**Ustilago maydis** Infection Strongly Alters Organic Nitrogen Allocation in Maize and Stimulates Productivity of Systemic Source Leaves \(^{1[W][OA]}\)

Robin J. Horst, Gunther Doeblemann, Ramon Wahl, Jörg Hofmann, Alfred Schmiedl, Regine Kahmann, Jörg Kämper, Uwe Sonnewald, and Lars M. Voll *

Friedrich-Alexander-Universität Erlangen-Nürnberg, Lehrstuhl für Biochemie, 91058 Erlangen, Germany (R.J.H., J.H., A.S., U.S., L.M.V.); Max Planck Institute for Terrestrial Microbiology, D-35043 Marburg, Germany (G.D., R.K.); and University of Karlsruhe, Institute of Applied Biosciences, Department of Genetics, 76187 Karlsruhe, Germany (R.W., J.K.)

The basidiomycete **Ustilago maydis** is the causal agent of corn smut disease and induces tumor formation during biotrophic growth in its host maize (*Zea mays*). We have conducted a combined metabolome and transcriptome survey of infected leaves between 1 d post infection (dpi) and 8 dpi, representing infected leaf primordia and fully developed tumors, respectively. At 4 and 8 dpi, we observed a substantial increase in contents of the nitrogen-rich amino acids glutamine and asparagine, while the activities of enzymes involved in primary nitrogen assimilation and the content of ammonia and nitrate were reduced by 50% in tumors compared to mock controls. Employing stable isotope labeling, we could demonstrate that **U. maydis**-induced tumors show a reduced assimilation of soil-derived \(^{15}\)NO\(_3\) and represent strong sinks for nitrogen. Specific labeling of the free amino acid pool of systemic source leaves with \([^{15}\text{N}]\)urea revealed an increased import of organic nitrogen from systemic leaves to tumor tissue, indicating that organic nitrogen provision supports the formation of **U. maydis**-induced tumors. In turn, amino acid export from systemic source leaves was doubled in infected plants. The analysis of the phloem amino acid pool revealed that glutamine and asparagine are not transported to the tumor tissue, although these two amino acids were found to accumulate within the tumor. Photosynthesis was increased and senescence was delayed in systemic source leaves upon tumor development on infected plants, indicating that the elevated sink demand for nitrogen could determine photosynthetic rates in source leaves.

Plant pathogens have evolved different strategies to colonize their plant hosts. While necrotrophic pathogens rapidly kill plant tissue, usually by the secretion of highly efficient toxins and cell wall-degrading enzymes (van Kan, 2006), and thrive on the dead plant material, biotrophic pathogens strictly rely on living tissue to survive and complete their life cycle (Divon and Fluhr, 2007).

**Ustilago maydis**, the causal agent of corn smut disease, is a biotrophic basidiomycete parasitizing maize (*Zea mays*) and its natural ancestor teosinte. It can induce the formation of tumors on all aerial organs (Banuett, 1995), resulting in stunted growth and yield losses (Martinez-Espinoza et al., 2002). **U. maydis** shows a dimorphic lifestyle (Kahmann and Kämper, 2004): while haploid sporidia are not infectious and grow saprophytically in a yeast-like manner, filamentous growth is initiated upon mating of two compatible sporidia on the plant surface. Filamentous hyphae form appressoria and directly penetrate host cells. Immediately upon host entry, the biotrophic interaction with intracellular fungal proliferation is initiated. About 4 d after penetration, the formation of hypertrophic host cells and concomitant tumor development are induced, while the fungal hyphae start proliferating in the apoplastic spaces that develop as a consequence of cell wall degradation and induced host cell enlargement (Doeblemann et al., 2008a, 2008b).

In infections by smut fungi, the plant plasma membrane gets invaginated and encases the growing hyphae (Doeblemann et al., 2009). This generates the so-called biotrophic interface, a compartment where fungal secretion leads to the formation of a vesicular matrix that comprises the enlarged contact zone typically found for members of the Ustilaginomycetes (Bauer et al., 1997; Begerow et al., 2006). The hyphae of **Ustilago** species often grow along the vasculature (Doeblemann et al., 2008b, 2009) that contains high concentrations of assimilates and nutrients (Lohaus et al., 1998, 2000). In general, infection sites of biotrophic fungi represent strong local metabolic sinks that drain nutrients from the host environment. Evidence

---

\(^{1}\) This work was supported by the Deutsche Forschungsgemeinschaft via the priority program FOR 666.

\(^{*}\) Corresponding author; e-mail lvoll@biologie.uni-erlangen.de.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantphysiol.org) is: Lars M. Voll (lvoll@biologie.uni-erlangen.de).

\(^{[W]}\) The online version of this article contains Web-only data.

\(^{[OA]}\) Open Access articles can be viewed online without a subscription.

www.plantphysiol.org/cgi/doi/10.1104/pp.109.147702
obtained for the rust fungus *Uromyces fabae* suggests that nutrients are mainly taken up as hexoses (generated by secreted fungal invertase) and amino acids (Hahn et al., 1997; Voegele et al., 2001; Struck et al., 2002, 2004). Very recently, a novel high-affinity *U. maydis* Suc transporter, Srt1, was characterized that is required for full virulence (R. Wahl, K. Wippel, J. Kämper, and N. Sauer, unpublished data).

Although *U. maydis* can infect all aerial parts of the plant, it has a high specificity for meristematic tissues (Wenzler and Meins, 1987), which represent sink organs and are dependent on the import of assimilates from source organs (i.e. germinating seeds or fully developed leaves in adult plants). The tumors that develop in infected leaves often span the entire leaf blade. Such leaf infections first hamper the establishment of C₃ photosynthesis, which results in reduced CO₂ assimilation upon tumor initiation (Horst et al., 2008). During tumor development, a further reduction in overall photosynthetic capacity of infected leaves is observed. Consequently, soluble carbohydrate accumulation in tumors is similar to that in young sink leaves (Doehlemann et al., 2008a; Horst et al., 2008).

The role of nitrogen metabolism in plant-pathogen interactions has not been investigated very intensively to date. There have been conflicting reports on whether plant-pathogenic fungi encounter nitrogen limitation in planta. Amino acid uptake transporters (Hahn et al., 1997; Struck et al., 2002, 2004; Divon et al., 2005; Takahara et al., 2009) and genes for the biosynthesis of major (Gln synthetase [GS]; Stephenson et al., 1997) and minor (Namiki et al., 2001; Both et al., 2005; Takahara et al., 2009) amino acids are induced during early plant colonization by most (hemi)biotrophs, indicating uptake and metabolization of organic nitrogen in planta by the intruder. Nitrate-nonutilizing mutants of various fungal (hemi)biotrophs, such as *U. maydis* and *Magnaporthe grisea* (Lau and Hamer, 1996; R.J. Horst, G. Doehlemann, R. Wahl, J. Hofman, A. Schmiedl, R. Kahmann, J. Kämper, U. Sonnewald, and L.M. Voll, unpublished data), did not exhibit any reduction in pathogenicity, indicating that nitrate is not essential for these pathogens in planta.

*U. maydis* exhibits the genetic equipment to synthesize all amino acids starting from inorganic nitrate (McCann and Snetselaar, 2008). Therefore, it should be able to use any nitrogen source that is available in maize leaves. While nitrate reductase knockout mutants are fully pathogenic on maize leaves (R.J. Horst, G. Doehlemann, R. Wahl, J. Hofman, A. Schmiedl, R. Kahmann, J. Kämper, U. Sonnewald, and L.M. Voll, unpublished data), nitrogen-auxotrophic mutants of *U. maydis* are reportedly unable to complete their infection cycle (Holliday, 1961). Similarly, a His-auxotrophic mutant of the hemibiotroph *M. grisea* (Sweigard et al., 1998) and an Arg-auxotrophic mutant of the hemibiotroph *Fusarium oxysporum* (Namiki et al., 2001) also exhibit reduced virulence, indicating the necessity for the biosynthesis of minor amino acids that are not readily available in planta.

In addition, the loss of AreA/NiT2 transcription factor homologs that coordinate the utilization of nitrogen sources via nitrogen catabolite repression in filamentous fungi (Marzluf, 1997) resulted in attenuated pathogenicity in the respective mutants of *F. oxysporum*, *Cladosporium fulvum*, and *Colletotrichum lindemuthianum* (Pellier et al., 2003; Divon et al., 2006; Thomma et al., 2006). This indicates that virulence genes are regulated by AreA-like transcription factors.

Is there physiological evidence for nitrogen limitation of fungal pathogens in planta? On the one hand, overfertilization of plants with nitrogen can lead to an increased susceptibility to fungal pathogens (Jensen and Munk, 1997; Hoffland et al., 2000; Agrios, 2005). For instance, the infection index of field-grown maize with *U. maydis* correlates with the amount and the timing of nitrogen application (Kostandi and Soliman, 1991). On the other hand, plants grown under nitrogen limitation often show increased susceptibility to pathogen infection, most likely caused by reduced general fitness (Snoeijers et al., 2000; Solomon et al., 2003). On the physiological level, free amino acid concentrations in the leaf apoplastic fluids of maize are up to 1.3 mM in maize, compared with apoplastic Suc contents of up to 2.6 mM (Lohaus et al., 2000). In addition, apoplastic amino acid concentrations even increased upon infection of tomato (*Solanum lycopersicum*) leaves with *C. fulvum* (Solomon and Oliver, 2001). In pea (*Pisum sativum*) leaves infected by powdery mildew, the transfer rate of radiolabeled Gln into fungal mycelium was found to be as high as 50% of the transfer rate of Glc (Clark and Hall, 1998). This indicates a similar capacity for sugar and amino acid uptake into haustoria. In summary, these observations suggest that nitrogen provision to apoplast-resident pathogens is not limited. However, nitrogen limitation for limited time periods cannot be excluded (e.g. during early pathogenic development on the host surface, when fungal cells depend on their own carbon and nitrogen reserves; for summary, see Solomon et al., 2003).

