Supporting Information

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SI Methods

Purification of Recombinant Cytokinin Oxidase/Dehydrogenases (CKXs). Six *AtCKX* cDNAs were amplified by PCR without their signal sequences with the primers listed in Table S3 and cloned into the AvrII-linearized vector pGAPZα::$His_{(9)}$ (Invitrogen) with the corresponding restriction enzymes (see Table S3). The constructs were subsequently stabilized into *Pichia pastoris* strain X33 by homologous recombination (1), and, as such, the genes were constitutively expressed from the strong *GAPDH* promoter.

The recombinant yeast strains were cultivated in YNB media buffered to pH 6.7 by potassium phosphate, supplemented with 2% glucose, at 28 °C with orbital shaking at 230 rpm for 3 to 5 days. The cells were removed by centrifugation at 15,000 $\times$ *g* and the pH of the cell-free medium was adjusted to pH 8.0 with 1 M Tris-base. The cell-free medium was concentrated to $\approx$60 mL by ultrafiltration on a MiniKros Sampler Module (Spectrum) with a 10-kDa cut-off. Ultrafiltration was repeated 3 times to substitute the buffered media for 50 mM Tris·HCl, pH 8.0, supplemented with 20% ammonium sulfate. The concentrated proteins were loaded on an octyl-Sepharose CL-4B hydrophobic column (GE-Healthcare) connected to a BioLogic LP liquid chromatograph equipped with UV and conductivity detectors (Bio-Rad). After applying the sample, the column was washed with a decreasing step-gradient of ammonium sulfate and the eluate was fractionated. The fractions with enzymatic activity were pooled and concentrated with a stirred ultrafiltration cell (Millipore) equipped with a YM 10 membrane (10-kDa cut-off). The concentrated sample was applied onto a Bio-gel hydroxyapatite (Bio-Rad) column equilibrated with 10 mM potassium phosphate buffer (pH 7.7). The proteins were eluted by a linear gradient of 10 mM and 1 M of potassium phosphate buffers (pH 7.7). Active fractions were concentrated to 2 mL and the buffer was replaced by 50 mM potassium phosphate (pH 7.4) containing 0.5 M NaCl with the ultra-filtration device. All CKX samples were finally purified on a Ni Sepharose HP (GE-Healthcare) equilibrated with 50 mM potassium phosphate buffer (pH 7.4) containing 0.5 M NaCl. His-tagged proteins were washed from the column by imidazole-containing (10 to 50 mM) loading buffer. Concentrated CKX proteins were checked for purity by SDS/PAGE followed by Western blotting with antibodies raised against barley CKX1 or CKX2 proteins. After purification, CKX proteins showed 20–80% homogeneity.

The CK dehydrogenase enzyme assay was based on the decolorization of the electron acceptor ferricyanide (500 μM) at 420 nm and normalized as other dehydrogenases (2). The reaction mixture contained 100 mM McIlvaine buffer, pH 6.0, and 50 μM CK substrate. The oxidase assay (3) could not be done for the *cis*-derivatives, due to cyclization of the degradation product (4).

1. Cregg JM, Barringer KJ, Hessler AY (1985) *Pichia pastoris* as a host system for transformations. *Mol Cell Biol* 5:3376–3385.
2. Appleby CA, Morton RK (1959) Lactic dehydrogenase and cytochrome b2 of baker's yeast. *Biochem J* 71:492–499.
3. Frébort I, *et al.* (2002) Cytokinin oxidase/cytokinin dehydrogenase assay: Optimized procedures and applications. *Anal Biochem* 306:1–7.
4. Galuszka P, *et al.* (2007) Biochemical characterization of cytokinin oxidases/dehydrogenases from *Arabidopsis thaliana* expressed in *Nicotiana tabacum* L. *J Plant Growth Regul* 26:255–267.

