Transcriptome Analysis of *Arabidopsis* Clubroots Indicate a Key Role for Cytokinins in Disease Development

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The clubroot disease of the family *Brassicaceae* is caused by the obligate biotrophic protist *Plasmodiophora brassicae*. Infected roots undergo a developmental switch that results in the formation of aberrant roots (clubs). To investigate host gene expression during the development of the disease, we have used the *Arabidopsis* ATH1 genome array. Two timepoints were chosen, an early timepoint at which the pathogen has colonized the root but has induced only very limited change of host cell and root morphology and a later timepoint at which more than 60% of the host root cells were colonized and root morphology was drastically altered. At both timepoints, more than 1,000 genes were differentially expressed in infected versus control roots. These included genes associated with growth and cell cycle, sugar phosphate metabolism, and defense. The involvement of plant hormones in club development was further supported; genes involved in auxin homeostasis, such as nitrilases and members of the *GH3* family, were upregulated, whereas genes involved in cytokinin homeostasis (cytokinin synthases and cytokinin oxidases/dehydrogenases) were already strongly downregulated at the early timepoint. Cytokinin oxidase/dehydrogenase overexpressing lines were disease resistant, clearly indicating the importance of cytokinin as a key factor in clubroot disease development.

Additional keywords: disease resistance, root gall.

The clubroot disease of the family *Brassicaceae*, caused by the obligate biotroph *Plasmodiophora brassicae*, is one of the most damaging diseases within this plant family and is difficult to control by either chemical or cultural means (Crisp et al. 1989). The growth of clubroot-infected plants is stunted compared with healthy plants, and the root system shows typical gall formation. Cross-sections of infected roots show that the galls are caused by cell elongation and cell division (Kobelt et al. 2000; Mithen and Magrath 1992). The life cycle of the obligate pathogen consists of two phases, the primary phase, which is restricted to root hairs of the infected plant, and the secondary phase, which occurs in the cortex and stele

of hypocotyl and roots (Ingram and Tommerup 1972). During the secondary phase, plasmodia are developing that later sporulate and form resting spores (Braserton 1995; Ludwig-Müller 1999).

The developing clubs form a strong metabolic sink on the host, although under optimal growth conditions, the host may tolerate club growth during early growth phases without reduction of shoot growth (Rausch et al. 1981). It was shown that plant hormones, in particular auxins and cytokinins, are correlated with the development of root galls (Butcher et al. 1974; Dekhuijzen and Overeem 1971). The increase of indole 3-acetic acid (IAA) might be due to the increased synthesis and turnover of putative host auxin precursors indole-3-acetaldoxime, indole-3-methylglucosinolate, and indole-3-acetonitrile in infected roots (Butcher et al. 1984; Grsic et al. 1999; Grsic-Rausch et al. 2000; Rausch et al. 1981, 1983; Searle et al. 1982).

In addition to auxin, increased concentrations of free and bound cytokinins were found (Dekhuijzen 1981; Dekhuijzen and Overeem 1971). Dekhuijzen (1981) also showed that the contents of bound and free cytokinins are different in the host cytoplasm and plasmodia of the pathogen. Müller and Hilgenberg (1986) isolated young secondary plasmodia and showed that they were able to incorporate ¹⁴C-adenine into *trans*-zeatin. It was thus assumed that the increase in cytokinins is at least partly derived from active synthesis of zeatin by plasmodia of *P. brassicae*. Due to the small amount of detectable cytokinin production, it was unclear whether this constituted an important pathogenicity factor (Müller and Hilgenberg 1986).

The morphological description of the disease in *A. thaliana* revealed two overlapping processes in the root cortex during the development of the club. At the beginning of club growth, cell division is the predominant process, whereas cell enlargement dominated the later developmental stages (Kobelt et al. 2000). This correlates with cytokinin production by the vegetative plasmodia of the pathogen during earlier disease stages (Dekhuijzen 1981) and increased levels of auxin during later disease stages, when plasmodia were forming sporangia and resting spores (Grsic-Rausch et al. 2000). Club growth might be an overall result of the ratios of these two classes of hormones during the disease, whereas cytokinin dominated the earlier and auxin the later phase.

To understand disease development on the molecular level, several approaches were followed to isolate genes differentially expressed during gall formation. RNA fingerprinting with arbitrary primed polymerase chain reaction (PCR) (Ito et

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al. 1999), a random priming approach to isolate cDNAs (Schuller and Ludwig-Müller 2002), and subtractive cDNA libraries (Arbeiter et al. 2002; Graf et al. 2004) were used to isolate gene fragments that could be related to the development of a club. However, all these studies revealed a mixture of plant and protist genes, and so far, only one unknown gene from the protist has shown correlation to pathogenesis (Ito et al. 1999).

In order to overcome these limitations in the molecular analysis of clubroot in this study, host gene expression was monitored during the development of the disease (10 and 23 days after inoculation [dai]), using the Arabidopsis ATH1 genome array. The transcriptome analysis has further elucidated the role of cytokinin regulation during clubroot development and has refined the decisive steps in establishing a strong metabolic sink in the root tissue by the pathogen. Moreover, we demonstrate a resistance phenotype to clubroot of cytokinin-deficient *AtCKX*-overexpressing plants.

RESULTS

Gene expression during clubroot development.

Two distinct developmental stages of clubroot disease were analyzed by gene expression profiling. At the early timepoint, no disease symptoms were visible and the pathogen was mostly at the stage of young vegetative plasmodia (Fig. 1B). The cell size was almost comparable to that of control roots (Fig. 1A). The second timepoint was characterized by clearly visible clubs and, in root sections, plasmodia, sporangia, and resting spores of the pathogen could be found (Fig. 1C). Between 1,000 and 2,000 genes were differentially expressed at each timepoint of 14,000 expressed genes at one of the analyzed timepoints in infected or control roots. The complete data set is available at ArrayExpress of the European Bioinformatics Institute, experiment number E-MEXP-254. About 1,500 and 1,900 genes were upregulated at timepoint 1 (TP1) and timepoint 2 (TP2), respectively, whereas approximately 1,400 and 2,000 genes were downregulated at the two timepoints. The same 483 genes were upregulated at TP1 and TP2, and 705 genes were similarly downregulated at the two timepoints (>2 fold change).

Grouping the differentially expressed genes according to their GO annotation on The Arabidopsis Information Resource (TAIR) website revealed few obvious categories. Mitochondrial-associated transcripts were upregulated at TP1 and chloroplast-associated genes at TP2. The latter is due to the fact that infected hypocotyls of older plants, which are found above ground, are green.

Major metabolic pathways (Fig. 2) and transport components (Fig. 3) were analyzed using MAPMAN software (Thimm et al. 2004). The visual display of MAPMAN-generated data organizes genes in contextual groups, which facilitates the discovery of general trends. In the current study, we used MAPMAN and compared control with infected roots for each timepoint to visualize gene expression data for complex processes. Visualization of metabolism resulted in the observation that, at the first timepoint, only a few pathways seem to be differentially regulated (Fig. 2A), including starch and S-metabolism. At the second timepoint (Fig. 2B), the photosynthetic apparatus is dramatically induced (discussed above). In addition, starch, lipids, and secondary metabolism, especially flavonoids, seem to be upregulated. Second, we found many major transport processes differentially regulated between the two timepoints (Fig. 3). At TP1, not many transport processes seem to be regulated at all, while at the second timepoint, several are increased, including sugars, lipids, ions, and other nutrients (N, S, P). This indicates high reserve-accumulation during TP2 in infected roots.

Comparing highly expressed genes at TP1 with those at TP2 in infected roots only supports those findings (Table 1). The genes induced more than 20-fold from infected roots at TP2 included many genes associated with reserve accumulation (e.g., lipid transfer proteins, starch synthesis) but also dehydrins/LEA proteins, which may indicate the stress situation of the infected roots at later timepoints. On the other hand, strongly downregulated genes in infected roots at TP2 compared with TP1 (Table 2) included many defense-related proteins (discussed below).

For more detailed analysis, we have chosen genes most likely involved in i) defense related genes, ii) cell division end elongation, and iii) hormone metabolism and perception (auxins and cytokinins) (Tables 3 and 4).

Consistent with the observation that *P. brassicae* can live inside its host for a long period without affecting vitality, the majority of annotated resistance and defense genes were not differentially expressed in clubs. This has been summarized for biotrophic pathogens as a hallmark in the interaction with plants (Panstruga 2003). Although a few defense genes were highly upregulated, a greater number of defense- and resistance-related genes were downregulated during the interaction. Of 312 genes identified as defense and disease-resistance related only 5% were upregulated at the first timepoint and 7% at the second timepoint. On the contrary, at the first timepoint, about 7% were downregulated and, at the second timepoint, this number doubled to 14%.

Several genes involved in growth and cell cycle control were strongly induced. Several cyclins were upregulated at the first timepoint, and two members of the alpha-expansin gene family were highly induced at the second infection timepoint. During later timepoints of pathogenesis, cell elongation is taking place, whereas cell division can be found mostly at earlier timepoints. Concomitantly, we found that cell cycle genes were upregulated during the earlier timepoint.