In maize, nitrogen uptake in the roots preferentially occurs in the form of nitrate, which is then transported to the leaves, where it is reduced and assimilated into organic forms (Oaks, 1994). Apart from the presence of several systematic studies on nitrogen remobilization in maize leaves (Hirel et al., 2005; Gallais et al., 2006, 2007; Martin et al., 2006), the analysis of mutants of cytosolic GSs (gs1-3 and gs1-4) has identified Gln as the major route of nitrogen export to growing ears, which represent a strong sink for nitrogen (Martin et al., 2006).

This work provides a systematic study of the dynamics in nitrogen metabolism during the interaction of maize with *U. maydis*. We address (1) the impact of *U. maydis*-induced tumor formation on local nitrogen metabolism, (2) its effect on nitrogen allocation in infected shoots, and (3) the impact of tumor presence on the performance of systemic source leaves.
RESULTS
Metabolome Analysis of Developing Tumors

We have conducted a combined transcriptome and metabolome analysis of infected leaf tissue at different time points after infection. Based on this data set, we previously described that U. maydis achieves a swift suppression of defense-related genes during the interaction and that photosynthetic gene expression is progressively reduced in infected leaves (Doehlemann et al., 2008a). In this report, we focus on the insights obtained from the corresponding metabolome data set.

Targeted metabolite analysis was performed for major carbohydrates, amino acids, antioxidants, phosphorylated intermediates, and organic acids at 12 h, 24 h, 4 d, 4.5 d, and 8 d after leaf infection with U. maydis in three independent experiments. Since our analysis was performed on infected leaf material, representing both host and fungal tissue, a prerequisite was to estimate the contribution of fungal cells to total extracted tissue. Assuming that the amount of fungal cells increases during infection, samples taken at 8 d post infection (dpi) were analyzed to examine the maximal portion of U. maydis in infected leaf tissue. While quantification of fungal genomic DNA by real-time PCR (see Supplemental Materials and Methods S1) indicated approximately equal amounts of fungal and host genome copies in the samples (data not shown), volume analysis of fluorescently labeled fungal and host cells in three-dimensional reconstructions of confocal image stacks revealed that only 2.3% of the total sample volume was of fungal origin (for a representative three-dimensional reconstruction, see Supplemental Movie S1). Comparing the data sets, the volume-nucleus ratio of the multinucleate fungal cells appears to be overestimated by real-time PCR analysis. As total fungal cell volume, but not the number of fungal nuclei, is relevant for estimating the pathogen’s contribution to metabolite contents of infected leaf tissue, we conclude that the amount of fungal material in the analyzed samples is largely negligible with respect to the conducted metabolite analysis.

A principal component analysis of all surveyed infection stages revealed that two major principal components coincide with leaf development (PC1, 40.7%) and tumor development (PC2, 24.5%; Fig. 1, A and B). Metabolites related to (C4) photosynthesis (i.e. pyruvate, phosphoenolpyruvate, malate, and 3-phosphoglycerate) had the strongest loading on PC1. These metabolites accumulate upon the establishment of photosynthesis in the developing leaf, which is not observed in infected leaves (Fig. 2C). For PC2, no distinct group of metabolites with particularly high loading could be defined. To this end, a more refined principal component analysis was performed, including only those time points at which tumor development had considerably progressed (4, 4.5, and 8 dpi; Fig. 1C). Here, the two major principal components correlated with U. maydis infection (PC1, 54.3%) and the time of day at which the material was harvested (PC2, 18.6%): 4- and 8-dpi samples were taken at the end of the light period, and 4.5-dpi samples were taken after the dark period. This analysis showed that the amino acids Gln, Phe, Tyr, His, and Thr as well as hexose phosphates strongly load onto PC1 (Fig. 1D). These metabolites remained constantly high in tumors, while their contents dropped during development of control leaves (Fig. 2A). Furthermore, a significant increase of total amino acids (i.e. Asn, Pro, Val, and Ile) and soluble sugars (Glc, Fru, and Suc) was observed during tumor development between 4 and 8 dpi (Fig. 2) that positively loaded on PC2 (Fig. 1D).

Organic Nitrogen Accumulates in Tumors

Measurement of free amino acid contents in maize leaves infected with U. maydis showed that the total free amino acid pool in tumors is elevated during the entire infection process (Fig. 2A), reaching 13.6 ± 0.6 μmol g⁻¹ fresh weight in tumors at 8 dpi compared with 10.4 ± 0.04 μmol g⁻¹ fresh weight in mock-infected leaves at 8 dpi. The contents of nearly all proteinogenous amino acids decreased during normal leaf development, except for Ala and Cys, which accumulate in mature leaves and Asp, Glu, and Pro that exhibit constant levels (Fig. 2A). In contrast to the situation in mock-infected leaves, the contents of most free amino acids remained high or increased progressively during tumor development in infected leaves.

Amino acids with a high nitrogen-carbon ratio were particularly abundant in tumors at 8 dpi. At this time point, the content of Asn was increased more than 3-fold compared with 4 dpi, while Arg contents rose steadily throughout the infection process and Gln contents remained constantly high at all time points analyzed (Fig. 2A). The comparison of the amino acid pool composition in tumors with fully developed control leaves (source) and very young leaves (sink) revealed that the tumor tissue strongly resembles juvenile sink leaves in this respect. The amino acid pool composition of sink leaves and tumors significantly differed from that of fully developed source leaves (Supplemental Fig. S1).

Primary Nitrogen Assimilation Is Down-Regulated in Tumors

To resolve whether the elevated organic nitrogen supply in tumors derives (1) from increased local primary nitrogen assimilation or (2) from stimulated import of amino acids from noninfected systemic leaves, we assessed transcript levels and activities of enzymes directly involved in primary nitrogen assimilation as well as the content of inorganic nitrogen forms in tumors. Analysis of the complementary microarray data set (Doehlemann et al., 2008a) showed transcript levels for all enzymes involved in primary
Figure 1. Principal component analysis of metabolite data from developing *U. maydis*-induced tumors between 0.5 dpi (leaf primordia) and 8 dpi (fully developed tumors). Metabolite data from infected and control leaves were obtained (*n* = 9–12) from three independent experiments, and the mean values were analyzed with the Markerview software as described in “Materials and Methods” using the autoscale option for scaling. A, Principal component analysis of metabolite data from all time points (12 h, 24 h, 2 d, 4 d, 4.5 d, and 8 dpi). Plotted is PC2 (24.5%) against PC1 (40.7%). PC1 correlates with leaf development in mock control leaves, while PC2 discriminates tumor formation at late infection time points. B, Loadings of the individual metabolites on PC1 and PC2 from all time points analyzed. C, Principal component analysis of metabolite data from late infection time points 4 and 8 dpi. PC1 correlates with tumor formation, whereas PC2 discriminates the time of day that the samples were taken. D, Metabolite loadings of PC1 plotted against metabolite loadings of PC2 for the data depicted in C. Acids: Amino acid; Asc, ascorbate; Cit, citrate; F6P, Fru-6-P; Frc, Fru; Fum, fumarate; Glc-1-P, G1P, G6P; gEC, g-glutamylcysteine; GSH, glutathione; Icit, isocitrate; 2OG, 2-oxoglutarate; PEP, phosphoenolpyruvate; 3PG, 3-phosphoglycerate; 6PG, 6-phosphogluconate; Pyr, pyruvate; Ru15BP, ribulose-1,5-bisP; Shik, shikimate; Succ, succinate; T6P, trehalose-6-P; aToc, α-tocopherol; gToc, γ-tocopherol; Toc, total tocopherol.
Figure 2. Metabolite analysis of *U. maydis*-induced leaf tumors. Time courses are shown for metabolite contents in developing leaf tumors (gray) and control leaves (black) at 1, 2, 4, and 8 dpi of 7-d-old maize plants. Samples were taken 1 h before the end of the light period. A, Contents of amino acids. B, Contents of carbohydrates. C, Contents of phosphorylated intermediates and organic acids. The shaded areas represent SE (n = 9–12).
nitrogen assimilation as down-regulated in tumors between 4- and 13-fold at 8 dpi compared with mock-infected leaves (Fig. 3A). Except for nitrate reductase (Nar1S), all the other genes were already down-regulated early during tumor development (4 dpi). Consistently, these transcriptional changes correlate with substantial reductions in the extractable enzyme activity of nitrate reductase (maximal and selective activity) as well as total GS in tumors at 4 and 8 dpi (Fig. 3B). Since all four cytosolic GS isoforms represented on the maize Genechip (gs1-1, gs1-2, gs1-4, and gs1-5) were not transcriptionally regulated upon infection with U. maydis, this drop in activity can be attributed to the plastidic GS2, which is involved in primary nitrogen assimilation in young leaves (Becker et al., 2000; Martin et al., 2006). Similarly, the contents of the inorganic nitrogen compounds nitrate and ammonium were also reduced in tumors, indicating a reduced availability of inorganic nitrogen (Fig. 3A).