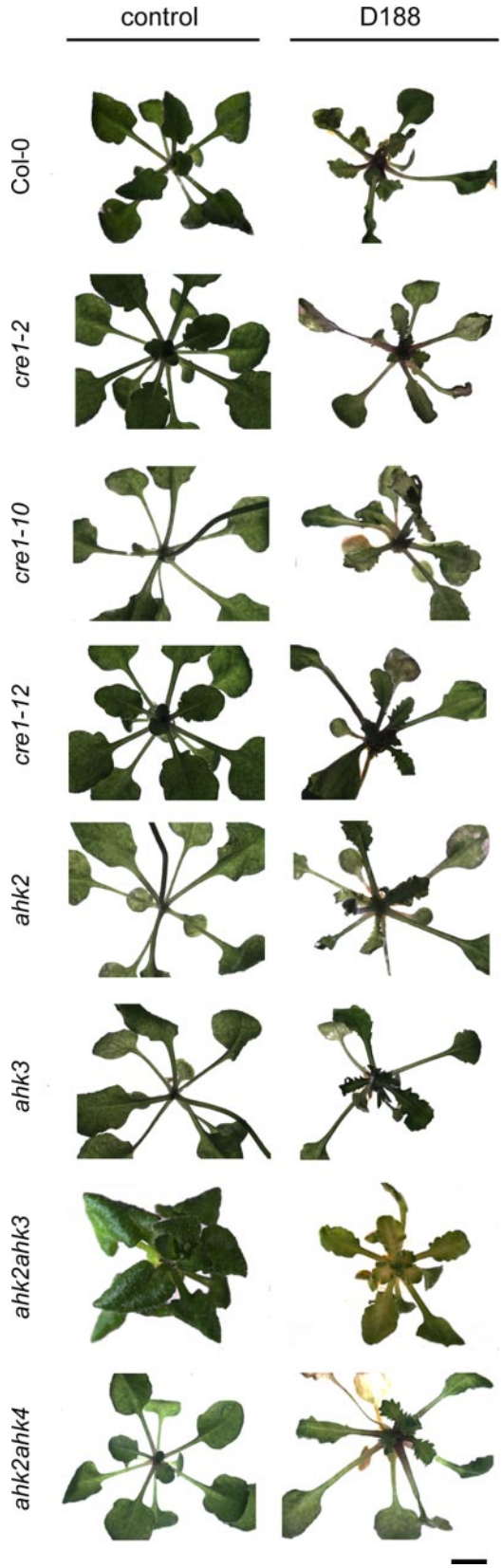

Fig. S1. Response of single and double CK receptor mutants upon mock inoculation and infection with *R. fascians* D188 at 17 days post infection (dpi). Images were taken at the same magnification (Scale bar, 1 cm.).

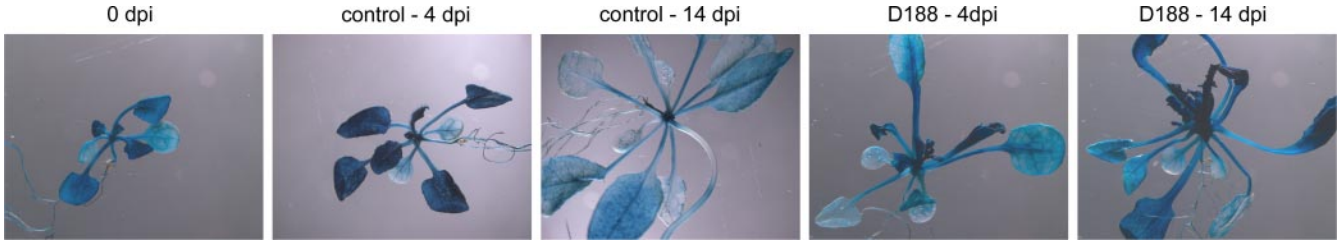

Fig. S2. Histochemical analysis of *AHK3:GUS* lines upon mock-inoculation (control) or infection with *R. fascians* D188. Expression patterns were visualized at 4 or 14 days post inoculation.

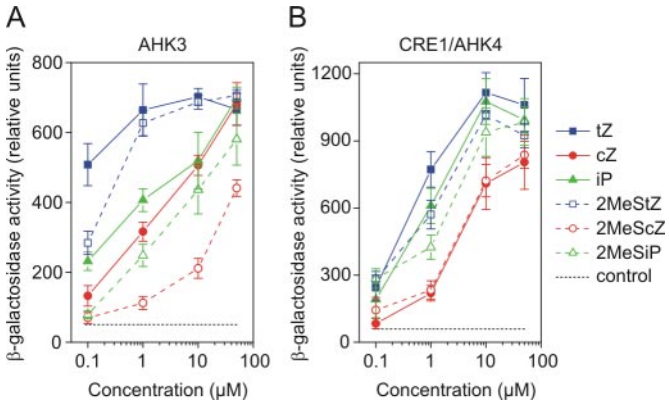

Fig. S3. Dose dependence of CK-induced β-galactosidase activity of *AHK3* and *AHK4* expressed in *Escherichia coli*. The β-galactosidase activity of noninduced strains (control) is indicated by the dotted line. Error bars represent SDs ($n = 3$).