Fig. 1. Longitudinal sections of *Arabidopsis* control roots and roots infected with *Plasmodiophora brassicae* at the two timepoints chosen for the microarray experiment. **A**, *Arabidopsis* control roots. **B**, Root cortex at timepoint 1 (10 days after inoculation). **C**, Middle of a club at timepoint 2 (23 days after inoculation). Bars represent 50 μ m. In **B**, typical young plasmodia are visible (YP) surrounding, in some cases, the host nucleus (N), which is also stained by methylene blue and basic fuchsin. In **C**, young plasmodia (YP), large secondary plasmodia (SP), and cells filled with resting spores (RS) can be seen. The plasmodia are more often found in the inner root cortex, whereas resting spores are more frequently found in the outer parts of the root.

Fig. 2. MAPMAN sketch of metabolic pathways comparing up- and downregulated genes from control and infected roots at timepoints 1 (10 days after inoculation [dai]) (upper panel) and 2 (23 dai) (lower panel).

Genes involved in the synthesis or signaling of plant hormones were also differentially regulated (Fig. 4). Gibberellin-related genes were mostly upregulated at the second timepoint during infection, which was also found for genes annotated with functions in brassinosteroid synthesis and signaling. As already shown in Table 1, the number of ABA-related genes was also upregulated at the second infection timepoint. For ethylene-related genes, it was found that a greater number were either up- or downregulated at the second timepoint as compared with the first timepoint. Those downregulated included several genes encoding for proteins involved in ethylene synthesis (ArrayExpress E-MEXP-254). This is in agreement with the finding that a constitutively ethylene-overaccumulating mutant (*eto2*) showed slightly reduced disease index and root fresh weight (data not shown). Salicylic acid-related genes were found to be more downregulated at TP2, whereas genes associated with jasmonate synthesis and signaling were equally up- or downregulated at the two timepoints. However, there was a large overlap between the most highly upregulated genes in clubroots at the early timepoint as compared with jasmonic acid (JA)-inducible genes analyzed by using Genevestigator (Zimmermann et al. 2004), indicating a role for JA during root-gall development (Siemens et al. in press).

The concentrations of auxins and cytokinins have been shown previously to be increased in a root gall after infection with *P. brassicae* (Grsic-Rausch et al. 2000; Müller and Hilgenberg 1986). Transcript levels of genes related to auxin and cytokinin

homeostasis were changed at the first timepoint, indicating a possible role of these hormones in disease development. The auxin-related genes nitrilase 1 and nitrilase 2 (*NIT1* and *NIT2*), auxin-induced *GH3* homologs, and putative auxin transport proteins were predominantly upregulated (>2 fold; Table 3). The enhanced expression of one or more nitrilases in *A. thaliana* during clubroot development confirmed a previous investigation (Grsic-Rausch et al. 2000). *NIT1* and *NIT2* were predominantly expressed in clubroot tissue, with an earlier peak during colonization of *NIT1* and a second peak of *NIT2* during maturation of pathogen spores (Grsic-Rausch et al. 2000). In Table 3, the two nitrilases are grouped together because the respective oligonucleotides on the ATH1 chip are not able to distinguish between the two isoforms. In addition, we found several *Aux/IAA* genes and auxin response factors to be differentially expressed and, although most of the *Aux/IAA* genes were downregulated (Table 3), there was no consistent pattern.

Strikingly, two of seven known putative cytokinin biosynthesis genes as well as cytokinin oxidases/dehydrogenases 1 and 6 (*AtCKX1*, *AtCKX6*) were downregulated (Table 4). In contrast, the expression of gene *AtCKX4* appeared to be upregulated by about twofold, but it was not possible to detect *CKX4* expression by β -glucuronidase (GUS) staining in promoter GUS lines (Fig. 5). The other *AtCKX* genes showed low expression in roots, without any indication for preferable expression in specific cell types or tissues and, also, no response to clubroot infection. The response regulator genes *ARR10* and

Fig. 3. MAPMAN sketch of transport-related genes up- and down-regulated from control and infected roots at timepoints 1 (10 days after inoculation [dai]) (inset) and 2 (23 dai). The arrows denote specifically highly upregulated gene families.

ARR5, which are involved in cytokinin signaling, and the cytokinin receptor gene *CRE1/AHK4* were upregulated by a factor of 4.7, 2.3, and 2.8, respectively, at the first timepoint. A reduction of the degradation capacity for cytokinins by inhibition of specific *CKX* genes could, in turn, result in an increased content of cytokinins. To further test the putative role of cytokinin oxidase/dehydrogenase genes, their expression during disease development was further analyzed.

Expression of cytokinin oxidase/dehydrogenase genes is inhibited during club development.

The expression patterns of *AtCKX1* and *AtCKX6* during pathogenesis were confirmed by qualitative reverse transcription (RT)-PCR and promoter-reporter gene analysis to validate the reduced expression levels estimated in the microarray experiment. For both *AtCKX* genes, a reduction in expression was found by RT-PCR in infected roots compared with controls (Fig. 5), especially at the second timepoint. In particular, *AtCKX1* expression was no longer detectable in infected roots. It has been shown in different publications that there is not a strict linear relationship between signal strength and the amount of transcripts for different genes with hybridization-based technologies, such as Affymetrix chips (Czechowski et al. 2004, 2005; Holland 2002).

Therefore, a second approach was used to verify the down-regulation of *AtCKX* genes during clubroot. Using promoter-reporter gene fusions, *AtCKX1* expression was found in control roots 24 and 37 days after germination (dag) only at root-branching points at the bases of lateral roots. In infected roots, the *AtCKX1* expression appeared to be reduced at early timepoints during disease development due to the reduced amounts of fine roots, and no expression was observed at late disease stages, since lateral roots were basically not present in infected roots (Fig. 6). For *AtCKX4*, the overall expression level was low and could therefore not been detected in roots using GUS reporter lines (Fig. 6), although according to the microarray data, gene expression was slightly upregulated at the second timepoint (Table 4). In contrast, *AtCKX1* is more prominently expressed in specific root tissues, such as root branching points (Fig. 6; magnification), and therefore, it was possible to observe GUS staining.

The *GUS* gene under the control of the *AtCKX6* promoter is strongly expressed throughout the whole root-vascular cylinder in control roots (Werner et al. 2003). In infected roots, an apparent slight reduction of GUS staining was already observed 7 dai, which was further reduced at 14 dai (Fig. 6). When the clubroot symptoms were fully developed (21 and 28 dai), GUS staining was only rarely observed in line *CKX6::GUS*, indicat-

Table 1. Strong upregulation of transcripts from infected roots of plants^a

AGI No.	Upregulation	Description
AT5G59310	663.576923	Lipid transfer protein 4 (LTP4)
AT2G38540	324.467391	Nonspecific lipid transfer protein 1 (LTP1)
AT3G17520	173.923077	Late embryogenesis abundant domain-containing protein
AT5G22460	141.323944	Esterase/lipase/thioesterase family protein
AT1G61800	124.41555	Glucose-6-phosphate/phosphate translocator
AT1G56600	108.659574	Galactinol synthase
AT2G16890	94.9148936	UDP-glucuronosyl/UDP-glucosyl transferase family protein
AT3G59060	89.5128205	Basic helix-loop-helix (bHLH) family protein
AT5G66400	81.0649137	Dehydrin (RAB18)
AT4G36700	71.1186441	Cupin family protein low similarity to preproMP27-MP32 from <i>Cucurbita</i>
AT3G29970	67.787234	Germination protein-related similar to HvB12D
AT2G35300	55.1891892	Late embryogenesis abundant group 1 domain-containing protein
AT1G80130	54.7892977	Expressed protein
AT2G45190	54.2093023	Axial regulator YABBY1
AT1G62510	54.0241935	Protease inhibitor/seed storage/lipid transfer protein (LTP) family protein
AT1G64110	53.772	AAA-type ATPase family protein
AT5G59320	53.2721823	Lipid transfer protein 3 (LTP3)
AT5G20790	52.5616438	Expressed protein predicted protein
AT1G52690	46.0797774	Late embryogenesis abundant protein, putative
AT4G15210	41.3146998	Beta-amylase (BM1)
AT4G02280	40.8377358	Sucrose synthase, putative
AT5G06760	38.9385965	Late embryogenesis abundant group 1 domain-containing protein
AT1G62290	38.518018	Aspartyl protease family protein
AT3G28220	35.4317618	Meprin and TRAF homology domain-containing protein/MATH domain-containing protein similar to ubiquitin-specific protease 12
AT5G25110	33.3690476	CBL-interacting protein kinase 25 (CIPK25)
AT5G25610	32.8583815	Dehydration-responsive protein (RD22)
AT3G50440	31.1242485	Hydrolase, alpha/beta fold family protein similar to ethylene-induced esterase
AT1G72260	30.5280595	Thionin (THI.1)
AT1G01140	30.2142857	CBL-interacting protein kinase 9 (CIPK9)
AT2G33380	29.6440367	Calcium-binding RD20 protein (RD20) induced by abscisic acid during dehydration
AT5G13170	28.2521994	Nodulin MtN3 family protein similar to MtN3; identical to senescence-associated protein (SAG29) mRNA
AT2G21820	26.9281915	Expressed protein
AT5G50360	26.5337423	Expressed protein
AT1G32900	26.080292	Starch synthase
AT1G73010	24.3142984	Expressed protein similar to phosphatase, orphan 1
AT3G21670	23.3426573	Nitrate transporter (NTP3)
AT2G47770	23.3059273	Benzodiazepine receptor-related
AT2G45130	22.6820276	SPX (SYG1/Pho81/XPR1) domain-containing protein weak similarity to NUC-2
AT1G16850	21.8180495	Expressed protein
AT3G17790;	21.63	[AT3G17790, acid phosphatase type 5 (ACP5)
AT3G17800		[AT3G17800, expressed protein]
AT1G22160	20.983209	Senescence-associated protein-related; similar to senescence-associated protein SAG102
AT2G18050	20.4244373	Histone H1-3 (HIS1-3)