Despite the apparent reduction of the capacity for inorganic nitrogen assimilation in tumors, Asp aminotransferase (Asp-AT), an indicator of high nitrogen availability (Lam et al., 1996), and two isoforms of Asn synthetase (AsnS) were induced at the transcriptional level compared with controls at 4 and 8 dpi (Fig. 3A). This indicates stimulated transformation of organic nitrogen into Asn. Consequently, the contents of Glu and Asp remained unchanged, while Gln and Asn accumulated (see above; Fig. 3A).

Taken together, we observed diminished availability of inorganic nitrogen and a reduced capacity for primary nitrogen assimilation in tumors on the one hand and elevated organic nitrogen content on the other. This discrepancy suggests that the primary nitrogen assimilation is not solely responsible for the accumulation of organic nitrogen in tumors.

Uptake and Distribution of Nitrate from the Soil

Since nitrate assimilation in young maize plants occurs predominantly in leaves (Oaks, 1992, 1994), we aimed at gathering direct experimental evidence demonstrating a reduction of nitrate assimilation in tumors. At 8 dpi, U. maydis-infected as well as mock control plants were watered with $^{15}$NO$_3^{-}$, and $^{15}$N uptake into tumor tissue was quantified during a 48-h time course. This experiment showed a reduced incorporation of $^{15}$N into the total nitrogen pool in tumor tissue relative to control leaves (Fig. 4A). However, saturation of the nitrogen pool with $^{15}$N was not reached in both conditions. In contrast, $^{15}$N incorporation into the free amino acid pool reached saturation as early as 10 h after treatment with $^{15}$NO$_3^{-}$ in control plants and remained at this level throughout the experiment (Fig. 4B). Incorporation of $^{15}$N into the free amino acid pool of tumor tissue was substantially reduced and did not reach saturation during the entire experiment. This is in line with our observation that the contents of most free amino acids increased 2- to 5-fold during the first 12 h after $^{15}$NO$_3^{-}$ application.
in control leaves, while the free amino acid pools remained fairly constant in tumors (Supplemental Fig. S2). In tumors, the level of $^{15}$N in the free amino acid pool dropped during the dark period, which was not observed in control leaves.

Incorporation of $^{15}$N into proteins followed a linear rate in control leaves and in tumors, but the $^{15}$N incorporation into proteins was reduced in infected leaves (Fig. 4C), which could be attributed to the decreased level of overall $^{15}$N labeling in tumors. To directly assess the rate of protein biosynthesis in fully developed tumors, we determined the incorporation of $[^{35}$S$]Met$ into the protein fraction in tumors and control leaves at 8 dpi. In control leaves, the ratio of the recovery of $^{35}$S in the protein fraction relative to $^{35}$Si in the free amino acid pool (0.14 ± 0.04) was significantly lower than in tumors (0.5 ± 0.1). This suggests a higher rate of amino acid incorporation into proteins in tumors compared with healthy leaves.

Analysis of protein biosynthesis-related transcripts suggests that cytosolic protein biosynthesis of tumor cells is up-regulated while plastidic protein biosynthesis is down-regulated (Supplemental Fig. S3).

In summary, our data suggest that the evident accumulation of organic nitrogen in tumors is not entirely fueled by local primary nitrogen assimilation but most likely relies on the import of reduced nitrogen compounds from systemic, noninfected leaves. The observed drop of $^{15}$N labeling in the free amino acid pool of tumors during the night might even reflect the contribution of organic nitrogen import from systemic plant organs.

**Tumors Represent Strong Sinks for N**

After labeling with $^{15}$NO$_3$-, we observed a reduced $^{15}$N uptake rate of systemic leaves in infected plants compared with control plants (data not shown). This indicates that, despite their diminished capacity for nitrogen assimilation, tumors represent a strong sink for nitrogen at the whole plant level. To verify this assumption, we calculated the systemic allocation of $^{15}$N in infected plants bearing tumors on leaf 4 (see “Materials and Methods” and final figure). At 9 dpi, tumor tissue constituted approximately 40% of the total shoot weight, whereas the corresponding leaf of a mock-infected plant amounted to maximum 15% of total shoot weight. On the one hand, this increase can be attributed to the reduced total shoot biomass of the infected plants compared with healthy plants; on the other hand, tumors exhibited a strongly increased total weight compared with normal leaf tissue: leaf segments carrying tumors had four to five times more biomass than healthy leaves of the same segment length. Consequently, when regarding the distribution of nitrogen among the entire shoot, the allocation of nitrogen to tumors was two to three times increased compared with the corresponding leaf of a mock-infected plant (Fig. 4D), even though primary nitrogen assimilation was reduced in tumors.

**Figure 4.** Incorporation kinetics of nitrogen originating from $^{15}$NO$_3^-$ into different pools of nitrogen-containing compounds. Plants were watered with 40 mL of 20 mM K$^{15}$NO$_3$ (containing a molar ratio of $^{15}$N/$^{14}$N of 0.2) at 8 dpi, and samples were taken at the time points indicated. Black lines represent control plants, and gray lines represent infected plants. A, Incorporation of $^{15}$N into the total nitrogen pool (A), into the pool of free amino acids (AA; B), and into protein-bound amino acids (C) in percentage of total nitrogen. The shaded areas represent ±SE ($n=4$). D, Allocation of $^{15}$N derived from soil nitrate to tumors. The allocation of $^{15}$N to fourth leaves of infected plants bearing tumors (right) or healthy control leaves (left) at 48 h after treatment with $^{15}$NO$_3^-$ relative to the total amount of $^{15}$N retrieved in all aerial organs is shown. Error bars represent ±SE ($n=4$).
We also assessed the influence of tumor development on assimilate supply to and metabolism in upper systemic sink leaves, but only a few changes were observed relative to controls (Table I; Supplemental Fig. S4). Nevertheless, contents of total soluble carbohydrates were reduced by approximately 20%, while contents of Asn, Asp, Arg, Pro, and Gln were substantially lowered between 40% and 50% in upper sink leaves of infected plants compared with the same leaves of mock-infected plants. The reduced accumulation of carbon and, especially, nitrogen assimilates supports the hypothesis that import of assimilates into upper systemic leaves is reduced upon the establishment of the tumor as a strong additional sink organ.

Reallocation of Organic Nitrogen from Systemic Leaves

As suggested by our previous results, we next investigated whether the export of organic nitrogen compounds from systemic source leaves toward *U. maydis*-induced tumors was increased. The organic nitrogen pool of the source leaf below the tumors (leaf 3 when leaf 4 was infected; see final figure) was labeled by [15N]urea at 8 dpi. Thirty hours after treatment, the reallocation of [15N] between partitions of the maize shoot was analyzed comparing (1) infected and corresponding control leaves (leaf 4), (2) the two leaves below the labeled leaf (leaves 1 and 2), and (3) the younger (upper) systemic leaves above the infection site (for illustration, see final figure). Export of organic nitrogen to the roots was not assessed. The recovery of total exported nitrogen in tumors was increased approximately 3-fold compared with corresponding leaves from mock-infected plants, indicating an increased import of organic nitrogen from lower source leaves into the tumors (Fig. 5A). In turn, less of the exported nitrogen was recovered in upper systemic leaves of infected plants. However, the allocation of nitrogen from leaf 3 to the two older leaves was not altered upon infection.

An analysis of the total pool size of [15N]-labeled amino acids revealed that tumors accumulated nearly five times more labeled amino acids than the corresponding leaves from control plants (Fig. 5B) on a fresh weight basis. Considering the elevated total fresh weight of the tumors compared with control leaves, tumors accumulate 10 times more free amino acids compared with corresponding control leaves (data not shown). Furthermore, healthy leaves mainly accumulated [15N]Glu (Fig. 5B) and [15N]Ala (data not shown), indicating that [15N] label is finally retrieved in amino acids whose pools are subject to a high turnover in photosynthetically active maize leaves. In contrast, tumors accumulated mainly [15N]Asn as well as [15N]Gln and [15N]Glu (Fig. 5B), which likely relates to high rates of overall amino acid import.

Taken together, these data suggest that tumors rely on the import of organic nitrogen from noninfected, systemic source leaves. This, in turn, implies a higher export rate of these compounds from systemic leaves.

Export of Assimilates from Systemic Leaves Increases upon Infection

To test whether lower source leaves from infected plants (with tumors on leaf 4) export more amino acids than the corresponding leaves from control plants, phloem exudates were collected from leaf 3. Exudates were collected from leaves pretreated with urea 1 d prior to sampling to simulate the conditions of the [15N]urea-labeling experiment (high nitrogen availability). To rule out artifacts caused by this treatment, exudates were also collected from nontreated leaves (normal nitrogen availability).