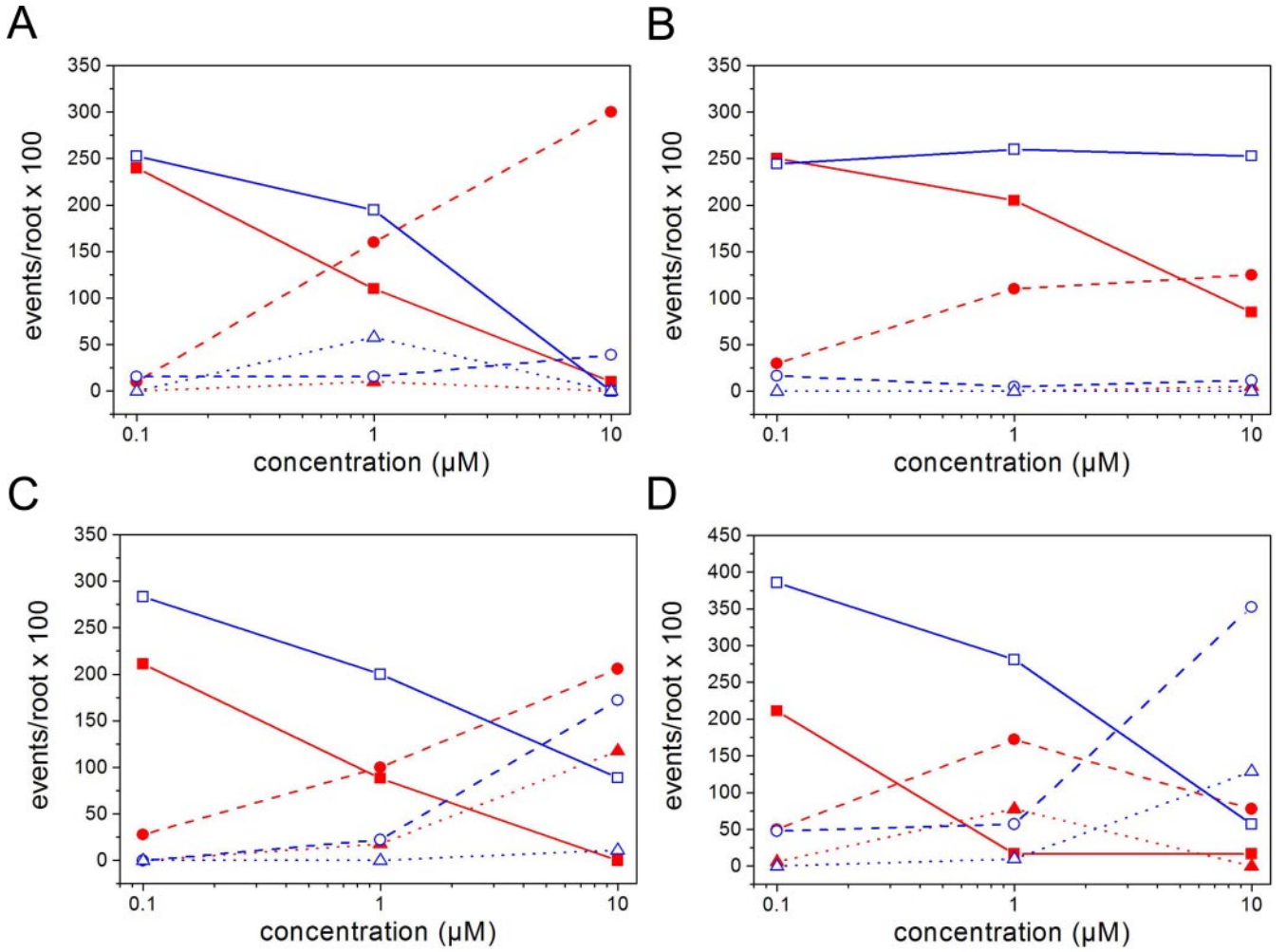

Fig. S4. Dose-dependent regeneration capacity of the individual and equimolar mixes of *R. fascians*-produced CK bases. Root inhibition (triangle), and callus (square) and shoot induction (circle) events observed in the regeneration assays with classical (red symbols) and 2MeS (blue symbols) CKs. (*A*) Isopentenyladenine (iP) and 2-methylthio (2MeS)iP. (*B*) *cis*-zeatin (cZ) and 2MeScZ. (*C*) *trans*-zeatin (tZ) and 2MeStZ. (*D*) eq4 (iP, 2MeSiP, cZ, and 2MeScZ; blue symbols) and eq6 (iP, 2MeSiP, cZ, 2MeScZ, tZ, and 2MeStZ; red symbols).