^a Upregulation >20 fold from infected roots of plants at 23 vs. 10 days postinfection.

ing a strong downregulation of the gene. In cross-sections of root galls, only occasionally stained cells were observed in *CKX6::GUS* plants, whereas in control plants the central cylinder showed staining (Fig. 7). To exclude the possibility that we have missed subtle changes in other *CKX* genes, we have investigated all available *CKX::GUS* lines (Werner et al. 2003). Only the lines *CKX1::GUS* and *CKX6::GUS* gave staining patterns in control and infected roots, whereas all other lines did not show any GUS staining (data not shown). This is in agreement with the observations that *AtCKX2*, *AtCKX3*, and *AtCKX5* were not differentially expressed in the microarray experiment (data not shown).

Since, at this timepoint, a large proportion of cells might be affected by the pathogen so that gene function is already generally disturbed, we compared GUS activity in lines harboring a cytokinin-responsive promoter (*ARR5::GUS*) and an auxin-responsive promoter (*DR5::GUS*). After infection, these lines

showed clearly an increase in GUS activity in infected versus control roots, indicating an increased responsiveness to cytokinin and auxin as well as an increased content of the two hormones in infected roots as compared with controls during early and late infection, respectively (Fig. 6). Cross-sections showed an increase in the number of GUS-stained cells in line *ARR5::GUS* (Fig 7). This also demonstrated that the transcription and translation machinery of infected cells at this time-point was still intact.

Enhanced degradation of cytokinin causes pathogen resistance.

The clubroot disease symptoms of different transgenic lines overproducing *AtCKX* genes (*35S::AtCKX1*, *35S::AtCKX2*, *35S::AtCKX3*) were compared with the susceptible ecotype Col-0 (Table 5). The lines used in this study were described in detail by Werner and associates (2003), and lines with high ex-

Table 2. Strong downregulation of transcripts from infected roots^a

AGI No.	Downregulation	Description
AT2G01520	0.00494234	Major latex protein-related; Pathogenesis-related protein Bet v I family
AT4G11190	0.00535777	Disease resistance-responsive family protein
AT4G33720	0.00715789	Pathogenesis-related protein (PR-1)
AT4G13290	0.00742804	Cytochrome P450 71A19
AT3G01190	0.00819912	Peroxidase 27 (PER27) (PRXR7)
AT4G11310	0.00830424	Cysteine proteinase, similar RD21A (thiol protease)
AT1G14120	0.01290265	2-Oxoglutarate-dependent dioxygenase, putative similar to adventitious rooting related oxygenase ARRO-1, 2OG-Fe(II) oxygenase superfamily
AT2G05510	0.01421622	Glycine-rich protein
AT1G12010	0.01580135	1-Aminocyclopropane-1-carboxylate oxidase
AT4G15390	0.01602223	Transferase family protein similar to alcohol acyltransferase
AT4G11290	0.01701149	Peroxidase, putative identical to peroxidase ATP19a
AT3G26460	0.02437474	Major latex protein-related/MLP-related low similarity to major latex protein
AT1G35910	0.02508711	Trehalose-6-phosphate phosphatase
AT1G13420	0.02543413	Sulfotransferase family protein similar to steroid sulfotransferase 1
AT3G17690	0.02552553	Cyclic nucleotide-binding transporter 2/CNBT2
AT4G22212	0.02697634	Expressed protein
AT1G49860	0.02754846	Glutathione S-transferase
AT5G53250	0.02897083	Arabinogalactan-protein, putative (AGP22)
AT1G77380	0.02913843	Amino acid carrier, putative
AT3G49620	0.02923602	2-Oxoacid-dependent oxidase, putative (DIN11)
AT4G30140	0.03103892	GDSL-motif lipase/hydrolase family protein
AT1G66280;	0.03331564	[AT1G66280, glycosyl hydrolase family 1 protein ;
AT1G66270		[AT1G66270, beta-glucosidase (PSR3.2), similar to thioglucoside glucohydrolase
AT5G62550	0.03354065	Expressed protein
AT1G70830;	0.0336562	[AT1G70830, Bet v I allergen family protein similar to Csf-2;
AT1G70850		[AT1G70850, Bet v I allergen family protein similar to Csf-2
AT1G32970	0.03367232	Subtilase family protein
AT1G02900	0.03454384	Rapid alkalization factor (RALF) family protein
AT4G29690	0.03469415	Type I phosphodiesterase/nucleotide pyrophosphatase family protein
AT1G30760	0.03512088	FAD-binding domain-containing protein similar to SP1P30986 reticuline oxidase precursor (Berberine-bridge-forming enzyme) (BBE)
AT5G63590	0.03700167	Flavonol synthase 1 (EC 1.14.11.-) (FLS 1))
AT5G59090	0.03835467	Subtilase family protein contains similarity to prepro-cucumis
AT5G26280	0.03989849	[AT5G26280, meprin and TRAF homology domain-containing protein/MATH domain-containing protein low similarity to ubiquitin-specific protease 12
AT5G26260		[AT5G26260, meprin and TRAF homology domain-containing protein
AT1G16530	0.0400165	LOB domain protein 3/lateral organ boundaries domain protein 3 (LBD3); identical to ASYMMETRIC LEAVES2-like protein 9
AT3G48360	0.04030483	Speckle-type POZ protein-related
AT4G29270	0.0418041	Acid phosphatase class B family protein
AT1G31710	0.04204532	Copper amine oxidase
AT5G45700	0.04235808	NLI interacting factor (NIF) family protein
AT5G35940	0.04326599	Jacalin lectin family protein similar to myrosinase-binding protein homolog
AT4G24340	0.04386844	Phosphorylase family protein
AT4G15100	0.04434949	Serine carboxypeptidase S10 family protein
AT1G45130	0.04495557	Beta-galactosidase, putative/lactase
AT4G04830	0.04546711	Methionine sulfoxide reductase domain-containing protein/SeIR domain-containing protein low similarity to pilin-like transcription factor
AT5G63600	0.04591183	Flavonol synthase
AT4G15700	0.04725139	Glutaredoxin family protein (thioltransferase)
AT3G23890	0.04916653	DNA topoisomerase, ATP-hydrolyzing/DNA topoisomerase II/DNA gyrase (TOP2)

^a Downregulation <0.05 fold from infected roots of plants 23 vs. 10 days postinfection.

pression levels of the transgene were used (data not shown). Although the microarray data suggested a specific role for *AtCKX1* and *AtCKX6* only, we reasoned that overexpression of any *CKX* gene or protein would result in the degradation of cytokinins and thus in a lower cytokinin content. The *CKX* genes used in this experiment showed different cellular localization (Werner et al. 2003), making those interesting tools to investigate the effect of the cellular localization of cytokinins for clubroot development.

Resistance to clubroot is defined by the reduction of root weight that is correlated to gall size and reduction of resting spores and a lower disease index (DI) (Siemens et al. 2002). Ecotype Col-0 showed the typical disease indices (DI > 90) and root indices (roots of infected plants/roots of not infected plants (R_i/R_{ni}) > 2) of susceptible lines (Siemens et al. 2002). The plants showed clubs that were extended over the whole hypocotyl and also, in part, into the rosette ground (Fig. 8). In contrast, the overproduction of *AtCKX1* (35S::*AtCKX1*) and *AtCKX3* (35S::*AtCKX3*) caused an apparent resistance to *P. brassicae* isolate 'e_H', but the overexpression of *AtCKX2* (35S::*AtCKX2*) had only very limited influence on clubroot

disease. Severe swellings were rarely observed after inoculation in the roots of line 35S::*AtCKX1*, resulting in low disease indices. The plants appeared similar to noninoculated control plants (Fig. 8). After infection the main root of 35S::*AtCKX1* often showed reduced growth and undulation. The pathogen was sometimes able to induce a small hypocotyl gall (Fig. 8). In line 35S::*AtCKX3*, scoring of clubroot symptoms always revealed a significant difference from those of Columbia; clubs were induced less frequently and infected roots showed greater variation of disease symptoms (Table 5). In contrast, line 35S::*AtCKX2* showed only slight differences as compared with Columbia, which were, in most experimental series, not significant even with reduced inoculation pressure (data not shown). However, although the root indices indicate susceptibility of this line, the mean root index was significantly lower than the index of Col-0. In summary, the root indices of the investigated transgenic lines gave indications of induced tolerance by overexpression of *CKX* genes.