Under both experimental conditions, the amino acid exudation rate of leaf 3 from infected plants was about 2-fold increased compared with the exudation rate of leaf 3 from control plants (Table II). Treatment with urea resulted in a proportional increase of amino acid exudation rate in both infected and control plants. Under both nitrogen regimes, a slight shift was observed in the amino acid composition of the phloem exudates from leaf 3 of infected plants compared with controls. Exudates of systemic leaves of infected plants contained more Ala and less minor amino acids compared with the controls (Table II). Under regular nitrogen availability, leaf exudates from infected plants also contained more Gln and less Gly compared with exudates from control plants. The amino acid composition of phloem exudates differed strongly from those of leaf and tumor extracts (for comparison, see Supplemental Fig. S1). This indicates that amino acids are specifically loaded into the phloem and that the free amino acid pool in the phloem does not reflect the amino acid composition in source or sink organs.

| Sample      | Total Amino Acids | Asn | Asp | Gln | Glu | Sugars |
|-------------|-------------------|-----|-----|-----|-----|--------|
|             | µmol g⁻¹ fresh wt |     |     |     |     |        |
| Control     | 13.8 ± 1.1        | 236 ± 26 | 1.5 ± 0.2 | 282 ± 21 | 3.0 ± 0.2 | 34.3 ± 2.8 |
| Infected    | 11.3 ± 0.7        | 134 ± 19* | 0.8 ± 0.1* | 156 ± 19* | 2.8 ± 0.2 | 27.2 ± 1.6* |
Phloem-Mobile Minerals Accumulate in Tumors

To further investigate whether phloem transport from source leaves to U. maydis-induced tumors is increased compared with noninfected tissue, we assessed the mineral composition of tumors. It is known that the accumulation of mainly phloem-mobile minerals (e.g. potassium, phosphorus, and magnesium) is a consequence of increased phloem flow, since these minerals are basically not transported in the xylem sap (Marschner, 1997). Compared with control leaves, the potassium and phosphorus contents in tumors at 8 dpi were 1.8- and 3.1-fold increased, respectively, while the magnesium content was slightly decreased (Table III). In contrast, contents of the predominantly xylem-mobile minerals iron and calcium were decreased in tumors by 40% and 75%, respectively. These data suggest an increased phloem flow and a decreased xylem flow into tumors.

Photosynthetic Capacity Is Higher in Systemic Leaves of Infected Plants

The increased export of nitrogen assimilates from lower systemic leaves of infected plants should be fueled by an increased CO2 assimilation rate, since photosynthesis provides the carbon backbones for amino acid biosynthesis. Thus, we determined the photosynthetic CO2 assimilation rate (A) and the electron transport rate (ETR) from leaf 3 at different time points after infection with U. maydis at ambient (400 µE m⁻² s⁻¹) and saturating (2,200 µE m⁻² s⁻¹) light intensity.

Most obvious at saturating light intensity, the photosynthetic capacity of leaf 3 was significantly higher in U. maydis-infected plants compared with mock controls (Fig. 6). Both assimilation rate and ETR of leaf 3 from control plants decreased with leaf age starting between 5 and 7 dpi (i.e. between 15 and 17 d post sowing), while the reduction in photosynthetic capacity of leaf 3 was delayed by approximately 3 d in infected plants (Fig. 6, A and B). In plants that had developed mature tumors on leaf 4, both assimilation rate and ETR of leaf 3 from infected plants were increased by 20% to 25% (7 dpi), 30% (8 dpi), and approximately 60% (11 dpi) compared with the respective control leaves. The transcript levels of the senescence markers See1 and See2b (Smart et al., 1995) increased with leaf aging in leaf 3 of noninfected plants between 8 and 14 dpi, while, in comparison, the induction of these two genes was markedly reduced in leaf 3 of infected plants at 11 and 14 dpi (Supplemental Fig. S5). Furthermore, visual inspection showed that senescence-associated chlorophyll loss of the leaves was strongly delayed in infected plants (Fig. 6C), suggesting that increased export rates and maintenance of photosynthetic performance are achieved by a delay in the onset of senescence of lower source leaves of infected plants.

Developing tumors have a reduced photosynthetic capacity compared with uninfected leaves of the same position (Horst et al., 2008), and infected plants are stunted (see above). Thus, the signal for the delay in senescence could be caused either by a concomitant increase in total sink strength due to tumor development or by the reduction of green, photosynthetically active biomass of infected plants, both leading to a general change in the source-to-sink balance of the entire plant. Therefore, assimilation rate and ETR of leaf 3 were determined from plants, of which all younger leaves above leaf 3 were shielded from light with aluminum foil, mimicking a drop in the source-to-sink ratio. While assimilation rate (A) and ETR in control plants dropped to 12.5 ± 1.4 and 39 ± 3 µmol m⁻² s⁻¹ at 14 dpi, respectively, photosynthetic
performance remained high in infected plants \( (A = 19.6 \pm 1.2 \ \mu \text{mol m}^{-2} \text{s}^{-1}) \) and ETR = 59 \pm 6 \ \mu \text{mol m}^{-2} \text{s}^{-1} \) and covered plants \( (A = 21 \pm 0.6 \ \mu \text{mol m}^{-2} \text{s}^{-1} \) and ETR = 74 \pm 6 \ \mu \text{mol m}^{-2} \text{s}^{-1} \). Covering young leaves not only reduced the source strength of the whole plant but also created a strong sink, since the covered leaves still grew, suggesting that these leaves also rely on the import of assimilates from older source leaves. Photosynthetic performance of lower source leaves remained higher than in plants with all upper leaves covered, suggesting that both the reduction in total source capacity and the occurrence of an additional strong sink organ together effectuate the retention of photosynthetic capacity in lower source leaves of maize plants carrying tumors.

**DISCUSSION**

Biotrophic plant pathogens represent strong local sinks for nutrients. While extensive work has been done on nutrient acquisition and metabolism of the individual pathogens, only a few studies have addressed the question how overall host nitrogen metabolism is affected upon infection with biotrophic fungi.

In this report, we provide a systematic study of (1) the dynamics in nitrogen metabolism during the biotrophic interaction between maize and the corn smut fungus *U. maydis* in infected leaves and (2) nitrogen allocation in the host plant. Our results have revealed three fundamental insights into the biology of this interaction. First, *U. maydis*-induced tumors represent a strong sink for organic nitrogen that, second, is provided by systemic source leaves in which, third, photosynthetic capacity and amino acid export are increased, which is accompanied by a suppression of senescence. Furthermore, we show that the amino acids transported in the phloem are metabolized to amino acids with a high nitrogen-carbon ratio after the uptake into tumors.

**Table II. Phloem exudation rate and amino acid composition of phloem exudate collected from leaf 3 of plants infected with *U. maydis* (leaf 4 harbored tumors) or from control plants at 9 dpi**

The experiment was conducted under normal (no urea treatment; left two columns) and high nitrogen availability (leaves treated with 200 mM urea; right two columns) conditions. SE values \( (n = 6) \) are indicated, and statistically significant changes upon infection are marked by asterisks (Welch-Satterthwaite \( t \) test; \( P < 0.05 \)).

| Amino Acid | Normal Nitrogen Availability | | High Nitrogen Availability | |
| --- | --- | --- | --- | --- |
| | Control | Infected | Control | Infected |
| Amino acid exudation rate (nmol g\(^{-1}\) fresh wt h\(^{-1}\)) | 26 \pm 5 | 47 \pm 7* | 36 \pm 6 | 83 \pm 17* |
| % total amino acids | | | | |
| Asp | 9.5 \pm 0.5 | 8.2 \pm 0.5 | 9 \pm 0.4 | 9 \pm 2 |
| Glu | 20 \pm 2 | 19 \pm 2 | 13 \pm 1 | 16 \pm 1 |
| Asn | 0.9 \pm 0.1 | 1.2 \pm 0.1 | 0.9 \pm 0.1 | 1.1 \pm 0.1 |
| Ser | 11 \pm 1 | 11 \pm 1 | 15.6 \pm 0.8 | 14 \pm 1 |
| Gln | 2.1 \pm 0.2 | 4.4 \pm 0.9* | 3.5 \pm 0.3 | 4.9 \pm 0.8 |
| Gly | 14 \pm 1 | 8.9 \pm 0.5* | 13 \pm 1 | 11.1 \pm 0.9 |
| His | 0.7 \pm 0.1 | 0.7 \pm 0.2 | 0.9 \pm 0.1 | 0.5 \pm 0.1* |
| Thr | 2.6 \pm 0.4 | 4.3 \pm 0.7 | 2.8 \pm 0.3 | 2.4 \pm 0.4 |
| Arg | 5 \pm 0.3 | 3.5 \pm 0.5* | 5.4 \pm 0.2 | 3.1 \pm 0.3* |
| Ala | 12 \pm 2 | 24 \pm 2* | 10 \pm 2 | 25 \pm 3* |
| Pro | 1.7 \pm 0.2 | 1 \pm 0.1* | 1.4 \pm 0.2 | 0.9 \pm 0.1* |
| Tyr | 2.1 \pm 0.2 | 1.4 \pm 0.1* | 2.6 \pm 0.2 | 1.2 \pm 0.2* |
| Val | 5 \pm 0.6 | 2.5 \pm 0.3* | 5.2 \pm 0.3 | 2.2 \pm 0.3* |
| Met | 0.7 \pm 0.2 | 0.34 \pm 0.05 | 1 \pm 0.1 | 0.27 \pm 0.02* |
| Ile | 3.5 \pm 0.6 | 1.7 \pm 0.3* | 3.9 \pm 0.3 | 1.3 \pm 0.3* |
| Lys | 3.4 \pm 0.4 | 3.9 \pm 0.9 | 4.3 \pm 0.2 | 2.7 \pm 0.5* |
| Leu | 3.8 \pm 0.5 | 2.1 \pm 0.3* | 4.4 \pm 0.4 | 2 \pm 0.5* |
| Phe | 2.1 \pm 0.3 | 1.4 \pm 0.2 | 2.1 \pm 0.2 | 1.1 \pm 0.2* |

