Simultaneous $^{13}$CO$_2$ and $^{15}$NH$_4$NO$_3$ labeling with hydroponically grown seedlings was used to quantify the relative metabolic phenotyping. The established technology displays a breakthrough for quantitative high-throughput plant analysis of herbicides, the approach showed severe disturbance in the synthesis of branched-chain amino acids upon treatment with imazapyr. The technology was applied to rice ($Oryza sativa$) plants at different growth stages. For adult plants, $^{13}$CO$_2$ labeling revealed enhanced carbon assimilation of the flag leaf from flowering to late grain-filling stage, linked to efficient translocation into the panicle. Simultaneous $^{13}$CO$_2$ and $^{15}$NH$_4$NO$_3$ labeling with hydroponically grown seedlings was used to quantify the relative distribution of carbon and nitrogen. Two hours after labeling, assimilated carbon was mainly retained in the shoot (69%), whereas 7% entered the root and 24% was respired. Nitrogen, taken up via the root, was largely translocated into the shoot (85%). Salt-stressed seedlings showed decreased uptake and translocation of nitrogen (69%), whereas carbon metabolism was unaffected. Coupled to a gas chromatograph, labeling analysis provided enrichment of proteinogenic amino acids. This revealed significant protein synthesis in the panicle of adult plants, whereas protein biosynthesis in adult leaves was 8-fold lower than that in seedling shoots. Generally, amino acid enrichment was similar among biosynthetic families and allowed us to infer labeling dynamics of their precursors. On this basis, early and strong $^{13}$C enrichment of Embden-Meyerhof-Parnas pathway and pentose phosphate pathway intermediates indicated high activity of these routes. Applied to mode-of-action analysis of herbicides, the approach showed severe disturbance in the synthesis of branched-chain amino acids upon treatment with imazapyr. The established technology displays a breakthrough for quantitative high-throughput plant metabolic phenotyping.

Metabolically engineered crops are highly desired to ensure the global food supply (Khush, 2003) and to produce various chemicals and materials (Fischer and Emans, 2000; Sharma and Sharma, 2009). Without doubt, tailored plant metabolic engineering requires a good knowledge of the underlying metabolism. This explains the strong interest in tools and technologies to analyze plants on the systems level. Particularly, analysis of plant metabolic pathways and intracellular fluxes by means of isotope experiments, coupled to mass spectrometry (MS) and NMR (Ratliffe and Shachar-Hill, 2006; Young et al., 2011), has predictive power for metabolic engineering (Kruger and Ratcliffe, 2009; Shachar-Hill, 2013). Such analyses, reported for cell suspension cultures (Ronten et al., 2002; Kruger et al., 2007; Williams et al., 2008) and isolated plant organs like leaves (Schafer et al., 1980; Cegelski and Schaef, 2005; Hasunuma et al., 2010), tubers (Roessner-Tunali et al., 2004), and seeds (Schwender et al., 2003; Junker et al., 2007), provide valuable insight into metabolism but fail to describe the behavior of intact, whole plants (Allen et al., 2009), which is necessary to give a systemic picture of metabolic functions under physiologically relevant conditions (Cliquet et al., 1990; Römisch-Margl.
et al., 2007). This is overcome by performing isotope-labeling experiments in entire plants, preferably using \(^{13}\)CO\(_2\), a far safer tracer compound than its radiolabeled equivalent \(^{14}\)CO\(_2\) (Römisch-Margl et al., 2007), combined with labeling analysis by MS and NMR (Chen et al., 2011). Such experiments, however, suffer from extended labeling time periods up to several hours or even days (Hutchinson et al., 1976; Nouchi et al., 1995; Wu et al., 2009) and CO\(_2\) concentrations beyond natural abundance to reach detectable amounts of enrichment (Römisch-Margl et al., 2007). As an example, the application of elevated CO\(_2\) levels can induce changes in plant metabolism (e.g. sink-source ratios, photosynthetic activity, and respiration), thereby affecting carbon sequestration and growth (Arp, 1991; Zhu et al., 2014). Compared with conventional MS (0.05 atom %) and NMR, combustion-isotope ratio mass spectrometry (C-IRMS) provides a far higher precision (0.0002 atom %; Meier-Augenstein, 1999b), which makes this technique particularly attractive to analyze plants at low enrichment. The high precision of C-IRMS even allows for metabolic studies without preceding tracer application (Tcherkez et al., 2003; Yousfi et al., 2012, 2013). Selected applications, which coupled C-IRMS with elemental analysis (EA), have been used to trace turnover and incorporation processes in plants (Clquiet et al., 1990; Dyckmans et al., 2000; Nogués et al., 2004) as well as to analyze plant interactions with the soil microbial community (Griffiths et al., 2004; Leake et al., 2006; Wu et al., 2009). With the availability of gas chromatography (GC)-coupled C-IRMS, enrichment analysis could be targeted to individual metabolic compounds, like sugars, fatty acids, and amino acids (Derrien et al., 2004; Ölsion et al., 2005; Molero et al., 2011; Lattanzi et al., 2012).