Three additional isolates were used to test clubroot tolerance of *CKX* overexpressors. Lines 35S::*AtCKX1* and 35S::*AtCKX3*

Table 3. Down- (<0.5×) and upregulated genes (>2×) related to auxin synthesis, metabolism, transport, and gene regulation^a

AGI No.	Gene annotation	Expression level ^b				Mean ratio of induction	
		Timepoint 1		Timepoint 2			
		Control (24 dag)	Inoc. (10 dpi)	Control (37 dag)	Inoc. (23 dpi)	Timepoint 1 ^c	Timepoint 2 ^d
At2g23170	Auxin-responsive <i>GH3</i> family protein	1.8	162.5	1.7	229.2	92.0 ± 61.5	137.5 ± 40.5
At1g72540	Dual specificity cytosolic kinase	0.8	38.1	6.1	124.9	47.6 ± 8.2	20.5 ± 13.1
At5g13360	Auxin-responsive <i>GH3</i> family protein	19.8	67.1	16.4	164.2	3.4 ± 0.9	10.0 ± 3.8
At4g34760	Auxin-induced protein X15	19.6	65.6	43.1	64.1	3.4 ± 1.2	1.5 ± 19.8
At1g77690	Amino acid permease, similar to <i>AUX1</i>	92.7	290.9	225.7	27.6	3.1 ± 0.5	0.1 ± 0.3
At1g73590	Auxin efflux carrier protein, putative (<i>PIN1</i>)	56.2	176.5	123.8	76.0	3.1 ± 0.5	0.6 ± 0.3
At2g32410	Auxin-resistance protein (<i>AXR1</i>)	23.7	61.2	35.5	28.1	2.6 ± 0.4	0.8 ± 0.1
At4g34750	Auxin-responsive protein	23.4	58.2	38.2	21.3	2.5 ± 0.6	0.6 ± 20.2
At1g28130	Auxin-responsive <i>GH3</i> family	37.2	91.9	43.7	70.9	2.5 ± 0.4	1.6 ± 19.7
At2g33860	Auxin-responsive factor (<i>ARF3</i>)	70.7	161.7	99.7	245.8	2.3 ± 0.3	2.5 ± 1.0
At5g37020	Auxin-responsive factor (<i>ARF8</i>)	84.7	178.4	134.8	206.3	2.1 ± 0.3	1.5 ± 0.4
At1g70940	Auxin transport protein, putative (<i>PIN3</i>)	83.2	173.5	138.7	200.9	2.1 ± 0.4	1.4 ± 0.5
At4g02980	Auxin-binding protein 1 (<i>ABP1</i>)	73.3	150.8	90.2	166.7	2.1 ± 0.2	1.8 ± 0.4
At2g14960	Auxin-responsive <i>GH3</i> family	21.4	43.4	14.2	17.1	2.0 ± 1.5	1.2 ± 0.6
At1g48630	Activated protein kinase C receptor	514.7	1021.0	583.5	262.7	2.0 ± 0.2	0.5 ± 0.2
At3g12830	Auxin-responsive family protein	84.6	154.4	35.9	137.5	1.8 ± 0.4	3.8 ± 2.9
At3g44310;	AT3g44310. nitrilase 1 (<i>NIT1</i>)						
At3g44300	AT3g44300. nitrilase 2 (<i>NIT2</i>) *	410.7	632.1	348.4	2565.7	1.5 ± 0.3	7.4 ± 2.8
At5g01990	Auxin efflux carrier family protein	46.3	65.1	37.1	178.5	1.4 ± 0.1	4.8 ± 1.6
At4g37390;	AT4g37390. auxin-responsive <i>GH3</i> family						
At1g59500	AT1g59500. auxin-responsive <i>GH3</i> family protein	27.8	36.1	2.3	70.9	1.3 ± 0.5	31.3 ± 9.1
At1g77850	Transcriptional factor B3 family protein	34.9	43.0	51.7	22.6	1.2 ± 0.5	0.4 ± 0.3
At4g38840	Auxin-responsive protein	16.6	19.5	18.1	38.4	1.2 ± 0.3	2.1 ± 19.5
At4g14550	Auxin-responsive protein (<i>IAA14</i>)	21.4	24.3	15.3	76.1	1.1 ± 0.2	5.0 ± 1.9
At4g03400	Auxin-responsive <i>GH3</i> family	17.1	18.9	13.3	97.4	1.1 ± 0.3	7.3 ± 2.9
At5g35580	Protein kinase	243.9	250.2	399.6	41.5	1.0 ± 0.3	0.1 ± 0.4
At1g19850	Transcription factor MONOPTEROS (<i>IAA24</i>)	183.8	181.6	225.0	83.9	1.0 ± 0.0	0.4 ± 0.3
At4g00880	Auxin-responsive family protein	154.1	141.7	355.0	52.3	0.9 ± 0.2	0.1 ± 0.3
At2g21050	Amino acid permease, similar to <i>AUX1</i>	93.1	87.6	246.8	54.7	0.9 ± 0.1	0.2 ± 0.3
At3g23050	Auxin-responsive protein/indoleacetic acid-induced protein 7 (<i>IAA7</i>)	45.9	37.7	26.7	135.6	0.8 ± 0.2	5.1 ± 1.7
At2g22670	Auxin-responsive protein/indoleacetic acid-induced protein 8 (<i>IAA8</i>)	1319.9	1038.7	1503.3	641.4	0.8 ± 0.1	0.4 ± 0.2
At4g17280	Auxin-responsive family protein	46.1	36.1	51.8	16.6	0.8 ± 0.1	0.3 ± 0.3
At4g27260	Auxin-responsive <i>GH3</i> family protein	66.1	48.8	34.2	190.0	0.7 ± 0.2	5.6 ± 2.0

(Continued on following page)

^a Differentially influenced by *Plasmodiophora brassicae* in infected and not inoculated plants of ecotype Col-0 at two timepoints after inoculation. All values are normalized to the expression level of not inoculated Col-0 plants at the corresponding timepoint. Mean values of expression levels of three independent experiments and the mean values of induction and standard deviations are given. **NIT1* and *NIT2* can not be distinguished on the microarray by the oligonucleotides used.

^b dag = days after germination; dpi = days postinfection.

^c 10 dpi/24 dag ± SD (standard deviation).

^d 23 dpi/37 dag ± SD.

exhibited significant tolerance to all tested isolates (Table 5). On the contrary, the scoring and the root index of the line 35S::CKX2 showed an induced tolerance, significantly only to isolate 'k₁' but not to isolates 'ICK' and 'e₂' (Table 5).

To further analyze the effect of *AtCKX* overexpression in root galls with respect to endogenous cytokinins, we have conducted an experiment using a homozygous line of the gene constructs 35S::*AtCKX1* and *ARR5::GUS* to demonstrate the downregulation of endogenous cytokinins in infected roots. In comparison with the *ARR5::GUS* line, this line showed a strongly reduced expression pattern of GUS in control roots as well as in infected roots, indicating a reduced cytokinin level in the roots (Fig. 9). As described for 9-day-old young seedlings (Werner et al. 2003), the detectable GUS expression in roots of this line was also restricted to root meristems and to the central cylinder in 24- and 35-day-old plants and the corresponding plants 14 and 21 dai (Fig. 9).