**Table III. Contents of mainly xylem-mobile (iron and calcium) and mainly phloem-mobile (magnesium, phosphorus, and potassium) minerals in tumors and control leaves at 8 dpi**

| Sample | Iron | Calcium | Magnesium | Phosphorus | Potassium |
| --- | --- | --- | --- | --- | --- |
| Control | 0.060 \pm 0.004 | 18.1 \pm 0.8 | 1.8 \pm 0.1 | 3.8 \pm 0.2 | 31 \pm 1 |
| Tumor | 0.037 \pm 0.004* | 2.7 \pm 0.2** | 1.4 \pm 0.1* | 11.8 \pm 0.5** | 54 \pm 3** |

*SE values are indicated \( (n = 4) \), and statistically significant changes in contents are marked by asterisks (Welch-Satterthwaite \( t \) test; \( * P < 0.05 \), ** \( P < 0.005 \)).
Tumor Development on Maize Leaves Establishes Nitrogen Sink Metabolism

Our study has demonstrated that tumors have elevated contents of nitrogen-rich amino acids, such as Gln, Asn, and Arg, when compared with mock-infected leaves. A transient accumulation of nitrogen-rich amino acids has also been observed during the first 24 to 48 h of potato (Solanum tuberosum), maize, and barley (Hordeum vulgare) infection with Phytophthora infestans, Colletotrichum graminicola, and Blumeria graminis f. sp. hordei, respectively (Grenville-Briggs et al., 2005; R.J. Horst, G. Doehlemann, R. Wahl, J. Hofmann, A. Schmiedl, R. Kahmann, J. Kämper, U. Sonnewald, and L.M. Voll, unpublished data). During early biotrophy, these changes in the free amino acid pool may indicate increased nitrogen assimilation that is either (1) required to launch defense responses or (2) reflects the demand for organic nitrogen by the parasite. Accumulation of nitrogen-rich amino acids has also been observed during late necrotrophic growth of Pseudomonas syringae in tomato (Olea et al., 2004) and C. lindemuthianum in bean (Phaseolus vulgaris) leaves (Tavernier et al., 2007). In these two studies, a reduction in primary nitrogen assimilation was accompanied by an increase in cytosolic GS1 and Glu dehydrogenase (GDH) transcript abundance. These two genes are typical senescence markers and are thought to be involved in the recycling of nitrogen that is released during protein degradation (Kamachi et al., 1991; Masclaux et al., 2000). Therefore, GS1 and GDH may facilitate the export of nitrogen from infected leaves in these two interactions, thereby decreasing nitrogen availability to the pathogens (Tavernier et al., 2007).

In U. maydis-induced tumors, none of the cytosolic GS isoforms was differentially regulated, and only one GDH (Zm.17860) was slightly up-regulated (see corresponding microarray data, deposited by Doehlemann et al. [2008a], as mentioned in “Materials and Methods”). In addition, other reliable senescence markers like See1 (Cys protease) and See2 (legumain-like protease; Smart et al., 1995) were not transcriptionally up-regulated in tumors. These comparisons with other pathosystems suggest that senescence is not induced in tumors and that the nitrogen metabolism of tumor tissue differs strongly from the nitrogen metabolism observed in other plant-pathogen interactions.

In addition to the accumulation of nitrogen-rich amino acids, we found an induction of Asp and Asn metabolism at the transcript level, while we could determine that primary nitrogen assimilation was reduced in tumors at the transcriptional and enzymatic as well as the physiological level. Asn usually accumulates under C limitation (Lam et al., 1996). Since carbon may become limiting to the host due to increased respiration of the infected tissue (see transcript data) and/or due to carbon uptake by the pathogen (Snoeijers et al., 2000), our results could be explained by carbon limitation in tumors. However, we have previously shown that carbon accumulates in the form of soluble carbohydrates in U. maydis-infected leaves (Doehlemann et al., 2008a; Horst et al., 2008). To consider carbon limitation as the trigger for the accumulation of Asn thus seems unlikely. Instead, we favor that the high sink strength of tumors for nitrogen could be the cause of the observed effects on the amino acid pool.

It is difficult to estimate the contribution of the pathogen to tumor nitrogen metabolism, especially as only less than 3% of the total tumor volume is of fungal origin.
origin. Compared with sporidia harvested immediately after maize infection, fungal genes involved in nitrogen utilization were not induced in fully developed tumors, despite three genes involved in Trp biosynthesis (R.J. Horst, G. Doehlemann, R. Wahl, J. Hofmann, A. Schmiedl, R. Kahmann, J. Kämper, U. Sonnewald, and L.M. Voll, unpublished data). However, this result may indicate that nitrogen supply to \textit{U. maydis} is similar in planta and on synthetic media. Consequently, it remains elusive to what extent the hypertrophic growth of tumor cells and pathogen metabolism contribute to the massive increase in sink strength of \textit{U. maydis}-triggered tumors.

Nevertheless, our data provide some indication that the increased import of nitrogen into tumors might predominantly fuel protein biosynthesis of \textit{U. maydis}. Plant cytosolic ribosomes produce large amounts of pathogenesis-related proteins during pathogen attack (Stintzi et al., 1993), and many of these defense genes were also strongly induced in fully developed tumors at 8 dpi (Doehlemann et al., 2008a). Although cytosolic protein biosynthesis of the host was strongly up-regulated in tumors at the transcriptional level, the photosynthetic machinery (Doehlemann et al., 2008a) and, thus, genes involved in plastidic protein biosynthesis were strongly down-regulated in tumors compared with controls. Irrespective of the transcriptional induction of cytosolic protein synthesis, we can assume that total host protein biosynthesis is diminished in tumors compared with healthy leaves, because the photosynthetic apparatus represents the vast majority of cellular protein in mature maize leaves, with Rubisco being up to 50% of the total leaf protein (Leegood et al., 2000). However, we have determined an increased incorporation of $^{35}$S]Met into the protein fraction of tumors. Although we cannot directly discriminate between host and pathogen, taken together, these data suggest that most organic nitrogen acquired by the tumors is directed toward fungal protein biosynthesis.

**Organic Nitrogen Is Rerouted to Tumors from Systemic Source Leaves**

Although we could demonstrate that primary nitrogen assimilation is reduced in tumors, we have also observed that nitrogen derived from soil nitrate preferentially accumulated in tumors compared with corresponding leaves of noninfected plants. Although apparently contradictory, this result can be explained, as tumors represent a large biomass fraction of the entire shoot. Second, organic nitrogen import from systemic source leaves into tumors was strongly increased compared with corresponding leaves of healthy plants, suggesting that after nitrate feeding, organic nitrogen import from systemic leaves into tumors is favored over a direct import of supplied nitrate from the soil (Fig. 7). The reallocation of nitrogen from systemic leaves into tumors defines the tumor as a sink organ for nitrogen, as was already shown for carbon (Billett and Burnett, 1978; Doehlemann et al., 2008a; Horst et al., 2008). As the increased import of organic nitrogen into tumors leads to an accumulation of nitrogen-rich amino acids, it appears extremely unlikely that \textit{U. maydis} suffers from nitrogen limitation in planta, especially as hyphae can grow alongside the vascular bundles in mature tumors (Doehlemann et al., 2008b). In addition, nitrate does not seem to represent a favored nitrogen source of \textit{U. maydis} in planta, as nitrate reductase mutants in the SG200 background are fully pathogenic (R.J. Horst and L.M. Voll, unpublished data).

The amino acid Gln has been shown to play a major role in nitrogen mobilization to the kernels during the grain-filling period of maize cv B73 (Martin et al., 2006). Although organic nitrogen derived from systemic leaves predominantly accumulates as Asn and Gln in tumors, these amino acids constitute only a minor fraction in the phloem sap of the systemic leaves. This, together with the transcriptional induction of Asn biosynthetic genes, suggests that these amino acids are specifically synthesized in tumors and not directly transported via the phloem.

Our findings are in sharp contrast to previous observations in host leaves during necrotrophic growth of \textit{P. syringae} (Olea et al., 2004) and \textit{C. lindenmuthianum} (Tavernier et al., 2007), where organic nitrogen is mobilized and exported from the infection site to noninfected plant organs via Asn synthetase, cytosolic GS, and GDH. In addition, it was recently observed that, upon infection of spotted knapweed (\textit{Centaurea maculosa}) with the root herbivore \textit{Agapeta zoegana}, total nitrogen uptake was reduced and nitrogen was translocated from the root to the shoot of the plant (Newingham et al., 2007). These examples suggest that plants have evolved a way of limiting nutrient supply at the infection site. \textit{U. maydis} seems to

---

Figure 7. Model comparing the nitrogen flow in healthy and \textit{U. maydis}-infected plants. The flow of inorganic nitrogen (black arrows) and amino acids (AA; gray arrows) is depicted for healthy plants (left) and \textit{U. maydis}-infected plants bearing tumors (right). The thickness of the arrows indicates the flow rate. Plant development and leaf numbering correspond to the material sampled in our experiments. In addition, the inhibition of lower source leaf senescence by increased sink demand is highlighted in the right sketch.
overcome nutrient limitation in infected leaves by inducing tumor formation, thereby generating a strong artificial sink that outcompetes other systemic sink tissues (Fig. 7).