Here, we describe a novel approach, which combines \(^{13}\)C- and \(^{15}\)N-based whole-plant studies with subsequent labeling analysis of entire plants, plant tissues, and individual metabolites using EA-C-IRMS and GC-C-IRMS for in vivo metabolic fingerprinting of the model crop rice (Oryza sativa) under physiologically relevant conditions. For the isotope experiments, specific labeling reactors were designed and constructed, which allowed precise \(^{13}\)C-pulse labeling of plants as well as simultaneous \(^{13}\)C and \(^{15}\)N tracing under well-defined environmental conditions. This offered a major benefit compared with previous studies: the applicability of physiological concentrations of \(^{13}\)CO\(_2\) and short labeling periods under highly controlled conditions regarding light, temperature, and humidity. The developed approach was applied to compare rice plants at different developmental stages, concerning assimilation, translocation, and incorporation of label into metabolic intermediates after single or double labeling with \(^{13}\)CO\(_2\) and \(^{15}\)NH\(_4\)NO\(_3\). Particularly, studies combining \(^{13}\)C and \(^{15}\)N label detection in plants are rare so far (Clquiet et al., 1990; Dyckmans et al., 2000). Thereby, the interaction of carbon/nitrogen metabolism and relevant sink-source relations of individual plant organs were studied. Furthermore, a stress-induced phenotype was investigated by exposing rice seedlings to high salinity, a major abiotic stress. The examination of the mode of action of herbicide treatment further underlined the high potential of the technique to reliably quantify metabolic variation. The developed technology provides plant metabolic engineers with a sophisticated tool for fast screening and metabolic profiling of distinct phenotypes, which perfectly complements more demanding isotopically nonstationary metabolic flux analysis (Szecowka et al., 2013; Ma et al., 2014) to get a comprehensive picture of plant metabolic functions.