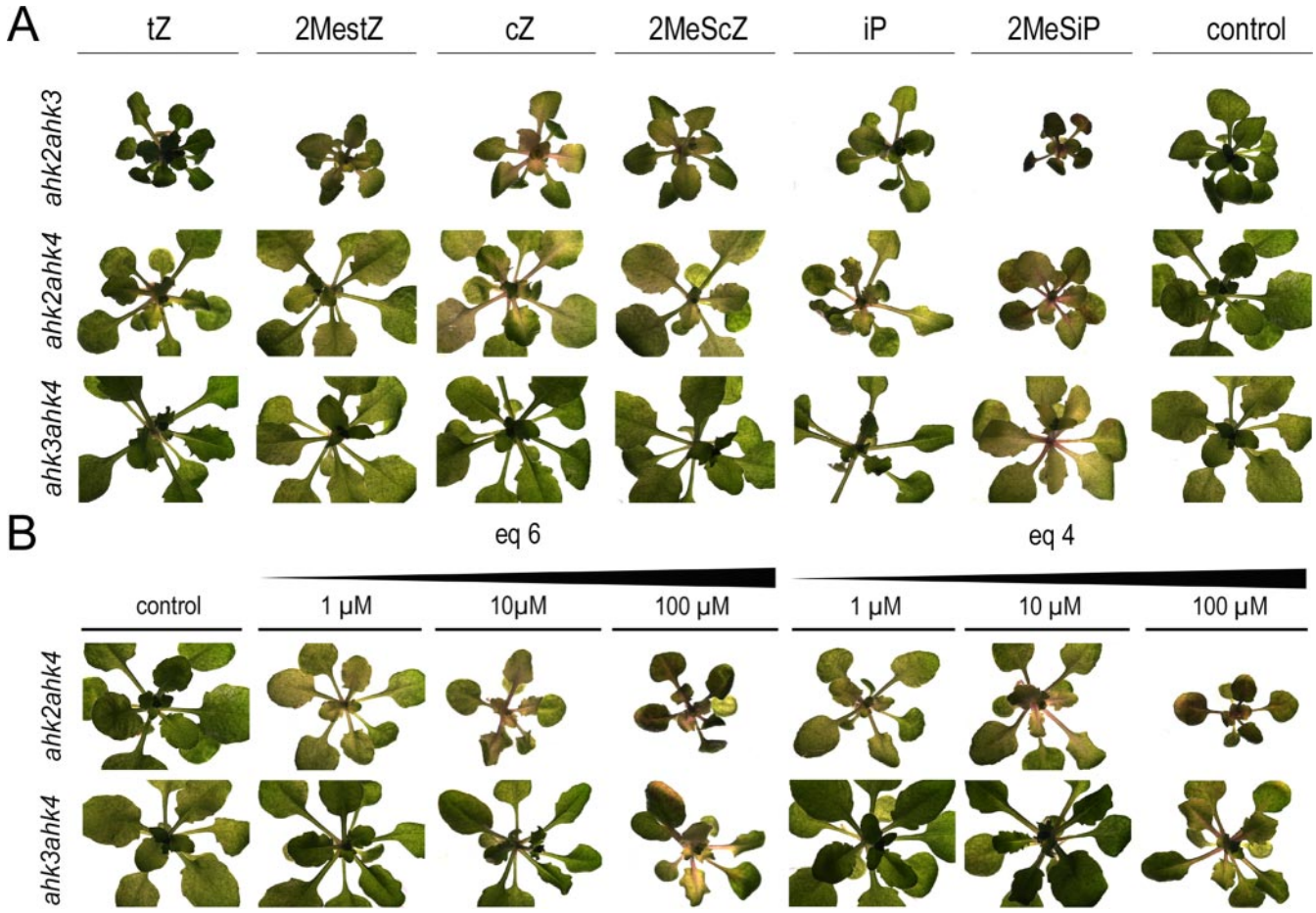

Fig. S5. Phenotypic response of the CK receptor double mutants toward individual CK treatment at 10 μM (*A*) and different concentrations of equimolar mixes of the *R. fascians*-produced CK bases (*B*). In *B*, *ahk2ahk3* is not shown, but the response was similar to that of *ahk2ahk4*; eq4 (iP, 2MeSiP, cZ, and 2MeScZ); eq6 (iP, 2MeSiP, cZ, 2MeScZ, tZ, and 2MeStZ).