Photosynthesis and Organic Nitrogen Export Are Stimulated in Systemic Source Leaves upon Tumor Formation

We have demonstrated that import of organic nitrogen into tumors from systemic leaves is strongly increased and that amino acid exudation from systemic source leaves is specifically elevated in infected plants compared with noninfected plants (for summary, see Fig. 7). This suggests an increased phloem flow from systemic leaves to infected leaves, as phloem-mobile minerals accumulated in tumors as well. Thus, the induction of tumor formation seems to be a very effective way of rerouting all phloem-mobile nutrients to the infection site. On the other hand, lower contents of xylem-mobile minerals were detected in tumors, which can be explained by the diminished stomatal conductance for water vapor of tumors compared with healthy leaves, which could account for a decreased mineral transport capacity toward tumors (Horst et al., 2008).

Furthermore, we have observed that photosynthesis is elevated in lower systemic source leaves of infected plants and that these leaves exhibit a delay in senescence (Fig. 7). Due to the fact that shielding all younger, growing source leaves from light resulted in similar effects on lower source leaves as tumor formation, our results suggest that a strong shift in the sink-source balance of the entire plant, decreasing source and increasing sink capacity at the same time, triggers the delayed senescence of older source leaves of infected plants (Fig. 7). A delay in senescence of lower source leaves has also been observed upon infection of *Ricinus communis* by the plant parasite *Cuscuta reflexa* (Jeschke and Hilpert, 1997), which taps directly into the phloem of the host, where it acquires carbohydrates and amino acids. It is long known that the sink-source balance can influence the onset of senescence (Nooden and Guiamet, 1989), and it has previously been hypothesized that the drop of photosynthesis below a critical threshold can induce senescence (Smart, 1994; Bleecker and Patterson, 1997). In *U. maydis*-infected maize plants, increased demand for nitrogen and carbon by the tumors provokes a stimulation of carbon and nitrogen export in systemic source leaves, which consequently necessitates increased carbon and nitrogen assimilation. As a result, photosynthetic performance in systemic leaves of infected plants will remain above the critical threshold for a longer time than that of corresponding leaves from uninfected plants, thereby delaying senescence induction. Conflicting results on the significance of altered hexose contents for the induction of senescence have been obtained (Dai et al., 1999; Lara et al., 2004; Kocal et al., 2008). In the *U. maydis*-maize interaction, however, hexose contents in lower systemic source leaves did not change until the onset of leaf senescence in control plants (data not shown), indicating that hexose contents do not modulate the onset of senescence in the system studied here.

In the past, transgenic approaches to increase plant productivity by manipulation of source and sink strength have resulted in the finding that biomass production and yield of many crop plants are not source limited but restricted by the target organ’s sink strength for nitrogen and carbon (Herbers and Sonnewald, 1998; Okita et al., 2001). Increasing sink strength for nitrogen by seed-specific overexpression of an amino acid permease in pea (Rolletschek et al., 2005) resulted in increased seed storage protein synthesis of up to 25% and in the accumulation of nitrogen-rich amino acids (Asn, Gln, and Arg) in seeds. The uptake of ammonium from the soil and vegetative growth were also stimulated in these transgenics (Rolletschek et al., 2005), indicating the stimulation of systemic nitrogen metabolism by elevated sink strength for nitrogen, similar to the influence of tumor formation on nitrogen metabolism in systemic leaves reported here.

Our study links the induction of senescence and source leaf productivity to elevated sink demand in infected plants. This renders the *U. maydis*-maize pathosystem a promising system to address (1) the role of source-sink relations for the induction of senescence and (2) how sink strength influences source metabolism.

MATERIALS AND METHODS
Plant and Fungal Cultivation and Infection Conditions

For combined transcript and metabolite profiling experiments, maize (*Zea mays* ‘Early Golden Bantam’) was cultivated as described (Doehlemann et al., 2008a). For all other experiments, the same maize cultivar was grown on P-type soil (Frahstorfer Erde) in phytochambers at a 14-h-light (28°C, relative humidity 50%–60%)/10-h-dark (20°C, relative humidity 80%–90%) regime including a 1-h ramping of light and temperature at the beginning and end of the light period. Light was provided at a photon flux density of 350 μmol m⁻² s⁻¹. The solopathogenic *Ustilago maydis* strain SG200 (Kämper et al., 2006) was cultivated on potato dextrose plates or in YEPSO, liquid medium (Tsukuda et al., 1988) at 28°C, and plant infection was conducted as described (Gillissen et al., 1992). A suspension with fungal sporidia or the same volume of water for mock controls was syringe injected into the stems of 8- to 10-d-old maize seedlings, which resulted in local infection of leaf 4 and sometimes also leaf 5. To study nitrogen allocation, only those plants showing similar tumorization were used.

Gene Expression Analysis by DNA Microarray

Gene expression data from *U. maydis*-induced tumors was obtained from the same set of material described by Doehlemann et al. (2008a), which is deposited in the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) under the accession number GSE10023.

Metabolite Quantification and Analysis

Metabolite contents were determined in three independent experiments from subsets of the leaf material pools that were employed for transcriptome analysis (Doehlemann et al., 2008a), such that material of four independent
samples for metabolite analysis were pooled to generate one sample pool for transcription analysis per time point. Due to the high reproducibility of the three replicate experiments with four replicate samples each, metabolite contents were computed based on all 12 analyzed samples per time point. Principal component analysis of metabolite data was performed with the MarkerView software (version 1.1.0.7; Applied Biosystems) using the autoscale algorithm for scaling.

The contents of carbohydrates and free amino acids (except Thr and Cys) were determined exactly as described (Abbasi et al., 2009), while Cys contents were determined by the same HPLC-based method used for glutathione quantification by Abbasi et al. (2009).

The contents of organic acids and phosphorylated intermediates were determined as follows. Maize leaf material was weighed in liquid nitrogen, ground to a fine powder, divided into aliquots, and stored at −80 °C. Powder aliquots of approximately 50 mg were homogenized in 1 ml of 1 M ice-cold perchloric acid using a precooled mortar and pestle. The remaining was reextracted with 1 ml of 1 M KOH (eluent A) and 100 mM KOH (eluent B) within a Dionex (Dionex) in combination with Analyst 1.4.1 (Applied Biosystems) operated in multiple reaction monitoring mode. The system was controlled by the software Chromelon V5 6.8 and DCMS-Link V5/1.1 (Dionex) in combination with Analyst 1.4.1 (Applied Biosystems). Metabolites were separated on two IonPac AS11HC column (2 × 250 mm; Dionex) protected by an AG11HC guard column (2 × 50 mm). The elution gradient was generated with water (elucent A) and 100 mM KOH (elucent B) within a total run time of 80 min at a flow rate of 0.25 ml min⁻¹ and a column temperature of 35 °C as follows: 0 min; 4%; 0 to 1 min; 4%; 1 to 6 min; 15%, 6 to 12 min; 19%; 12 to 22 min; 20%; 22 to 24 min; 23%; 24 to 27 min; 35%; 27 to 37 min; 38%; 37 to 39 min; 45%; 39 to 44 min; 100%; 44 to 71 min; 100%; 71 to 76 min; 4%; and 76 to 80 min, 4% eluent B. Scan ranges were from mass-to-charge ratio 87 to 606 (precursor ions) and mass-to-charge ratio 59 to 385 (product ions). The electrospay ionization source parameters were −4,500 eV at 600 °C, N₂ gas pressures were 20 p.s.i. (curtaingas), 30 p.s.i. (gas1), and 20 p.s.i. (gas2), and collision gas was set to medium. The dwell time for ions was 75 ms, and scan time per cycle was 3.7 s. Compound-specific parameters are listed in Supplemental Table S1. The contents of metabolites were calculated based on peak areas for precursor/product ion transitions relative to standards.

**Enzyme Activity Assays**

The selective and maximal activities of nitrate reductase and GS activity were determined as described (Gibon et al., 2004) using aliquots of the leaf powder pools that were also employed for gene expression and metabolite profiling.

**Determination of Photosynthetic Parameters**

Photosynthetic parameters from lower systemic leaves (leaf 3 when tumors developed on leaves 4 and 5) were determined between 4 to 11 dpi using a combined infrared gas exchange-chlorophyll fluorescence imaging system (GFS-3000 and MINI-Imaging-PAM Chlorophyll Fluorometer; Walz) at an actinic illumination of 400 and 2,200 μmol m⁻² s⁻¹, 28°C leaf temperature, 350 μmol L⁻¹ CO₂, and 100,000 L⁻¹ water at a background ambient illumination of 350 μmol m⁻² s⁻¹ photon flux density. CO₂ assimilation rates (A) were calculated according to (von Caemmerer and Farquhar, 1981), and ETR was calculated as described (Horst et al., 2008).

**Collection of Phloem Exudates**

At the time points indicated, the lower systemic leaf (leaf 3) was cut with a razor blade while being submerged in 5 ml EDTA, pH 8.0, to prevent clogging of the sieve tubes by callose. Leaves were placed in 1.5 ml of 5 mM EDTA, and the exudates of the first 15 min were discarded. Then, every 30 min, the leaves were transferred to fresh EDTA solution, and the exudates were snap frozen and used for amino acid quantification as described above. Exudation rates were calculated from the linear phase of the exudation.