RESULTS

Construction of Labeling Reactors and Experimental Design

Three sizes and types of labeling reactor were designed, constructed, and validated to evaluate their suitability for in vivo \(^{13}\)C labeling studies (Fig. 1): two tube reactors for \(^{13}\)CO\(_2\) studies with soil-grown adult plants (250 L) and seedlings (100 L), respectively, and a box reactor for combined \(^{13}\)CO\(_2\) and \(^{15}\)NH\(_4\)NO\(_3\) labeling studies with hydroponic seedlings (125 L). For all reactors, the wall material used allowed full transmission of light (Supplemental Fig. S1), and temperature and humidity could be maintained at desired values (Supplemental Fig. S2). Hence, the reactors allowed plants to be labeled under the same light, temperature, and humidity regimen that they were exposed to during growth in a research plant growth cabinet (phyto chamber). Immediate replacement of ambient CO\(_2\) by an equimolar level of \(^{13}\)CO\(_2\) was realized by an absorber unit connected to the reactor. A first set of experiments with soil-grown rice seedlings was conducted to identify the optimum conditions regarding the supply of tracer (400 or 700 \(\mu\)L L\(^{-1}\) \(^{13}\)CO\(_2\)) and the time period of incubation with \(^{13}\)CO\(_2\), \(^{13}\)NH\(_4\)NO\(_3\), and \(^{14}\)CO\(_2\)) (10, 60, and 180 min; Supplemental Fig. S3). Short incubation times of 10 min at ambient levels of \(^{13}\)CO\(_2\) (400 \(\mu\)L L\(^{-1}\)) were sufficient to allow precise estimation of the assimilated carbon, due to the high precision of the EA-C-IRMS measurement (Supplemental Fig. S3A). Labeled under these conditions, the shoots of rice seedlings showed marked \(^{13}\)C enrichment (i.e. a \(\delta\) value of 170\(^{\circ}\) ± 6\(^{\circ}\)). The low deviation underlines that the assimilation was quantified with excellent reproducibility, particularly considering the high complexity of the studied plant system. Enrichment values were in an equal range after simultaneous labeling of one, three, six, or 12 plants in the same reactor for 60 min, indicating that even larger sets of plants and longer incubation times did not result in CO\(_2\) limitation (Supplemental Fig. S3B). In order to examine diurnal effects on photosynthesis and CO\(_2\) assimilation, isotopic labeling studies with seedlings were conducted every 1 h in the time frame of 2.5 to 9.5 h after sunrise. Labeling of plants at different times did not reveal significant differences from plants labeled at midday (6.5 h after sunrise; Supplemental Fig. S3C). Accordingly, the
Figure 1. Equipment designed and constructed for in vivo $^{13}\text{C}$ and $^{15}\text{N}$ labeling studies. A, Large tube reactor (0.5 m diameter, 1.3 m height, and 255 L volume) for $^{13}\text{CO}_2$ labeling of soil-grown adult rice plants. B, Small tube reactor (0.5 m diameter, 0.5 m height, and 98 L volume) for $^{13}\text{CO}_2$ labeling of soil-grown rice seedlings. C, Box reactor (0.5 × 0.5 × 0.5 m, 125 L volume) for simultaneous $^{13}\text{CO}_2$ and $^{15}\text{NH}_4\text{NO}_3$ labeling of hydroponic rice seedlings. All reactors were equipped with a temperature control.
Routine work flow was as follows. Rice plants were grown in a phyto chamber under ambient air, until they were placed inside the enclosure shortly before the labeling experiment. Ambient CO₂ was then removed from the reactor within 30 s and replaced by an equimolar amount of ¹³CO₂ (400 μL L⁻¹). After 10 min of incubation in this atmosphere, plants were removed from the reactor and either harvested directly for assessment of carbon assimilation or cultivated further in the phyto chamber for assessment of carbon translocation. For simultaneous tracing of ¹⁵N, roots of hydroponically grown seedlings were supplied with ¹⁵NH₄NO₃. The isotopic enrichment of harvested plant material and extracted amino acids was determined using EA-C-IRMS and GC-C-IRMS, respectively.

Combined Assessment of Carbon and Nitrogen Metabolism in Rice Seedlings

The developed approach was next used for combined ¹³CO₂ and ¹⁵NH₄NO₃ labeling of hydroponically grown rice seedlings, in order to obtain an integrated picture of carbon and nitrogen metabolism (Fig. 2). Isotopically labeled ¹⁵NH₄NO₃ was provided to the roots, while at the same time, ¹³CO₂ was supplied to the shoot of a rice seedling, using the designed box reactor (Fig. 1C). Immediately after the pulse, maximum ¹³C enrichment was detected in the shoot (450‰ ± 45‰; Fig. 2A), while ¹⁵N labeling was highest for the root (4,130‰ ± 25‰; Fig. 2C). However, the transport of carbon and nitrogen seemed fast, because significant ¹⁵N enrichment at this time point was already found in the shoot (190‰ ± 25‰; Fig. 2B). Likewise, the root contained slight amounts of ¹³C (15‰ ± 5‰; Fig. 2D). Within 2 h after assimilation, ¹⁵N and ¹³C levels were evenly equilibrated. During the ongoing chase period, ¹³C and ¹⁵N enrichments decreased continuously in root and shoot. By integration of the measured ¹³C and ¹⁵N enrichment data, it was now possible to determine the percentage reallocation of label within 2 h after the labeling pulse (Fig. 3), thereby providing fast and quantitative access.