DISCUSSION

The clubroot disease of the family *Brassicaceae* is an important and damaging disease within this plant family. To obtain

insight in the pathogenesis process, we have investigated the transcriptome of *Arabidopsis thaliana* after infection with *Plasmodiophora brassicae* at two developmentally distinct timepoints. Several observations were made that relate to the general biology of this interaction.

i) Genes involved in cell division and expansion, such as cell cycle genes and expansins, are upregulated, especially at the first analyzed timepoint of the disease. This is in accordance with the morphology of a club and the timing of cell division and cell enlargement during the development of a club (Kobelt et al. 2000).

ii) Although club cells are heavily infected with *P. brassicae*, there are surprisingly few resistance responses observed. The microarray data indicate that most known defense- or resistance-related genes are either not differentially expressed or downregulated. Ito and associates (1996) described proteins with homology to pathogenesis-related (PR) protein group 5 that were enhanced during a susceptible response. A study on PR-proteins during pathogenesis showed no clear correlation between the activity of peroxidase and chitinase isoenzymes in roots of a susceptible and a resistant cultivar of Chinese cabbage and resistance or tolerance to clubroot (Ludwig-Müller et

Table 3. Continued from preceding page

AGI No.	Gene annotation	Expression level ^b				Mean ratio of induction	
		Timepoint 1		Timepoint 2		Timepoint 1 ^c	Timepoint 2 ^d
		Control (24 dag)	Inoc. (10 dpi)	Control (37 dag)	Inoc. (23 dpi)		
At1g51780	Indole 3-acetic acid (IAA)-amino acid hydrolase 5/auxin conjugate hydrolase (<i>ILL5</i>)	236.2	173.2	288.0	104.1	0.7 ± 0.0	0.4 ± 0.3
At4g36110	Auxin-responsive protein	29.1	21.2	15.4	38.3	0.7 ± 0.2	2.5 ± 0.8
At2g33310	Auxin-responsive protein/indoleacetic acid-induced protein 13 (<i>IAA13</i>)	409.8	277.3	669.0	323.6	0.7 ± 0.1	0.5 ± 0.2
At5g20730	Auxin-responsive factor (<i>ARF7</i>)	170.3	108.4	178.7	80.4	0.6 ± 0.1	0.4 ± 0.2
At4g27450	Expressed protein similar to auxin down-regulated protein <i>ARG10</i>	690.5	426.3	385.0	1969.9	0.6 ± 0.2	5.1 ± 2.5
At1g04250	Auxin-responsive protein/indoleacetic acid-induced protein 17 (<i>IAA17</i>)	441.4	249.3	466.9	221.7	0.6 ± 0.1	0.5 ± 0.2
At3g16500	Auxin-responsive Aux/IAA family protein	254.9	139.5	284.4	123.6	0.5 ± 0.1	0.4 ± 0.2
At2g04850	Auxin-responsive protein-related	57.5	29.2	133.1	6.9	0.5 ± 0.2	0.1 ± 0.0
At2g46370	Auxin-responsive <i>GH3</i> family protein	357.1	179.7	313.7	142.0	0.5 ± 0.2	0.5 ± 0.2
At1g56220	Dormancy/auxin associated family protein	551.7	264.1	205.9	705.6	0.5 ± 0.1	3.4 ± 1.7
At2g46690	Auxin-responsive family protein	533.7	251.1	941.6	76.4	0.5 ± 0.1	0.1 ± 0.4
At5g53590	Auxin-responsive family protein	1787.8	813.1	1497.7	405.9	0.5 ± 0.0	0.3 ± 0.3
At4g12980	Auxin-responsive protein	133.1	60.0	179.9	36.2	0.5 ± 0.1	0.2 ± 0.3
At3g15460;	AT3G15460. brix domain-containing protein						
At3g15450	AT3g15450. expressed protein	2935.7	1246.0	1650.0	472.0	0.4 ± 0.1	0.3 ± 0.3
At1g75590	Auxin-responsive family protein	29.9	11.8	23.6	19.1	0.4 ± 0.1	0.8 ± 0.5
At1g04100	Auxin-responsive protein/indoleacetic acid-induced protein 10 (<i>IAA10</i>)	160.7	56.0	115.2	69.3	0.3 ± 0.0	0.6 ± 0.5
At5g25890	Auxin-responsive protein/indoleacetic acid-induced protein 28 (<i>IAA28</i>)	319.6	101.7	288.6	80.7	0.3 ± 0.1	0.3 ± 0.3
At5g20820	Auxin-responsive protein-related	70.4	21.7	82.1	48.5	0.3 ± 0.1	0.6 ± 0.6
At1g04240	Auxin-responsive protein/indoleacetic acid-induced protein 3 (<i>IAA3</i>)	131.2	37.8	130.1	39.6	0.3 ± 0.1	0.3 ± 0.3
At3g23030	Auxin-responsive protein/indoleacetic acid-induced protein 2 (<i>IAA2</i>)	529.6	150.5	587.9	218.7	0.3 ± 0.0	0.4 ± 0.3
At4g29080	Auxin-responsive Aux/IAA family protein	959.6	262.1	1469.5	99.1	0.3 ± 0.0	0.1 ± 0.4
At1g51950	Auxin-responsive protein/indoleacetic acid-induced protein 18 (<i>IAA18</i>)	441.3	117.9	675.5	137.8	0.3 ± 0.1	0.2 ± 0.3
At1g15580	Auxin-responsive protein/indoleacetic acid-induced protein 5 (<i>IAA5</i>)	32.2	8.1	69.8	4.1	0.3 ± 0.2	0.1 ± 0.1
At3g47620	TCP family transcription factor	475.2	110.5	364.4	374.1	0.2 ± 0.0	1.0 ± 0.2
At5g19140	Auxin/aluminum-responsive protein	898.3	196.7	875.7	925.6	0.2 ± 0.1	1.1 ± 0.1
At4g22620	Auxin-responsive family protein	172.3	35.7	180.7	41.0	0.2 ± 0.1	0.2 ± 0.3
At3g15540	Auxin-responsive protein/indoleacetic acid-induced protein 19 (<i>IAA19</i>)	276.2	56.2	330.8	56.3	0.2 ± 0.1	0.2 ± 0.3
At3g04730	Auxin-responsive protein/indoleacetic acid-induced protein 16 (<i>IAA16</i>)	765.4	98.8	501.8	188.8	0.1 ± 0.0	0.4 ± 0.3
At3g60690	Auxin-responsive family protein	673.5	86.7	190.5	49.5	0.1 ± 0.0	0.3 ± 0.5
At2g45210	Auxin-responsive protein	314.1	36.0	58.7	62.4	0.1 ± 0.0	1.1 ± 0.6

al. 1994). However, in the susceptible interaction, a novel chitinase and peroxidase isoenzyme was induced (Ludwig-Müller et al. 1994). Since a few genes are also upregulated, it can be speculated that there is an initial equilibrium between pathogen attack and host plant defense that is later shifted in favor to pathogen. It has been demonstrated in incompatible interactions in *Arabidopsis* that, during this stage of secondary colonization of the root gall, resistance mechanisms (such as hypersensitive response) to clubroot so far described are functional (Fuchs and Sacristán 1996; Kobelt et al. 2000).

iii) The balance between cytokinin and auxin may be of crucial importance. An involvement of plant hormones as pathogenicity factors is strongly indicated by the correlation of clubroot disease symptoms with an increase of auxin (Butcher et al. 1974; Ludwig-Müller et al. 1993, 1996) and cytokinin (Dekhuijzen and Overeem 1971), resulting in increased cell division and cell elongation. While it was previously shown that the host auxin metabolism is redirected towards the synthesis of more auxin via the nitrilase pathway in *Arabidopsis* (Grsic-Rausch et al. 2000), it was speculated that the increase in cytokinins is due to the formation of zeatin by the pathogen itself (Dekhuijzen 1981; Müller and Hilgenberg 1986). Concomitant measurement of auxin and cytokinin in young root galls of Chinese cabbage led to the hypothesis that a very early increase in IAA and IAA-conjugate synthesis during primary infection is followed by changes in the cytokinin pool by which *P. brassicae* creates its own sink (Devos et al. 2005). The induction of auxin correlates with an increase in seedling growth and xyloglucan endotransferase/hydrolase leading to cell elongation (Devos et al. 2005).

In the microarray experiment, it was found that nitrilases 1 and 2, which could not be separated by the oligonucleotides on the ATH1 array, were induced during club development, especially at the second timepoint in our investigation. This is in accordance with the increase in endogenous IAA content in infected *Arabidopsis* plants (Grsic-Rausch et al. 2000; Ludwig-Müller et al. 1999) as well as the increase in GUS-staining of

the *DR5::GUS* line (Fig. 5). *NIT1* and *NIT2* are the nitrilase homologs predominantly expressed in clubroot tissue, and nitrilase was mainly localized in infected cells containing sporulating plasmodia (Grsic-Rausch et al. 2000). A nitrilase 1 mutant (*nit1*) was found to have reduced root-gall size and lower free IAA content in clubs (Grsic-Rausch et al. 2000; Neuhaus et al. 2000), underpinning the relevance of enhanced auxin synthesis for pathogenicity. In addition, the upregulation of several members of the *GH3* gene family of *Arabidopsis* was observed. Several members of this family, including the *GH3* genes upregulated during clubroot, are able to synthesize IAA amino-acid conjugates via adenylation (Staswick et al. 2002, 2005). Therefore, we hypothesize that not only the level of free IAA but also the total auxin pool is important for gall development.

On the contrary, most of the auxin-inducible *Aux/IAA* genes, with the exception of two members, were either downregulated (Table 3) or not regulated at all. Mutants in auxin transport and auxin response did not show clear effects concerning a higher tolerance against clubroot (Siemens et al. 2002). Genes connected to signal transduction, such as the putative extracellular auxin receptor *ABPI* (Chen et al. 2001a and b; Steffens et al. 2001) and the intracellular auxin receptor *TIR1* (Dharmasiri et al. 2005; Kepinski and Leyser 2005), were not differentially regulated according to our criteria of twofold induction or decrease during club development. *AXR1*, which is part of the auxin-induced *Aux/IAA* degradation machinery via the 26S proteasome (Leyser et al. 1993), was also not differentially expressed. These findings indicate that the integration of the auxin signal must be via other pathways. Maybe the downregulation of *Aux/IAA* repressors of auxin-induced gene expression during pathogenesis is enough to increase the intracellular auxin response.