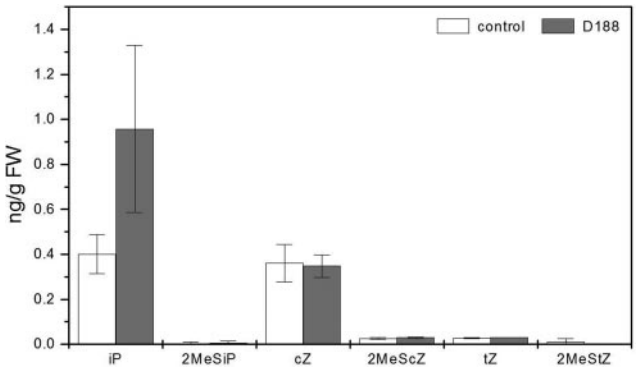

Fig. S6. Cytokinin profiles in mock inoculated (control) tobacco plants and leafy galls induced upon *R. fascians* D188 infection. Measurements were performed 4 weeks post infection. Error bars represent SDs ($n = 3$).

Table S1. Substrate specificity of apoplastic CKX enzymes of *Arabidopsis* in dehydrogenase (ferricyanide, FC) and oxygen (O_2) mode at pH 6.0 given as absolute activities (pkat/mg)

	CKX2		CKX4		CKX6	
Substrate	FC	O_2	FC	O_2	FC	O_2
iP	250	2.7	47.4	0.29	750	6.1
2MeSiP	72	0.5	22.9	0.15	403	1.3
cZ	23	—	11.2	—	262	—
2MeScZ	5	—	4.5	—	130	—
tZ	75	3.0	39.4	0.28	224	3.4
2MeStZ	130	0.4	8.1	0.11	318	4.2

All data represent mean values of at least two biological replicates. Deviations between replicates did not exceed 10%; dashes, oxidative degradation of *cis*-derivatives cannot be determined (see *Materials and Methods*).

Table S2. Substrate specificity of the vacuolar CKX enzymes and CKX5 of *Arabidopsis* in dehydrogenase (FC) and oxygen (O_2) mode at pH 6.0 given as absolute (pkat/mg) and relative activities

	CKX1				CKX3				CKX5			
	FC		O_2		FC		O_2		FC		O_2	
Substrate	pkat/mg	%	pkat/mg	%	pkat/mg	%	pkat/mg	%	pkat/mg	%	pkat/mg	%
iP	2,332	100	8.2	100	466	100	15.7	100	500	100	36	100
2MeSiP	3,597	154	5.8	71	508	109	13.8	88	529	106	15	42
cZ	3,306	142	—	—	0.5	0.1	—	—	191	38	—	—
2MeScZ	3,742	160	—	—	5.5	1.2	—	—	437	87	—	—
tZ	3,178	136	41.6	507	93	20	3.1	20	158	32	40	111
2MeStZ	3,838	165	23.7	289	513	110	5.0	32	482	96	79	219

All data represent mean values of at least 2 biological replicates. Deviations between replicates did not exceed 10%; dashes, oxidative degradation of *cis*-derivatives cannot be determined (see *Materials and Methods*). The sequence of CKX5 suggests an apoplastic localization, although this has not been experimentally proved. The phenotype of plants overexpressing *CKX5* resembles that of plants overexpressing vacuolar *CKX* genes (T.S., unpublished results), and the substrate specificity of CKX5 is more similar to that of the vacuolar CKX enzymes. Therefore, CKX5 was not included in Table 1 and Table S1.

Table S3. Primer sequences used for PCR cloning of the *AtCKX* cDNAs into *Pichia* transformation vectors

Gene	Primer sequence 5′ to 3′ end direction	Restriction site
CKX1	tccccgcggGTTCCAATCATTCTGTTAG	SacII
	acgcgtcgacTTATACAGTTCTAGG	SalI
CKX2	ggaattccatatgATTAAAATTGATTTACCTAAAT	NdeI
	gctctagaTCAAAAGATGTCTTGCCC	XbaI
CKX3	gtccatatgTCACACAACGAATTCGC	NdeI
	cggggtaccCTAACTCGAGTTTATTTTTTGA	Asp718
CKX4	tccccgcggCAGATGAGGGCATTGATG	SacII
	gctctagaTTAATTAAATATGTC	XbaI
CKX5	gtccatatgGTGGGTCTAAACGTG	NdeI
	ggggtaccTCACCATGAAGCCGC	Asp718
CKX6	gctctagaTCATGAGTATGAGACTGCCTTTTG	XbaI
	tccccgcggGCTTCTCTAGCAGCATTTC	SacII