Figure 1. (Continued.)

comprising a water-cooled ventilator at the bottom plate of the reactor and an external cryostat. In addition, an external CO₂ adsorption unit consisted of a high-power pump, an adsorber, and a fine dust filter. Prior to the experiments, the chosen plants were placed into the reactor, which were then closed gas tight by a rubber seal. Ambient CO₂ was removed from the reactor within 30 s. The experiments were started by injecting the desired amounts of ¹³CO₂ through an injection valve in the lid of each reactor.
to relative carbon and nitrogen fluxes in rice plants. The root-to-shoot ratio, calculated from the labeling data via Equation 4 (see “Materials and Methods”), was 0.173. This seemed a proper estimate according to previously reported values for rice between 0.05 and 0.3 (Yoshida, 1981). The majority of assimilated nitrogen (85%) was transported to the shoot (Fig. 3A), whereas only 15% remained in the roots. Regarding assimilated carbon, the major fraction (69%) was retained in the shoot, whereas only 7% was translocated into the roots. A total of 76% of assimilated carbon was thus recovered inside the plant 2 h after assimilation, which indicated a loss of 24% via respiration. The retainment of carbon in the shoot was slightly higher than that for maize (Zea mays) plants, in which 53% of 13C is recovered inside the shoot at elongation (Meng et al., 2013). The same calculation, using labeling data from samples taken 24 h after the pulse (i.e. including a dark period), revealed an increased loss of carbon through respiration by 52% (data not shown), which agreed well with the corresponding data obtained from the measurement of dry matter production, photosynthesis, and respiration (Tanaka and Yamaguchi, 1968).

**Impact of Salt Stress on the Assimilation and Translocation of Carbon and Nitrogen in Rice Seedlings**

Hydroponically grown rice seedlings were exposed to 100 mM sodium chloride for 6 d prior to the labeling experiment. Untreated plants served as a control. Immediately after the labeling pulse, root 15N enrichment was 4,190‰ ± 25‰ in the control plant and only 2,490‰ ± 160‰ in the stressed plant, corresponding to a decreased ammonium uptake of 40% (Fig. 2). In addition, the translocation of nitrogen from root to shoot was strongly impaired, leading to significantly less enrichment (43%-62%) in the shoot of stressed plants compared with control plants at all sampling time points. In contrast, carbon assimilation in the shoot was rather unaltered (Fig. 2A). As described above for untreated control plants, the distribution of carbon and nitrogen was inferred for a stressed plant by integrating 13C and 15N labeling data. The root-to-shoot ratio estimated via Equation 4 was only slightly increased under stress conditions (0.165 ± 0.01 for control plants compared with 0.173 ± 0.03 for stressed plants). Relative carbon distribution did not change in plants exposed to stress (Fig. 3). Nitrogen distribution, however, changed significantly, leading to the recovery of 30% of the assimilated nitrogen in roots compared with 15% in control plants. The amount of labeled nitrogen transported to the shoot was decreased accordingly, resulting in 69% compared with 86% in control plants (Fig. 3B).