The microarray analysis has yielded additional indications that cytokinin plays an essential role during clubroot disease. Several genes coding for cytokinin signaling proteins and cytokinin metabolic enzymes showed altered expression levels.

Table 4. Down- (<0.5×) and upregulated genes (>2×) related to cytokinin metabolism and signaling^a

AGI No.	Gene annotation	Expression level ^b				Mean ratio of induction	
		Timepoint 1		Timepoint 2			
		Control	Inoc.	Control	Inoc.	Timepoint 1 ^c	Timepoint 2 ^d
		(24 dag)	(10 dpi)	(37 dag)	(23 dpi)		
At4g31920	Two-component response regulator protein (<i>ARR10</i>)	28.9	136.5	54.3	87.4	4.7 ± 0.5	1.6 ± 0.5
At2g01830	Histidine kinase (<i>CRE1/AHK4</i>)	71.7	202.5	183.4	143.5	2.8 ± 0.3	0.8 ± 0.3
At3g48100	Response regulator 5 (<i>ARR5</i>)	75.3	175.3	295.4	94.2	2.3 ± 0.6	0.3 ± 0.3
At1g10470	Response regulator 4 (<i>ARR4</i>)	283.0	340.1	382.5	120.5	1.2 ± 0.1	0.3 ± 0.3
At1g74890	Response regulator 15 (<i>ARR15</i>)	23.6	25.5	34.3	15.1	1.1 ± 0.2	0.4 ± 0.2
At3g55950	Protein kinase family	234.5	251.6	292.0	95.8	1.1 ± 0.2	0.3 ± 0.3
At4g29740	Cytokinin oxidase (<i>CKX4</i>)	40.6	42.4	36.5	73.9	1.0 ± 0.1	2.0 ± 0.5
At1g28230	Purine permease (<i>PUP1</i>)	39.6	32.7	29.9	103.4	0.8 ± 0.2	3.5 ± 1.8
At4g16420	Transcriptional adaptor (<i>ADA2b</i>)	93.4	73.0	84.0	169.3	0.8 ± 0.2	2.0 ± 0.4
At3g19160	Cytokinin synthase (<i>IPT8</i>)	22.1	12.1	10.4	35.6	0.5 ± 0.1	3.4 ± 2.6
At3g57040	Response regulator 9 (<i>ARR9</i>)	79.2	42.2	70.6	25.8	0.5 ± 0.1	0.4 ± 0.3
At5g62920	Response regulator 6 (<i>ARR6</i>)	42.6	19.4	72.6	16.9	0.5 ± 0.1	0.2 ± 0.2
At4g16110	Response regulator (similar to <i>ARR2</i>)	85.8	38.2	43.8	37.6	0.4 ± 0.1	0.9 ± 0.2
At1g67710	Response regulator (<i>ARR11</i>)	53.3	20.3	53.3	3.6	0.4 ± 0.1	0.1 ± 0.1
At2g41510	Cytokinin oxidase (<i>CKX 1</i>)	36.7	13.8	42.8	10.7	0.4 ± 0.1	0.2 ± 0.2
At1g19050	Response regulator 7 (<i>ARR7</i>)	611.2	160.8	524.2	141.7	0.3 ± 0.1	0.3 ± 0.3
At3g63110	Cytokinin synthase (<i>IPT3</i>)	194.4	45.5	260.3	24.0	0.2 ± 0.1	0.1 ± 0.4
At5g05440	Cytokinin-specific binding protein	1171.6	259.4	979.9	280.5	0.2 ± 0.1	0.3 ± 0.3
At3g63440	Cytokinin oxidase (<i>CKX6</i>)	364.6	36.5	336.6	9.4	0.1 ± 0.1	0.1 ± 0.1

^a Influenced by *Plasmodiophora brassicae* in infected and not inoculated plants of ecotype Col-0 at two timepoint after inoculation. All values are normalized to the expression level of not inoculated Col-0 plants at the corresponding timepoint. Mean values of expression levels of three independent experiments and the mean values of induction and standard deviations are given.

^b dag = days after germination; dpi = days postinfection.

^c (10 dpi/24 dag) ± SD (standard deviation).

^d (23dpi/37 dag) ± SD.

Remarkably, one cytokinin receptor gene (*CRE1/AHK4*) was significantly upregulated, which could cause an increased sensitivity of the tissue toward cytokinin. Consistently, a higher expression level of the *ARR5* gene, a cytokinin-sensitive reporter gene, was found. In spite of cytokinin production by the pathogen, cytokinin-regulated genes, with the exception of *ARR5*, were not generally upregulated. It could be that the transcript levels of primary cytokinin response genes are not enhanced in response to long-lasting changes in cytokinin concentrations or that the response is tissue-specific and does not occur in clubs.

Moreover, the microarray analyses pointed to *AtCKX* genes as possible regulatory targets of the pathogen. The expression of two genes encoding cytokinin oxidases/dehydrogenases (*AtCKX1* and *AtCKX6*), responsible for cytokinin degradation, was reduced. A third gene (*AtCKX4*) was slightly upregulated at the second timepoint of infection, but the expression level of this gene was near the detection limit of the method and specific expression was not detectable in experiments using promoter-GUS lines (Fig. 6). Although, according to the microarray data, *AtCKX1* is also expressed at low levels, expression is focused to certain root tissues, such as root-branching points, and is therefore visible after GUS-staining of respective lines. Reduction of *AtCKX1* expression in *P. brassicae*-induced root galls might be due to the fact that lateral root formation was reduced. The downregulation of the cytokinin degradation capacity within colonized cells in combination with cytokinin production inside these cells by the pathogen could eventually turn colonized cells into a strongly dividing tissue by small amounts of pathogen-derived cytokinin.

Since whole roots were used, it might be possible that more subtle effects in specific areas of the root were not detected. The relative amounts of root tips in the overall tissue samples will clearly have an effect on the microarray data obtained. This is a general dilemma of this type of study. When the symp-

toms caused by infection lead to a pronounced change in organ development, it may be difficult to identify cause and effect.

Two root-specific cytokinin oxidases, *AtCKX1* and *AtCKX6*, are downregulated, which could be part of the strategy of the pathogen to enhance the effects of cytokinins in plasmodia-containing cells. The overproduction of *AtCKX1* and *AtCKX3* in the roots of *A. thaliana* induces an apparent resistance phenotype to *P. brassicae*, supporting the importance of the downregulation of cytokinin degradation during infection. In contrast, the overexpression of *AtCKX2* has only a limited effect on clubroot disease. Clearly, data have been previously presented that demonstrate a decrease of the cytokinin content on *AtCKX*-overexpressing transgenic plants in two different ways: i) by measuring the endogenous content and ii) by analyzing a cytokinin-sensitive reporter gene in the mutant background (Werner et al. 2003). The conclusion of a reduced cytokinin content in these plants is fully supported by additional analyses of gene expression by microarrays (Brenner et al. 2005). A reduction of the endogenous cytokinin content has been demonstrated in different tissues and in different developmental stages.

The subcellular compartmentation of different *AtCKX* proteins in *Arabidopsis* may be responsible for the different phenotypes seen in the different *AtCKX*-overexpressing lines. Enzymes *AtCKX1* and *AtCKX3* were localized in the vacuole, whereas *AtCKX2* is presumably localized in the apoplast of root cells, indicating an important role for subcellular compartmentation in cytokinin function (Werner et al. 2003). Schmülling and associates (2003) proposed that each cell during cell division produces its own cytokinin for its own exclusive use and that extracellular *AtCKX* may serve as protectant from cytokinins derived from neighboring cells, preserving the cytokinin

Fig. 4. Changes in hormone-related genes according to the MAPMAN classification. The number below the bars indicate total genes annotated in this category in MAPMAN. CK = cytokinin; GA = gibberellin; BR = brassinosteroid; ABA = abscisic acid; SA = salicylic acid; JA = jasmonic acid.

Fig. 5. Comparison of expression of *AtCKX1* and *AtCKX6* by reverse transcription-polymerase chain reaction in control (24 and 37 days after germination [dag]) and inoculated (10 and 23 days after inoculation [dai]) roots at early (TP1) and late (TP2) infection timepoints. Similar expression levels in all samples were shown by the amplification of actin 2. The inoculated roots are from plants of the same age as the control roots.

autonomy of each single cell. Vacuolar localization of *AtCKX* might create a stronger intracellular sink for cytokinins. In accordance with this hypothesis, *P. brassicae* induces the root gall presumably from the inside of pericycle cells (Kobelt et al 2000). Therefore, the overexpression of intracellularly localized *AtCKX* proteins may be more effective than the overexpression of the extracellular *AtCKX2*.

