (OPDA), *HpDTC1::GUS* reporter expression was visualized by X-gluc. (a) OPDA induced *HpDTC1::GUS* reporter expression, but JA did not. (b) JA and OPDA did not affect GUS expression in the control line. The values in parentheses represent the concentration (μM).



Supplementary Figure S7. The *HpDTC1::GUS* reporter gene was induced by *Pythium irregulare* infection in *P. patens* gametophores.

(a) Mycelia of *Pythium irregulare* MAFF 237501 were inoculated onto colonies of *HpDTC1::GUS* and vector control lines and then plates were incubated for 4 days. Scale bar: 5 mm. (b) The *HpDTC1::GUS* plants were histochemically stained by X-gluc. Scale bar: 1 mm. (c) The quantitative data for *HpDTC1::GUS* reporter induction by *P. irregulare* infection after 4 days incubation. The GUS activity was fluorometrically determined. Data are presented as mean \pm standard deviation of biological replicates. (*n*=5-8, *p<0.01.).



Supplementary Figure S8. Allelopathic activity of *H. plumaeforme* extracts on the growth of the moss *P. patens* and liverwort *M. polymorpha*.

The acetone extract was prepared from *H. plumaeforme* gametophores treated with or without 500 μ M CuCl₂. The plants were cultured on BCDATG agar medium containing the extract (200 μ g/mL) and then photographs were taken on the indicated days. Scale bars: 5 mm.



Supplementary Figure S9. Allelopathic activity of momilactone B on the growth of *H*. *plumaeforme* and liverwort *M*. *polymorpha*.

(a) Photograph of *H. plumaeforme* gametophores grown on BADATG agar plates containing momilactone B (10 and 50 μ M). (b) The growth of H. *plumaeforme* was recorded after 8 and 14 days incubation. The values shown are the relative length of gametophores at 0 days (100%). Inhibitory effects of momilactone B on the growth was not observed at 100 μ M. (c) *M. polymorpha* was grown on M51 agar medium containing momilactone B and cultured for 14 days. Momilactone B showed potent inhibitory activity on the growth of *M. polymorpha* at 2-5 μ M.



Supplementary Figure S10. The effects of dead gametophytes of *H. plumaeforme* on the growth of *B. cinerea*.

Mycelia were inoculated onto BCDATG agar plates containing the dried gametophores of *H*. *plumaeforme* (10% w/v) and then fresh *H. plumaeforme* gametophores were placed on the plate. The photograph was taken after 4 days incubation. Scale bar: 10 mm.



Supplementary Figure S11. Measurements of endogenous jasmonic acid in *H. plumaeforme*.

Moss samples (about 100 mg fresh weight) were homogenized and suspended in 2 mL 80% (v/v) methanol. After adding 5 ng [²H₂]-JA as an internal standard, the supernatant was loaded onto a Bond Elut C18 column (100 mg, 3 mL; Agilent, CA), which had been sequentially pre-washed with 3 mL each of methanol and water and equilibrated with 3 mL 80% (v/v) methanol, which was followed by elution with 2 mL 80% (v/v) methanol. The flow-through and eluted fractions were collected together and concentrated to a volume of about 0.5 mL *in vacuo*. The concentrated samples were subjected to liquid chromatography with electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) to quantify JA accumulation as previously described in Tamiru et al., A chloroplast-localized protein, LESION AND LAMINA BENDING, affects defence and growth responses in rice. *New Phytol.* 2016 *doi: 10.1111/nph.13864.* (a, c) SEM chromatogram (*m*/*z* 209>59) corresponding to the fragment ion from the internal standard [²H₂]-JA in 4h-treated sample for 4 h. A black arrow indicates the peak derived from JA. The endogenous JA level in *H. plumaeforme* was lower than the detection limit of our analysis system (<0.5 ng/gFW).



Supplementary Figure S12. *HpDTC1* expression and production of momilactones are specifically induced in the gametophores, but not in the protonema cells of *H. plumaeforme*.

(a) qRT-PCR was performed using HpDTC1 specific primers with total RNA from *H*. *plumaeforme* gametophores and protonema cells treated with chitosan (500 µg/mL) for 6 h. (b) Momilactones A and B in *H. plumaeforme* gametophores and protonema cells treated with chitosan (500 µg/mL) for 6 h were measured by LC-MS/MS. In protonema cells, HpDTC1 was not induced by chitosan treatment and consequently momilactones did not accumulate.



