

The Role of a Cytokinin Antagonist in the Progression of Clubroot Disease

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Supplementary Information:

Supplementary Methods

Supplementary Methods S1. Chemical synthesis and activity testing.

For the preparation of 6-(2-hydroxy-3-methylbenzylamino)purine (PI-55), 6-chloropurine was heated with 2-hydroxy-3-methylbenzylamine (to 90°C; 4 hours) in n-butanol with excess triethylamine. After lowering the reaction temperature, the precipitated product was filtered and rinsed with cold water and n-butanol and subsequently, it was crystalized from dimethylformamide. The melting points were determined on a Boetius stage and presented values are uncorrected. Analytical thin layer chromatography (TLC) was carried out using silica gel 60 WF254 plates (Merck). An EA1108 CHN analyzer (Fisons Instruments) was used for elemental analyses (C, H and N). ES+ mass spectra were recorded using a Waters ZMD 2000 mass spectrometer equipped with a direct probe, and a mass monitoring interval of 10 - 1500 amu. The spectra were collected using 3.0 second cyclical scans, applying a sample cone voltage of 25 V at a source block temperature of 150°C, desolvation temperature of 80°C and desolvation gas flow rate of 200 l/hour. The mass spectrometer was directly coupled to a MassLynx data system. NMR spectra were acquired in a Bruker Avance AV 300 spectrometer operating at a temperature of 300 K and a frequency of 300.13 MHz (¹H) or 75.48 MHz (¹³C). Samples were prepared by dissolving the compounds in DMSO-d₆. Tetramethylsilane (TMS) was used as the internal standard.

Elemental analysis (C, H, N) (Found: C, 60.85 ; H, 5.38 ; N, 27.29%. C₁₃H₁₃N₅O calc.: C, 61.17; H, 5.13; N, 27.43%); TLC (chloroform : methanol : ammonia (8:2:0.1, v/v/v) as the mobile phase): single spot; m.p. 278.4-279.5°C; ES+ MS: m/z 255 [MH]⁺; ¹H-NMR (DMSO-d₆, 300MHz): 13.03s (1H, N9H), 10.70s (1H, O1H), 8.45s (1H, N6H), 8.28s (1H, C8H), 8.16s (1H, C2H), 7.09d (1H, C13H), 7.00d (1H, C15H), 6.69tr (1H, C14H), 4.54s (2H, C9H), 2.16s (3H, C16H)

¹³C-NMR (DMSO-d₆, 75MHz): 153.94 (12C), 152.80 (6C), 152.00 (2C), 150.01 (4C), 140.01 (8C), 130.26 (15C), 128.63 (14C), 126.13 (10C), 125.94 (11C), 119.52 (13C), 119.10 (5C), 40.44 (9C), 16.93 (16C).

Standard bioassays based on stimulation of cytokinin-dependent tobacco callus growth, the retention of chlorophyll in excised wheat leaves and the dark induction of betacyanin synthesis in *Amaranthus* cotyledons were carried out as described previously (Holub et al. 1998). Specifically, the relative activity of the prepared derivative at optimal concentration was compared with the activity of 6-benzylaminopurine (BAP), (10⁻⁶ M BAP for the tobacco callus bioassay, 10⁻⁵ M BAP for the *Amaranthus* betacyanin bioassay, and 10⁻⁴ M BAP in the case of the senescence bioassay).

Holub J, Hanuš J, Hanke DE, Strnad M (1998) Biological activity of cytokinines derived from *Ortho*- and *Meta*-hydroxybenzyladenine. *Plant Growth Regul* 26: 109-115.

Supplementary Table S1. Primers for qRT-PCR.