In addition to the downregulation of *AtCKX*, several components of cytokinin signaling, such as *AHK4/CRE1* and *ARR5*, showed significantly altered expression patterns. This might, in turn, control downstream responses to cytokinin, such as alterations in the nutrient status and sink of the infected tissue.

In accordance with the hypothesis that the combined effects of auxin and cytokinin are causally involved in the pathogenesis of clubroot disease, microarray data and overexpression lines provided evidence that cytokinin is a crucial factor of gall development. The relevance of cytokinin for pathogenicity is highlighted by the resistance of *AtCKX*-overexpressing plants to *P. brassicae*. This finding might constitute a novel mechanism of disease resistance against *P. brassicae*. The limited cytokinin production rate of the pathogen may be compensated by downregulation of the cytokinin degradation capacity of the host cells. During further development of a club, the upregulation of nitrilases and the auxin-inducible *GH3* homologs point to the

crucial role of auxin homeostasis. Despite the importance of both phytohormones for club development, *P. brassicae* seems to be able to regulate a high number of other genes by a yet-unknown mechanism during the early movement to the vascular cylinder and the colonization of the root cortex.

Fig. 6. Roots of the promoter::GUS lines for cytokinin oxidases *CKX1::GUS*, *CKX4::GUS*, and *CKX6::GUS* differentially regulated according to the microarray experiment and, for comparison, cytokinin- (*ARR5::GUS*) and auxin (*DR5::GUS*)-responsive promoter:GUS lines were stained with 5-bromo-4-chloro-3-indolyl-β-D-glucuronide and were photographed at 10, 14, 17, 21, and 28 days (right) after inoculation with *Plasmodiophora brassicae* (c = control plants, i = inoculated plants). The β-glucuronidase staining at the beginning of the experiment was the same as for the first timepoint (data not shown). Bars represent 1 cm. The brownish color of the infected roots is due to the age of the roots and develops concomitantly with gall size. Destaining with ethanol or acetone does not reduce the brown color. The inset shows an enlarged control root of *CKX1::GUS* to visualize the specific staining at root-branching points.

Fig. 7. Histochemical analysis of longitudinal root sections. **A** and **B**, Lines *ARR5::GUS* and **C** and **D**, *CKX6::GUS* stained with 5-bromo-4-chloro-3-indolyl-β-D-glucuronide 28 days after inoculation with *Plasmodiophora brassicae* (**B** and **D**) and corresponding control roots (**A** and **C**). Bars represent 100 μm. The pictures were taken under dark-field conditions for better contrast; therefore, the β-glucuronidase staining is visible as magenta stain.

Table 5. Interaction of overexpression lines of *AtCKX1*, *AtCKX2*, *AtCKX3*, and ecotype Col-0 with different isolates of *Plasmodiophora brassicae* and different inoculation pressure^a

Line	Isolate	Disease index	R _i /R _{ni}
Col-0	e _H	99.2	4.78 ± 0.86
Col-0	1CK	100.0	4.19 ± 0.83
Col-0	k ₁	96.9	2.58 ± 0.60
Col-0	e ₂	91.4	7.70 ± 0.94
35S::AtCKX1	e _H	31.03 ^(s)	1.15 ± 0.28
35S::AtCKX1	1CK	50.6 ^(s)	0.66 ± 0.31
35S::AtCKX1	k ₁	40.8 ^(s)	0.99 ± 0.23
35S::AtCKX1	e ₂	62.5 ^(s)	1.29 ± 0.43
35S::AtCKX2	e _H	90.07 ^(n.s.)	2.61 ± 0.80
35S::AtCKX2	1CK	100.0 ^(n.s.)	1.97 ± 0.53
35S::AtCKX2	k ₁	77.9 ^(s)	1.36 ± 0.45
35S::AtCKX2	e ₂	83.3 ^(n.s.)	3.39 ± 0.82
35S::AtCKX3	e _H	61.6 ^(s)	1.23 ± 0.10
35S::AtCKX3	1CK	71.0 ^(s)	1.81 ± 0.65
35S::AtCKX3	k ₁	79.0 ^(s)	1.77 ± 0.51
35S::AtCKX3	e ₂	68.8 ^(s)	1.83 ± 0.49

^a The disease indices (DI) and the mean values and standard deviations of the root index (R_i/R_{ni}) of the different host-pathogen combinations are given. ^(s) or ^(n.s.) indicate a significant or not significant difference (α = 0.05) compared with Columbia, using the same isolate and infection pressure calculated by Kruskal-Wallis analysis and mean rank comparison.

MATERIALS AND METHODS

Plant and pathogen material.

Plant material. The ecotype Col-0 of *Arabidopsis thaliana* was originally provided by Nottingham Arabidopsis Stock Cen-

Fig. 8. Representative examples of infected plants of *35S::AtCKX1*, *35S::AtCKX2*, and *35S::AtCKX3* 28 days after infection with the *Plasmodiophora brassicae* isolate 'e_H' in comparison with roots of ecotype Columbia. All plants are shown at the same magnification. The bar represents 1 cm. The differences in rosette size are due to the fact that some of the *35S::AtCKX*-overexpressing plants have a dwarf phenotype.

tre (Nottingham, U.K.). *Brassica rapa* L. subsp. *pekinensis* cv. Granaat (ECD-05) was used for propagation of all isolates of *P. brassicae*. The transgenic lines overproducing cytokinin oxidases/dehydrogenases (*35S::AtCKX1*, *35S::AtCKX2*, *35S::AtCKX3*) and the transgenic lines carrying promoter-GUS fusions of the different *AtCKX* genes (*CKX1::GUS*, *CKX2::GUS*, *CKX3::GUS*, *CKX4::GUS*, *CKX5::GUS*, *CKX6::GUS*) as well as line *ARR5::GUS/CKX1::GUS* were described by Werner and associates (2003). The line carrying the *GUS* gene under control of an auxin-responsive promoter (*DR5::GUS*) and the line carrying the *GUS* gene under control of a cytokinin-responsive promoter (*ARR5::GUS*) were provided by T. Guilfoyle (University of Missouri, Columbia, MO, U.S.A.) and J. Kieber (University of North Carolina, Chapel, Hill, NC, U.S.A.), respectively.

Pathogen material. *P. brassicae* isolates 'e_H', 'e₃', 'e₂', and 'k₂' were described by Föhling and associates (2003) and Graf and associates (2004). The isolate '1-CK' is a single-spore isolate produced at INRA (Institut National de Recherche Agricolaire–Station d'Amélioration des Plantes, Le Rheu Cedex, France). Clubroot galls of all isolates were stored at –20°C until required. Resting spores were extracted by homogenizing mature clubroot galls of Chinese cabbage, followed by filtering through gauze (25 µm pore width) and two centrifugation steps (2,500 × g, 10 min) (Sacristán and Hoffmann 1979).

For propagation of *P. brassicae* isolates *B. rapa* cv. Granaat plants were inoculated with 4 ml of spore suspension (10⁷ spores/ml) 4 days after sowing and were cultivated for 8 weeks in the greenhouse.

Fig. 9. The expression of the cytokinin-responsive promoter::GUS construct *AtARR5::GUS* was analyzed in the genetic background of ecotype Columbia and of the CKX1-overproducing line *35S::CKX1*. Roots were stained with 5-bromo-4-chloro-3-indolyl-β-D-glucuronide and photographed at **A** and **B**, 14, and **C** and **D**, 21 days after inoculation with *Plasmodiophora brassicae* (c = control plants, i = inoculated plants). Due to infection, the root systems of the inoculated *35S::CKX1* plants are slightly reduced, but no clearly clubroot symptoms are visible. Bar represents 1 cm.

Cultivation and inoculation of plants. Cultivation and inoculation conditions of plants were performed according to Kobelt and associates (2000). Plants (14-day-old) cultivated in a controlled environment ($21 \pm 1^\circ\text{C}$, 16 h of light, $100 \mu\text{mol photons s}^{-1} \text{m}^{-2}$) were inoculated by injecting the soil around each plant with 2 ml of a resting spore suspension of the pathogen with a standard concentration of 10^6 spores per milliliter. Disease symptoms were assessed 28 dai, using a scale consisting of five classes and by estimating the R_i/R_{ni} indices according to Siemens and associates (2002). Disease symptoms were assessed using a scale consisting of five classes according to Klewer and associates (2001): 0 = no symptoms, 1 = very small clubs, mainly on lateral roots that do not impair the main root, 2 = small clubs covering the main root and a few lateral roots, 3 = medium-sized to bigger clubs, also including the main root, plant growth might be impaired, 4 = severe clubs in lateral, main root or rosette, fine roots completely destroyed, plant growth is affected. Disease index was calculated using the five-grade scale according to the formula $DI = (1n_1 + 2n_2 + 3n_3 + 4n_4) \times 100/4N_t$, where n_1 to n_4 represent the number of plants in the indicated class and N_t the total number of plants tested. For each isolate and line combination, at least 140 *Arabidopsis* plants were analyzed. These data were analyzed with the Kruskal-Wallis test, and subsequently, the mean rank differences were compared (Siegel and Castellan 1988).