**Quantitative Assessment of Carbon Assimilation in Adult Rice Plants**

In the flowering and grain-filling stages, rice plants exhibit different leaf types (i.e. normal leaves and flag leaves). The flag leaf is the uppermost leaf on a culm and the main carbon provider of the panicle when fully developed (Rawson and Hofstra, 1969). Assimilation patterns of plants in different developmental stages, sampled immediately after a 10-min labeling pulse, were compared (Fig. 4). Most of the label was found in the leaves. Plants in the flowering stage showed similar 13C enrichment in normal leaves (260‰ ± 40‰) and flag leaves (240‰ ± 50‰; Fig. 4B). This was also observed for the early grain-filling stage. During late grain filling, however, the flag leaf was more important for
the assimilation of $^{13}$CO$_2$, indicated by significantly higher enrichment ($170\% \pm 6^\circ$ in normal leaves compared with $270\% \pm 30^\circ$ in flag leaves), which is similar to previous findings for wheat (Triticum aestivum; Rawson and Hofstra, 1969) and consistent with its function as a major source organ for carbon transport into the panicle. The stem did not exhibit any enrichment, while the panicle showed low $^{13}$C accumulation during early grain filling ($10\% \pm 5^\circ$; Fig. 4B).

**Quantitative Assessment of Carbon Translocation in Adult Rice Plants**

In order to assess carbon translocation, plants were postcultivated in the phyto chamber after a labeling period of 10 min. Samples taken after distinct chase periods provided a time-resolved $^{13}$C sequestration pattern. Immediately after the $^{13}$CO$_2$ pulse, most of the label was recovered in the leaves (Fig. 4D). Within 2 h of further growth, the $^{13}$C enrichment of flag leaves and normal leaves declined by 45% and 41%, respectively, while the enrichment in the stem and panicle increased. This trend continued until a maximal enrichment was reached after 24 h for the panicle (100% $\pm 20^\circ$) and after 48 h for the stem (50% $\pm 15^\circ$; Fig. 4D). As for the seedling experiments, the data for the adult plants showed high precision and reproducibility.

**Quantification of $^{13}$C and $^{15}$N Amino Acid Enrichment Using GC-C-IRMS**

Using GC-C-IRMS, the measurement of labeling enrichment was extended to amino acids. For each amino acid eluting from the GC column, the $^{13}$C and $^{15}$N
enrichment was given by the ion intensity at a mass-to-charge ratio (m/z) of 45 and 29, reflecting 13CO2 and 14N15N, respectively, formed by combustion in the instrument. For 16 amino acids, satisfying signal quality was generally obtained and provided precise estimates of their labeling status. Hereby, the amino acid pairs Glu/Gln and Asp/Asn were each quantified as lumped pools, due to conversion of the carboxamides Asn and Gln into their corresponding acids during the protein hydrolysis step of sample processing (Wittmann, 2007). Four amino acids (Cys, Met, Trp, and Arg) were not accessible because they were degraded during this treatment (Wittmann, 2007). Generally, 5 mg of lyophilized material was sufficient to provide high-quality data, independent of the type of tissue processed. The conditions used for extraction, precipitation, and derivatization of protein from lyophilized plant material was crucial with regard to the yield and purity of the amino acids obtained. In contrast to extraction in Tris buffer (pH 8.8), extraction in hot water (100°C, 15 min) yielded much less protein (data not shown). Similarly, precipitation of the extracted protein in ice-cold 10% (w/v) TCA was less efficient than precipitation in ice-cold acetone (data not shown). Additional tests with alternative derivatization agents revealed that trimethylsilyl derivatives of the amino acids (e.g. obtained with trimethylsilyl-trifluoroacetamide) were not fully separated by GC. Even a set of variations in the temperature profile did not allow full baseline separation for the amino acids Met/Asp, Ile/Pro, and Glu/Phe. Full baseline separation was necessary, however, as the combustion of analytes into CO2 and N2 prior to detection does not allow one to discriminate between overlapping analyte peaks. Derivatization with methyl-t-butyldimethylsilyl-trifluoroacetamide into t-butyldimethylsilyl derivatives finally led to full baseline separation of the target analytes. Some of the seedling samples, however, did not provide an unambiguous signal for Tyr, probably due to matrix overlay. Due to the fact that interference with background noise or matrix effects leads to false results in isotope experiments (Wittmann, 2007), this amino acid was partly excluded from further interpretation. It also turned out that the addition of dimethylformamide, commonly used to process microbial samples (Wittmann, 2007), was not compatible with the GC-C-IRMS instrument used, because the solvent was largely transferred into the combustion chamber. Accordingly, derivatization was conducted without the addition of solvents. Tests with different incubation times (30, 60, 90, and 120 min) and temperatures (80°C, 90°C, and 100°C) revealed that the combination of 60 min and 80°C provided the optimum signal-to-noise ratio.