Supplementary Figure S13. *H. plumaeforme* transcription factors induced by CuCl₂ treatment. Forty-two genes up-regulated more than 5-fold compared to untreated samples are categorized by types of families.

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HpDTC1_cDNA	HpDTC1-FR1-Fd	AGAAGACAAGAATACCTGGATAGAGACGAC
internal fragment	HpDTC1-FR1-Rv	
HpDTC1-RACE GSP primer for 5'-	HpDTC2-RACE5'-116	GGAAACTCGGGTTCGTGAACTCCAGATAGC
end	HpDTC2-RACE5'-266	TCTTTGCACACTGTCACTTCCCGTGTCCC
HpDTC1-RACE GSP primer for 3'-	HpDTC2-RACE3'-1929	TGTCGCAACTGGATTCCCGTGGTGAAGC
end	HpDTC2-RACE3'-2098	CGGCATTGACTACTGAAATCGAGAAGATGC
Cloning for	HpDTC1_cDNA Fd	TTATCGATGCAAAAGCAGTGGTTAG
HpDTC1_ORF	HpDTC1_cDNA Rv	AACGAACATTTTTGGGACTCTGGAACGCA
TAIL AD primers	TAIL-P1	NGTCGASWGANAWGAA
	GSP1-1	TAGAGTGTGAGTGATTCTGTCGTAA
TAIL GSP primers	GSP1-2	CCCAAGAACCATCTTCAAACTG
	GSP1-3	CATTGCAGAGCTTGAGGAAACT
TAIL AD primers	TAIL-P2	GTNCGASWCANAWGTT
	GSP2-1	TGAACCTACGAAAGTATCAACCGATC
TAIL GSP primers	GSP2-2	TACATTCCGACCCAATGTGGAG
	GSP2-3	CTGCGTAAGTTTCCTCAAGCTC
TAIL AD primers	TAIL-P1	NGTCGASWGANAWGAA
	GSP3-1	ATGAGAGCTGAGTGGCCATTG
TAIL GSP primers	GSP3-2	CACGGTTGGTTCATTCCTACTTCT
	GSP3-3	TTCCATAAAGGTCAGTCGGG
TAIL AD primers	TAIL-P2	GTNCGASWCANAWGTT
	GSP4-1	CCAAGGTCATTAAGGCAAGC
TAIL GSP primers	GSP4-2	GCTCCATTGACCGATAAACA
-	GSP4-3	GTGGATGATTCTATTGGTTGAGA
TAIL AD primers	TAIL-P3	WGTGNAGWANCANAGA
	GSP5-1	GAGAATTACAACAAGAGAATTACCT
TAIL GSP primers	GSP5-2	CCAAGGTCATTAAGGCAAGC
1	GSP5-3	GCTCCATTGACCGATAAACA
HpDTC1 promotor	PR-InF-HpDTC1-Fd	CTATAGGGGAAAGCTTAAAATAATGAAGAGTGGAGAGAGA
cloning	PR-InF-HpDTC1-Rv	GTGAATTCGAGCTCGGTACCGTCGTCTCTATCCAGGTATTCTTGTCTTC
HpDTC1-expression	HpDTC1-ORF-Fd	GGCATATGGAGCTCGGTACCGCCGTCGACACAGAAGACAA
vector	HpDTC1-ORF-Rv	CGACAAGCTTGAATTCTCAGGCCACAGGCTCGA
-	double-mutation-A Fw	GAAGAGGCCGCCACGAT
HpDTC1 mutant	double-mutation-A Rv	ATCGTGGGCGGCCCTCTTC
enzymes	double-mutation-B Fw	GGCGGTACTTCAATGGCTTTCC
	double-mutation-B Rv	TATATCTTGCAAATCACCATTCTTAGTCCATC
	HpACT3-Fd	CGAGCAGCATGAAGATCAAG
	HpACT3-Rv	GTACTCGCTCTTCGCAATCC
qRT-PCR primers	DTC1_for_RTPCR-Fd	TGCTGCTCAGCATGTATCGT
	DTC1_for_RTPCR-Rv	GGACTCTGGAACGCAAGACT
	PR-DTC1-GUS-Fd	ATTCATGTTTTCTAGAAAAATAATGAAGAGTGGAGAGAG
HpDTC1::GUS	PR-DTC1-GUS-Rv	CCGGGGGGGGGGGATCCTCCCTGGACATGAAGTTCGGTGTGC

Supplementary table S1 List of PCR primers