Gene	AGI No.	Primers 5' - 3'
Housekeeping genes (Endogenous controls)		
<i>Act2</i>	<i>At3g18780</i>	TGGTCGTACAACCGGTATTGTG ATCAGTAAGGTCACGTCCAGCAA
<i>Act7</i>	<i>At5g09810</i>	CTAGAGACAGCCAAGAGCAGTTC GTTTCATGGATTCCAGGAGCTTC
<i>EF1α</i>	<i>At5g60390</i>	TGAGCACGCTCTTCTTGCTTTCA GGTGGTGGCATCCATCTTGTTACA
MAPK/PTI marker genes		
<i>ARR4</i>	<i>At1g10470</i>	GGTGGTATCGGAGGAATTGA ACGCCATCCACTATCTACCG
<i>ARR5</i>	<i>At3g48100</i>	TGCCTGGGATGACTGGATATG CTCCTTCTTCAAGACATCTATCG
<i>ARR7</i>	<i>At1g19050</i>	TACTCAATGCCAGGACTTTCAGG TCTTTGAGACATTCTTGATACGAGG
<i>ARR8</i>	<i>At2g41310</i>	AGTAGATGACAACGACCCAAAT TATACTTCTAAACGCCGCTGAT
<i>ARR9</i>	<i>At3g57040</i>	ATG GGT ATG GCA GCA GAA TC AAG CCT TTG AGC CAG AAT CA
<i>ARR16</i>	<i>At2g40670</i>	CGTAAACTCGTTGAGAGGTTGCTC GCATTCTCTGCTGTTGTCACTTTG
<i>CKX1</i>	<i>At2g41510</i>	TAAGGGGAAGCAACCATATGTT AACGGTCAAATGAAGGTAGTCT
<i>CKX4</i>	<i>At4g29740</i>	TCTTCTAAACCAAACCTCAACTTCTG TGCGGTTGTTCCATTTGTTTC
<i>CKX5</i>	<i>At1g75450</i>	GGCGTTTTCAAGGGCATTTC TCGTCCCATTGTCTTTGTTCA
<i>CKX6</i>	<i>At3g63440</i>	GGGTACATGTGTCTGAGGTAAA ATTTGTTCACTGGGTAGACGAT
<i>IPT1</i>	<i>At1g68460</i>	GAATCGGAAAGACAAAGTCGTC ATGATCTCTGAAGGGAAACGAG
<i>IPT3</i>	<i>At3g63110</i>	CTCTCCATTGAATCCGTCCTAA TCTTGTCAACTCTCTCAGACAC
<i>IPT5</i>	<i>At5g19040</i>	GACACTTACGAAGATTTACGG GCCTCGATGTAAGAATTGGAAC
<i>IPT7</i>	<i>At3g23630</i>	CAAGCAATCTCGAACTCTCAG AGACAAAGGAGTTAAGTACGGG
<i>LOG1</i>	<i>At2g28305</i>	GGGTTTGATTTCTCAAGCTGTT CATCAGAGTGCTTAGCCATTTTC
<i>LOG6</i>	<i>At5g03270</i>	GGTTTAATGGGTTTGGTGTCTC CATCAGAATGCTTAGCCATCAC

Supplementary Table S2.

Down and upregulated genes related to cytokinin signaling and metabolism. Expression changes were followed by qPCR and expressed as fold changes between mock-treated (0.1% DMSO) controls and infected/non-infected Col-0 or *ahk2 ahk3* double mutant with or without PI-55 treatment at a concentration of 10 $\mu\text{mol l}^{-1}$. All data were obtained 28 days after infection (dai) of control (ctrl) or clubroot-infected (inf) plants in three independent biological replicates.

AGI No.	Description	Fold Change					
		Col-0 ctrl.	Col-0 inf.	Col-0 inf.	<i>ahk2ahk3</i> ctrl.	<i>ahk2ahk3</i> inf.	<i>ahk2ahk3</i> inf.
		PI-55	mock	PI-55	PI-55	mock	PI-55
AT1G10470	Response regulator 4 (<i>ARR4</i>)	1.05	1.61	1.48	1.58	0.90	1.12
AT3G48100	Response regulator 5 (<i>ARR5</i>)	1.18	1.40	2.03	0.35	0.41	1.11
AT1G19050	Response regulator 7 (<i>ARR7</i>)	1.22	1.10	2.08	1.64	0.68	1.92
AT2G41310	Response regulator 8 (<i>ARR8</i>)	0.57	1.27	0.77	0.80	0.65	0.41
AT3G57040	Response regulator 9 (<i>ARR9</i>)	0.56	1.20	0.78	0.56	0.58	0.62
AT2G40670	Response regulator 16 (<i>ARR16</i>)	1.04	1.62	0.47	0.99	3.64	0.75
AT2G41510	Cytokinin oxidase/dehydrogenase 1 (<i>CKX1</i>)	0.72	0.88	0.41	1.20	0.48	1.07
AT4G29740	Cytokinin oxidase/dehydrogenase 4 (<i>CKX4</i>)	1.67	2.42	5.14	0.54	0.31	1.63
AT1G75450	Cytokinin oxidase/dehydrogenase 5 (<i>CKX5</i>)	1.22	0.85	1.64	0.95	0.91	0.77
AT3G63440	Cytokinin oxidase/dehydrogenase 6 (<i>CKX6</i>)	1.04	0.88	1.14	0.32	0.32	0.40
AT1G68460	Isopentenyltransferase 1 (<i>IPT1</i>)	0.88	0.78	1.02	0.78	1.36	1.75
AT3G63110	Isopentenyltransferase 3 (<i>IPT3</i>)	0.80	1.10	0.49	0.74	0.90	0.70
AT5G19040	Isopentenyltransferase 5 (<i>IPT5</i>)	0.44	1.27	0.65	0.63	0.82	0.94
AT3G23630	Isopentenyltransferase 7 (<i>IPT7</i>)	0.61	0.95	0.96	1.02	1.09	1.39
AT2G28305	LONELY GUY 1 (<i>LOG1</i>)	1.03	0.91	1.37	0.74	0.72	1.61
AT5G03270	LONELY GUY 6 (<i>LOG6</i>)	0.44	1.54	1.87	0.37	0.50	0.95