Isotope Distribution Patterns

Dynamic Incorporation of Carbon into Protein Amino Acids

The developed protocol was now used to quantify 13C distribution patterns in rice related to amino acid metabolism (Fig. 5). A first study with seedlings revealed that enrichment increased with time, as 13C was incorporated continuously into cell protein. Amino acids differed strongly with regard to label incorporation, which seemed to correlate to their biosynthetic origin. Particularly, Tyr and Phe, stemming from intermediates of the Embden-Meyerhof-Parnas (EMP) pathway (phosphoenolpyruvate) and of the non-oxidative pentose phosphate (PP) pathway (erythrose 4-phosphate), were enriched rather fast and to a greater extent than all other amino acids and contributed up to 9% and 15% of the entire enrichment, respectively (Fig. 5A). Immediately after the pulse, significant 13C incorporation also was observed for amino acids stemming from pyruvate, another intermediate of the EMP pathway (i.e. Ala, Ser, Gly, Val, and Leu). In contrast, labeling enrichment was delayed for amino acids synthesized from 2-oxoglutarate and oxaloacetate, intermediates of the tricarboxylic acid cycle (Fig. 5A). Generally, highest enrichments were detected 24 h after the labeling pulse. After 48 h, the enrichment declined for most amino acids, probably due to a dilution with 13CO2 taken up during the postlabeling incubation. The 13C labeling profiles of Gly and Ser were similar, indicating that they originate from the same metabolic precursor, 3-phosphoglycerate (Wittmann, 2007). The same trend also was found for other amino acid families, like the Asp family of amino acids, with Thr, Lys, Ile, and Asp itself. In comparison with rice seedlings, the general amino acid enrichment was, on average, 8 times lower in the leaf of an adult plant during the late grain-filling stage (Fig. 5A). This was interesting because the total assimilation of carbon of both tissue types was in the same range (Fig. 4B; Supplemental Fig. S3) and matches perfectly with the changing role of leaves from a strong sink during development to a major source during reproduction, assimilating but not incorporating carbon dioxide (Thrower, 1962; Turgeon, 1989). Low enrichment was equally observed for normal leaves (60 ± 10% total amino acid enrichment), flag leaves (70% ± 10% total amino acid enrichment), and stem (40% ± 25% total amino acid enrichment) within 1 d of label translocation (Fig. 5B). In contrast, the panicle of adult rice plants was much more active regarding amino acid metabolic pathways (280% ± 30% total amino acid enrichment). Here, highest enrichment was detected for Phe (39% ± 3%), and Tyr (30% ± 2%), followed by Ala (28% ± 4%), Glu (23% ± 2%), and Leu (22% ± 3%), which was in accordance with the pattern detected for rice seedlings. The other amino acids showed lower enrichment (Fig. 5B).