**Elemental and Mineral Analysis**

Determination of carbon and nitrogen (¹⁴N and ¹⁵N) was performed from finely ground and freeze-dried material with an elemental analyzer (Vario EL; Elementar Analysensysteme). Nitrogen uptake/nitrogen import into a specific tissue from the start of the labeling experiment to the time of harvest (new N) was calculated using the formula:

\[ \text{new N (g g}^{-1} \text{ dry weight)} = (\text{¹⁵N}_{\text{new}} - \text{¹⁵N}_{\text{natural}}) / (\text{¹⁴N}_{\text{total}} - \text{¹⁵N}_{\text{natural}}) \times \text{N}_{\text{natural}} \]

where ¹⁵N_{new} is the atom % of N relative to total nitrogen in the tissue, ¹⁵N_{natural} is the natural abundance of ¹⁴N (0.363 atom %), ¹⁴N_{total} is the atom % of N used to label the plants (see below), and N_{natural} is the nitrogen content of the tissue (g g⁻¹ dry weight). Quantification of mineral nutrients was performed after dry ashing (480°C, 8 h) and dissolving the ash in 6 M HCl with 1.5% (w/v) hydroxylammonium chloride, measuring aqueous 1:10 (v/v) dilutions. Measurements were carried out by inductively coupled plasma optical emission spectroscopy (Spectro Analytical Instruments) as described (Fuehrer et al., 2008).

**¹⁵N Labeling and Determination of ¹⁵N Abundance in Amino Acid Pools**

The incorporation of soil nitrate was determined as follows. Maize plants were watered with 40 ml of 20 mM KNO₃ enriched with 20% [⁰¹⁵N]NO₃ at 8 dpi. Samples of infected and control leaves as well as from the remaining, symptomless aerial organs were taken at 4, 7, 10, 24, and 48 h after administration of [⁰¹⁵N]NO₃. The plant material was ground to a fine powder and used for elemental analysis (see above) and for the determination of ¹⁵N abundance in the free and protein-bound amino acid pools. Proteins were extracted, precipitated with methanol-chloroform-water, and hydrolyzed for 16 h in 6 N HCl at 110°C. The released amino acids from the protein fraction were determined as described below.

For the determination of the reallocation of reduced nitrogen from systemic leaves, 100 μl of 200 mM urea (with 0.85% Silwet L-77 as surfactant) enriched with 98% [⁰¹⁵N]urea was spread evenly onto leaf 3 of maize plants at 8 dpi. After 30 h, samples were taken from the labeled, the infected, and the older and younger systemic leaves and prepared for elemental and amino acid analysis. Amino acids were extracted from leaf material as described above, and ¹⁴N and ¹⁵N amino acid contents were assayed using the HPLC-mass spectrometry (MS) system described for metabolite quantification. Ten microliters of the extracts was separated on a C18RP Acclaim OA guard column (5 μm, 4 × 25 mm; Dionex) protected by an Acclaim OA guard column (5 μm, 4 × 25 mm; Dionex). The elution gradient was generated with 50 mM ammonium acetate, pH 3.5 (elucent A), water (elucent C), and acetonitrile (elucent D) within a total time of 75 min at a flow rate of 0.25 ml min⁻¹ and a column temperature of 30°C as follows: 0 to 2 min, 5% A/95% C; 2 to 20 min, 30% A/70% C; 20 to 25 min, 35% A/65% C; 25 to 30 min, 70% A/30% C; 30 to 35 min, 85% A/15% C; 35 to 40 min, 90% A/10% C; 40 to 45 min, 95% A/5% C; 45 to 50 min, 5% A/95% C/90% D; 50 to 65 min, 5% A/95% C/40% D; 65 to 70 min, 5% A/95% C/80% D; 70 to 75 min, 5% A/95% C. Ionization was performed at +5,500 eV at 500°C. N₂ gas pressures were 10 p.s.i. (curtaingas), 40 p.s.i. (gas1), and 45 p.s.i. (gas2). The interface heater was on, and collision gas was set to medium. Compound-specific parameters were determined by tuning with standard solutions of 1 to 10 mM. Dwell time was set to 50 ms, while the scan time was divided into three periods of 0 to 9.1 min, 9.2 to 13 min, and 13.1 to 53 min with 0.9 s, 1.8 s, and 0.6 s per cycle, respectively. Precursor/product ion transitions that allow the calculation of the abundance of ¹³N and ¹⁵N of selected nitrogen atoms in the respective amino acids were derived from multiple reaction monitoring spectra of standard solutions and are listed in Supplemental Table S2. ¹⁵N-labeled amino acids were recorded with tuning parameters derived from unlabeled isotypes. To calculate ¹⁴N enrichment (%) in a particular amino acid, the peak area of the ¹⁴N-containing fragment was divided by the total peak area of ¹⁵N and ¹⁵N fragment. To account for the natural abundance of ¹⁴C and ¹⁵N, the obtained values were corrected against the background in unlabeled extracts.

**Incorporation of [³⁵S]Met into Proteins**

To determine the incorporation of amino acids into proteins, 7-d-old maize seedlings were watered three times at 2-d intervals with 2 mM [³⁵S]Met. These seedlings were infected as described above. Samples from tumors and control leaves were taken at 8 dpi. Proteins and free amino acids were extracted from finely ground material (100–300 mg) with 500 μl of extraction buffer (50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 0.01% Triton X-100, and Complete Protease
Inhibitor Cocktail [Rochel] before proteins were precipitated by the addition of an equal volume of 20% TCA. The pellet was washed twice with 1 mL of 10% TCA, the supernatants were combined, and radioactivity in the pellet and supernatant fractions was determined by liquid scintillation counting.

**Statistical Analysis of Nonmicroarray Data**

Statistical analysis of metabolite and physiological data was performed with the VANTED software (Junker et al., 2006) using the integrated Welch-Satterthwaite t test.

**Supplemental Data**

The following materials are available in the online version of this article.

**Supplemental Figure S1.** Amino acid composition of sink leaves, source leaves, and tumors.

**Supplemental Figure S2.** Amino acid contents in tumors/control leaves after treatment with $^{15}$N-

**Supplemental Figure S3.** MapMan representation of transcript levels of genes involved in maize protein biosynthesis at 8 dpi.

**Supplemental Figure S4.** Metabolite analysis of upper systemic, symptomless sink leaves of plants infected with *U. maydis*.

**Supplemental Figure S5.** Expression levels of senescence markers in leaf 3.

**Supplemental Table S1.** Ion transitions used for the detection and quantification of metabolites by ion exchange chromatography-MS/MS.

**Supplemental Table S2.** Ion transitions used for the detection and quantification of labeled and unlabeled amino acids by liquid chromatography-MS/MS.

**Supplemental Movie S1.** Confocal projections of *U. maydis*-colonized maize leaf tissue at 8 dpi.

**Supplemental Materials and Methods S1.**

**ACKNOWLEDGMENTS**

We thank Walter Horst (Leibniz University, Hanover, Germany) for suggestions and advice for the $^{15}$N-labeling experiments and for critical reading of the manuscript, Hartmut Wieland (Leibniz University) for the elemental and mineral analyses, and Doreen Zajic (Friedrich-Alexander-Universität Erlangen-Nuremberg) for figure artwork.

Received September 22, 2009; accepted November 12, 2009; published November 18, 2009.

**LITERATURE CITED**

Abbasi AR, Saur A, Hennig P, Tschiersch H, Hajirezaei M, Hofius D, Sonnewald U, Voll LM (2009) Tocopherol deficiency in transgenic tobacco (*Nicotiana tabacum* L.) plants leads to accelerated senescence. Plant Cell Environ 32: 144–157

Agrios GN (2005) Plant Pathology, Ed 5. Elsevier Academic Press, London

Banuett F (1995) Genetics of *Ustilago maydis*, a fungal pathogen that induces tumors in maize. Annu Rev Genet 29: 179–208

Bauer R, Oberwinkler F, Vanky K (1997) Ultrastructural markers and systematics in smut fungi and allied taxa. Can J Bot 75: 1273–1314

Becker TW, Carrayol E, Hirel B (2000) Glutamine synthetase and glutamate dehydrogenase isoforms in maize leaves: localization, relative proportion and their role in ammonium assimilation or nitrogen transport. Planta 211: 800–806

Begerow D, Stoll M, Bauer R (2006) A phylogenetic hypothesis of *Ustilaginomycotina* based on multiple gene analyses and morphological data. Mycologia 98: 906–916

Billet EE, Burnett JH (1978) Host-parasite physiology of maize smut fungus, *Ustilago maydis*. 2. Translocation of C-14-labeled assimilates in smutted maize plants. Physiol Plant Pathol 12: 103–112

Bleecker AB, Patterson SE (1997) Last exit: senescence, abscission, and meristem arrest in Arabidopsis. Plant Cell 9: 1169–1179

Both M, Csukai M, Stumpf MPH, Spanu PD (2005) Gene expression profiles of *Blumeria graminis* indicate dynamic changes to primary metabolism during development of an obligate biotrophic pathogen. Plant Cell 17: 2107–2122

Clark JIM, Hall JL (1998) Solute transport into healthy and powdery mildew-infected leaves of pea and uptake by powdery mildew mycelium. New Phytol 140: 261–269

Dai N, Schaffer A, Petreikov M, Shahak Y, Giller Y, Ratner K, Levine A, Granot D (1999) Overexpression of *Arabidopsis* hexokinase in tomato plants inhibits growth, reduces photosynthesis, and induces rapid senescence. Plant Cell 11: 1253–1266

Divon HH, Fluhr R (2007) Nutrition acquisition strategies during fungal infection of plants. FEMS Microbiol Lett 266: 65–74