For quantitative estimation, the plants were cut at the top of the hypocotyl into shoots and roots. The infected roots were measured as galls, ignoring occasional remaining noninfected lateral roots. Root fresh weight was used to calculate the root index according to Siemens and associates (2002). For each isolate and line combination, at least 35 *Arabidopsis* plants were analyzed and compared by Student *t*-test.

RNA preparation, cRNA synthesis, and microarray hybridization.

RNA preparation, cRNA synthesis, and microarray Affymetrix GeneChip array hybridization was performed at the John Innes Genome Laboratory, using the outlined modifications of protocols described in the Affymetrix expression analysis technical manual II (Affymetrix, Santa Clara, CA, U.S.A.).

At two timepoints after inoculation, roots of inoculated plants (10 and 23 dai) and roots of the corresponding noninoculated plants (24 and 37 dag) were harvested. Soil residue was carefully removed from roots using brushes, and the roots were immediately frozen in liquid nitrogen and were grounded with mortar and pestle. Briefly, total RNA was extracted from three independent experiments by the RNeasy plant mini kit (Qiagen, Venlo, The Netherlands) according to the manufacturer's instructions. RNA samples were further cleaned using Qiagen RNeasy mini-columns, and their quality was assessed by running 1 μl of each RNA sample on Agilent RNA6000nano LabChips (2100 Bioanalyzer Version A.01.20 SI211; Agilent Technology, Palo Alto, CA, U.S.A.). First-strand cDNA synthesis was performed according to the Affymetrix manual II, using 10 μg of total RNA. Second-strand cDNA synthesis was performed with minor modifications to the Affymetrix manual II. cDNA termini were not blunt-ended and the reaction was not terminated using EDTA. Instead, double-stranded cDNA products were immediately purified following the "Cleanup of Double-Stranded cDNA" protocol (Affymetrix manual II). cDNA was resuspended in 22 μl of RNase free water.

cRNA preparation was performed with minor modifications to the Affymetrix manual II. cDNA (11 μl) was used as a template to produce biotinylated cRNA, using half the recommended volumes of the ENZO bioarray high yield RNA transcript labeling kit (ENZO Life Sciences, Farmingdale, NY, U.S.A.). Labeled cRNAs were purified following the "Cleanup

and Quantification of Biotin-Labeled cRNA" protocol (Affymetrix manual II). cRNA quality was assessed on Agilent RNA6000nano LabChips (2100 Bioanalyzer Version A.01.20 SI211, Agilent Technology). cRNA (20 μg) was fragmented according to the Affymetrix manual II.

High-density oligonucleotide arrays containing probes of more than 22,000 different *Arabidopsis* genes (*Arabidopsis* ATH1 arrays; Affymetrix) were used for gene expression detection. Hybridization overnight at 45°C and 60 rpm (Hybridization Oven 640), washing and staining (GeneChip Fluidics Station 450, using the EukGEws2_450 Antibody amplification protocol), and scanning (GeneArray 2500) was carried out according to the Affymetrix manual II.

Microarray suite 5.0 (Affymetrix) was used for image analysis and to determine probe signal levels. The average intensity of all probe sets was used for normalization and scaled to 100 in the absolute analysis for each probe array. For each microarray, overall intensity normalization for the entire probe sets was performed using Affymetrix Microarray Suite 5.0. Using the GeneChip Suite 5.0 default parameters, the detection *P*-value and the signal value were calculated for each probe set from each independent timepoint and condition. The detection *P*-value generated by this analysis allowed us to determine whether a transcript is reliably detected (present, *P*-value < 0.04), and the signal value assigns a relative measure of abundance to the transcript. The data were then passed through a quality filter using Access (Microsoft, Redmond, WA, U.S.A.), through which transcripts were selected for further analyses according to the following ad hoc criteria. A significantly expressed transcript must have been significantly expressed in at least two samples obtained under the same condition (*P* for present, detection *P*-value < 0.04). Note that low-abundance transcripts may actually encode important cellular proteins, even if statistical analyses define these as absent (A). To monitor and assess different genes from three independent samples, expression induction (ratio > 2 $\times$, ratio > 20 $\times$) or repression (ratio < 0.5 $\times$, ratio < 0.05 $\times$) were calculated as mean values and standard deviation of the ratios of expression values of a particular sample (10 and 23 dai) and its corresponding control sample (24 and 37 dag) (inoculated versus control) using Excel (Microsoft, Redmond, WA, U.S.A.). Annotation of transcripts presented in the tables was done by using the Affymetrix probe set IDs for the respective genes and annotation and AGI numbers from the Salk Institute genomic analysis laboratory database SIGnAL were retrieved. The annotation of highly regulated genes was constantly updated using TIGR and TAIR databases.

Data analysis using the MAPMAN software.

Ratios of control to infected roots were calculated for each timepoint (TP1, TP2) for all values, and the values were converted to $\log_2$ in Excel files. For the data import into MAPMAN, the protocol given in the instructions was followed.

RT-PCR.

Total RNA was prepared using the RNeasy kit (Qiagen). Fresh weight of control and infected root material (100 mg) was harvested, washed thoroughly, dried between filter paper and homogenized in 450 μl of extraction buffer according to the manufacturer's instructions. To minimize contamination with genomic DNA, RNA was digested with RNase-free DNase (1 U μl^{-1}) (Stratagene, La Jolla, CA, U.S.A.). First-strand cDNA was prepared from total RNA using M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA, U.S.A.).

Amplification of partial *AtCKX1* and *AtCKX6* cDNA fragments by PCR was performed using the following primers: sense primer *AtCKX1*, 5'-GGCAATTCTACATCCAAGGTC AG-3'; antisense primer *AtCKX1*, 5'-CAAGCCCGCCAAGAA

CACT-3'; sense primer *AtCKX6*, 5'-ATTTGGAAACATACTA ACGGATACAAG; and antisense primer *AtCKX6*, 5'-TACTAC ATATATGGATACGCATCATCG. These sequences are perfect match primers corresponding to a nonhomologous region to other *AtCKX* genes in the coding region of *AtCKX1*, resulting in a 430-bp amplicon, and to a sequence with low homology in the coding sequence (sense) as well as the 3' untranslated region (antisense) of *AtCKX6*, resulting in a 496-bp amplicon, respectively. As control, all cDNA samples were amplified with actin 2 (At3g18780) primers with the following sequences: sense primer actin, 5'-GAAGATTAAGGTCGTTG CACCACCTG; and antisense primer actin, 5'-ATTAACATTG CAAAGAGTTTCAAGGT. PCR was performed according to standard procedures, using the following program for actin amplification. Initial denaturation at 94°C for 5 min, followed by 40 cycles of 94°C for 30 s, 62°C for 60 s, 72°C for 60 s, and final elongation at 72°C for 5 min. The same program was used for the amplification of *AtCKX1* and *AtCKX6*, but the annealing temperature was 54°C.

The PCR fragments were excised from the gel, were eluted with the StrataPrepDNA gel extraction kit (Stratagene, La Jolla, CA, U.S.A.), and were directly submitted to sequencing (SeqLab, Göttingen, Germany). Sequence alignments were performed using BLAST (Altschul et al. 1997). All other standard molecular methods were performed according to Sambrook and associates (1989).

GUS staining.

Histochemical GUS staining was performed by incubating control and *P. brassicae*-infected plants with their roots in a solution containing 0.5 mg of 5-bromo-4-chloro-3-indolyl- β -D-glucuronide per milliliter dissolved in 0.1 M phosphate buffer, pH 7.4, containing 10 mM Na₂EDTA, 0.5 mM K₃[Fe(CN)₆], 0.5 mM K₄[Fe(CN)₆], and 0.5% (wt/vol) Triton-X-100 (Jefferson 1987). The incubation was stopped by washing the roots in phosphate buffer, pH 7.4, without substrate and storing the roots in 70% (vol/vol) ethanol. Stained roots were embedded in glycolmethacrylate (Technovit 7100; Heraeus-Kulzer, Wehrheim, Germany) according to Kobelt and associates (2000), and sections of 5 to 10 μ m thickness were analyzed in dark field or in bright field after staining with methylene blue and basic fuchsin.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

Affymetrix expression analysis technical manual II:
www.affymetrix.com/support/technical/manuals.affx
 ArrayExpress, European Bioinformatics Institute:
www.ebi.ac.uk/arrayexpress
 GabiPD MAPMAN webpage: gabi.rzpd.de/projects/MapMan
 John Innes Genome Laboratory: www.jicgenomelab.co.uk
 Salk Institute genomic analysis laboratory database SIGnAL:
signal.salk.edu/about.html
 The Arabidopsis Information Resource (TAIR) database:
www.arabidopsis.org
 The Institute for Genomic Research (TIGR) database:
www.tigr.org/tdb/e2k1/ath1/ath1.shtml

Erratum

A formatting error caused an incorrect column break in the final paragraph of the first column on page 481. The online version was corrected on December 21, 2006.