Divon HH, Rothan-Denoyes B, Davydov O, Di Pietro A, Fluhr R (2005) Nitrogen-responsive genes are differentially regulated in planta during Fusarium oxysporum f. sp lycopersici infection. Mol Plant Pathol 6: 459–470

Divon HH, Ziv C, Davydov O, Yarden O, Fluhr R (2006) The global nitrogen regulator, FNRI, regulates fungal nutrition-genes and fitness during Fusarium oxysporum pathogenesis. Mol Plant Pathol 7: 485–497

Doehlemann G, van der Linde K, Amann D, Schwammbach D, Hof A, Mohanty A, Jackson D, Kahmann R (2009) Pep1, a secreted effector protein of *Ustilago maydis*, is required for successful invasion of plant cells. PLoS Pathog 5: e1000290

Doehlemann G, Wahl R, Horst RJ, Voll L, Usadel B, Poree F, Stitt M, Pons-Kuehnenmann J, Sonnewald U, Kahmann R, et al (2008a) Reprogramming a maize plant: transcriptional and metabolic changes induced by the fungal biotroph *Ustilago maydis*. Plant J 56: 181–195

Doehlemann G, Wahl R, Vranes M, de Vries RP, Kämper J, Kahmann R (2008b) Establishment of compatibility in the *Ustilago maydis/maize* pathosystem. J Plant Physiol 165: 29–40

Fuehrs H, Hartwig M, Molina LEB, Heintz D, Van Dosselaer A, Braun HP, Horst WJ (2008) Early manganese-toxicity response in Vigna unguiculata L.: a proteomic and transcriptomic study. Proteomics 8: 149–159

Gallais A, Coque M, Le Gouis J, Prioul JL, Hirel R, Quillere I (2007) Estimating the proportion of nitrogen remobilization and of postsilking nitrogen uptake allocated to maize kernels by nitrogen-15 labeling. Crop Sci 47: 685–693

Gallais A, Coque M, Quillere I, Prioul JL, Hirel B (2006) Modelling postsilking nitrogen fluxes in maize (*Zea mays*) using N-15-labelling field experiments. New Phytol 172: 696–707

Gibson Y, Blaesing OE, Hannemann M, Carillo P, Hohn M, Hendriks JHM, Palacios N, Cross J, Selbig J, Stitt M (2004) A robot-based platform to measure multiple enzyme activities in Arabidopsis using a set of cycling assays: comparison of changes of enzyme activities and transcript levels during diurnal cycles and in prolonged darkness. Plant Cell 16: 3304–3325

Gillissen B, Bergemann J, Sandmann C, Schroeter B, Bolker M, Kahmann R (1992) A 2-component regulatory system for self-non-self recognition in *Ustilago maydis*. Cell 68: 647–657

Grenville-Briggs LJ, Avrova AO, Bruce CR, Williams A, Whisson SC, Birch PRJ, van West P (2005) Early amino acid biosynthesis in Phytophthora infestans during appressorium formation and potato infection. Fungal Genet Biol 42: 244–256

Hahn M, Neef U, Struck C, Gottfert M, Mendgen K (1997) A putative amino acid transporter is specifically expressed in haustoria of the rust fungus *Uromyces fabae*. Mol Plant Microbe Interact 10: 438–445

Herbers K, Sonnewald U (1998) Molecular determinants of sink strength. Curr Opin Plant Biol 1: 207–216

Hirel B, Andrieu B, Petreikov M, Shaha Y, Giller Y, Ratner K, Levine A, Granot D (1999) Overexpression of *Arabidopsis* hexokinase in tomato plants inhibits growth, reduces photosynthesis, and induces rapid senescence. Plant Cell 11: 1253–1266

Hoffland E, Jeger MJ, van Beusichem ML (2000) Effect of nitrogen supply rate on disease resistance in tomato depends on the pathogen. Plant Soil 218: 213–247

Holliday R (1961) Genetics of *Ustilago maydis*. Genet Res 2: 204–230

Horst RJ, Engelsdorf T, Sonnewald U, Voll LM (2008) Infection of maize leaves with *Ustilago maydis* prevents establishment of C-4 photosynthesis. J Plant Physiol 165: 19–28

Jensen B, Munk L (1997) Nitrogen-induced changes in colony density and spore production of *Erysiphe graminis* f sp hordei on seedlings of six spring barley cultivars. Plant Pathol 46: 191–202

U. *maydis* Infection Influences Nitrogen Allocation
Okita TW, Sun J, Sakurtingharjo C, Choi SB, Edwards GE, Kato C, Ito H, Matsui H (2001) Increasing rice productivity and yield by manipulation of starch synthesis. In: JA Goode, D Chadwick, eds, Rice Biotechnology: Improving Yield, Stress Tolerance and Grain Quality. John Wiley & Sons, New York, pp 135–152

Olea F, Perez-Garcia A, Canton FR, Rivera ME, Canas R, Avila C, Cazorla FM, Canovas FM, de Vicente A (2004) UCP-regulation and localization of asparagus synthetase in tomato leaves infected by the bacterial pathogen Pseudomonas syringae. Plant Cell Physiol 45: 770–780

Pellier AL, Lauge R, Veneault-Fourrey C, Langin T (2003) CLNRI, the AREA/NIT2-like global nitrogen regulator of the plant fungal pathogen Colletotrichum lindemuthianum is required for the infection cycle. Mol Microbiol 48: 639–655

Rolletschek H, Hosein F, Miranda M, Heim U, Gotz KP, Schleuth A, Borisjuk L, Saalbach J, Wobus U, Weber H (2005) Ecptic expression of an amino acid transporter (VFAAP1) in seeds of Vicia narbonensis and pea increases storage proteins. Plant Physiol 137: 1236–1249

Smart CM (1994) Gene expression during leaf senescence. New Phytol 126: 419–448

Smart CM, Hosken SE, Thomas H, Greaves JA, Blair BG, Schuch W (1995) The timing of maize leaf senescence and characterization of senescence-related CDNAs. Physiol Plant 93: 673–682

Snoeijers SS, Perez-Garcia A, Joosten MHAJ, De Wit PJGM (2000) The effect of nitrogen on disease development and gene expression in bacterial and fungal plant pathogens. Eur J Plant Pathol 106: 493–506

Solomon PS, Oliver RP (2001) The nitrogen content of the tomato leaf apoplastic increases during infection by Cladosporium fulvum. Planta 213: 241–249

Solomon PS, Tan KC, Oliver RP (2003) The nutrient supply of pathogenic fungi: a fertile field for study. Mol Plant Pathol 4: 203–210

Stephenson SA, Green JR, Manners JM, Maclean DJ (1997) Cloning and characterisation of glutamine synthetase from Colletotrichum gloeosporioides and demonstration of elevated expression during pathogenesis on Stenosiphon guineanum. Curr Genet 31: 447–454

Stintzi A, Heitz T, Prasad V, Wiedemann-Merdinoglu S, Kauffmann S, Geoffroy P, Legrand M, Fritig B (1993) Plant pathogenesis-related proteins and their role in defense against pathogens. Biochimie 75: 687–706

Struck C, Ernst M, Hahn M (2002) Characterization of a developmentally regulated amino acid transporter (AAT1p) of the rust fungus Uromyces fabae. Mol Plant Pathol 3: 123–129

Struck C, Mueller E, Martin H, Lohaus G (2004) The Uromyces fabae UfAAT3 gene encodes a general amino acid permease that prefers salt treatment with special emphasis on phloem retranslocation and ion leaching. J Exp Bot 55: 1721–1726

Marschner H (1997) Mineral Nutrition of Higher Plants, Ed 2. Academic Press, London

Martin A, Lee J, Kichey T, Gerentes D, Zivy M, Tatout C, Dubois F, Bailliant T, Valot B, Davanture M, et al (2006) Two cytosolic glutamine synthetase isoforms of maize are specifically involved in the control of grain production. Plant Cell 18: 3252–3274

Martinez-Espinoza AD, Garcia-Pedrajas MD, Gold SE (2002) The Ustilago ginasales as plant pests and model systems. Fungal Genet Biol 35: 1–20

Marzluf GA (1997) Genetic regulation of nitrogen metabolism in the fungi. Microbiol Mol Biol Rev 61: 17

Masclaux C, Valadier MH, Brugiere N, Moret-Gaudry JF, Hrelle D (2000) Regulation of the sink/source transition in tobacco (Nicotiana tabacum L) shoots in relation to nitrogen management and leaf senescence. Plant Cell 11: 510–518

McCann MP, Snetelaar KM (2008) A genome-based analysis of amino acid metabolism in the biotrophic plant pathogen Ustilago maydis. Fungal Genet Biol 45: 577–587

Namiki F, Matsunaga M, Okuda M, Inoue I, Nishi K, Fujita Y, Tsuge T (2001) Mutation of an arginine biosynthesis gene causes reduced pathogenicity in Fusarium oxysporum f. sp melonis. Mol Plant Microbe Interact 14: 580–584

Newingham BA, Callaway RM, Bassirirad H (2007) Allocating nitrogen away from a herbivore: a novel compensatory response to root herbivory. Oecologia 155: 803–818

Nooden LD, Guiamet JJ (2008) A genome-based analysis of amino acid metabolism in the biotrophic plant pathogen Ustilago maydis. Plant Cell 19: 267–274

Oaks A (1992) A reevaluation of nitrogen assimilation in roots. Bioscience 42: 103–111

Oaks A (1994) Primary nitrogen assimilation in higher plants and its regulation. Can J Bot 72: 739–750

Horst et al.