Combined 13C and 15N Incorporation into Protein Amino Acids

Amino acids of the shoot were analyzed for their 13C and 15N enrichment from combined 13CO2 and 15NH4NO3 labeling experiments with hydroponically grown rice seedlings (Fig. 6). For six selected amino
Figure 5. Incorporation of $^{13}\text{C}$ into protein amino acids upon $^{13}\text{CO}_2$ labeling. Enrichment of extracted amino acids was analyzed by GC-C-IRMS. A, Time-resolved pattern of a rice seedling (top, age of 12 d) and an adult rice plant at late grain filling (bottom, age of 88 d), for which soil-grown plants were labeled with 400 μL $^{13}\text{CO}_2$ for 10 min and then harvested directly or further cultivated at ambient air for 2, 4, 24, and 48 h prior to harvesting. Statistical analysis was conducted using Student’s $t$ test, whereby significant differences ($P \leq 0.05$) between seedling and adult leaves are marked with asterisks. B, Tissue-resolved pattern of an adult rice plant at late grain filling, for which plants were labeled with 400 μL $^{13}\text{CO}_2$ for 10 min followed by 24 h of cultivation at ambient air prior to harvesting of leaf, flag leaf, stem, and panicle. Statistical analysis was done by one-way ANOVA with Tukey’s test, whereby different letters (a, b, or c) indicate significant differences between means of the different tissues. C, Tissue-specific amino acid metabolism, visualized as Venn diagrams, which display the relation of amino acids based on the statistical significance between measured $^{13}\text{C}$ enrichments. Significant differences between means of amino acid $^{13}\text{C}$ enrichments ($P \leq 0.05$) were determined by one-way ANOVA with Tukey’s test. Amino acids that do not show significantly different $^{13}\text{C}$ enrichment are located in equally colored ellipses. The enrichment data are provided as mean values ($n = 3$) and reflect atomic percent excess corrected for natural isotopes. The full data sets are given in S4 to S6, respectively. To facilitate comparison, the data are normalized for the highest enrichment of each data set, which was set to 100%, and visualized by a color code between yellow (0%) and red (100%). CIT, Citrate; E4P, erythrose 4-phosphate; F6P, Fru-6-P; FUM, fumarate; GAP, glyceraldehyde 3-phosphate; G6P, Glc-6-P; MAL, malate; OAA, oxaloacetate; 2OG, 2-oxoglutarate; PEP, phosphoenolpyruvate; 3PG, 3-phosphoglycerate; PYR, pyruvate; RSP, ribose 5-phosphate; RUSP, ribulose 1,5-bisphosphate; SDL, seedling shoot; SUC, succinate.
acids (i.e. Ala, Gly, Pro, Ser, Asp, and Glu), root-based data with satisfying quality could be derived, which allowed a tissue-specific examination, at least for these molecules (Fig. 6). Immediately after labeling, strong 13C enrichment was detected for Ser, Asp, and Glu of root protein. At the same time, Ala, Gly, and Ser, extracted from shoot protein, revealed significant 13C enrichment (Fig. 6). Within 2 h, labeled compounds were distributed inside the plant, leading to combined 13C- and 15N-enriched amino acids in shoot and root. Although all amino acids exhibited significantly different 13C and 15N enrichment, the strongest differences were found for shoot amino acids. Ala and Ser exhibited higher 13C enrichment, whereas Pro, Asp, and Glu were more strongly enriched with 15N. Four hours after the labeling pulse, similar amino acid enrichment patterns of shoot and root indicated the equilibration of label between these organs (Fig. 6). Amino acid enrichment patterns were similar for soil-grown and hydroponically grown rice seedlings (Supplemental Fig. S4), indicating that hydroponic cultivation did not significantly influence at least this part of metabolism.

**Mode-of-Action Analysis: Effect of Imazapyr Treatment on Rice Seedlings**

Herbicide treatment was used as a proof of concept to demonstrate the potential of the established technology for mode-of-action studies. Imazapyr was chosen as a widely used herbicide, with a well-described mechanism of action (i.e. the inhibition of acetolactate synthase in branched-chain amino acid biosynthesis). The studies were conducted using 12-d-old, soil-grown rice plants, which were exposed to imazapyr treatment or to a control treatment. Plants were pulse labeled with 13CO2 4 h after herbicide application (Fig. 7). At the time point of the labeling experiment, no phenotypic alterations of plant morphology, as compared with the control plants, were observed (data not shown). The overall carbon assimilation at this time point was not disturbed, as indicated by equal 13C shoot enrichment of stressed and control plants, namely 586% ± 26% and 595% ± 16%, respectively (Fig. 7A). However, strongly diminished label incorporation was detected in proteinogenic amino acids, which was particularly pronounced for the branched-chain amino acids 2 h after the labeling pulse (Fig. 7B). Amino acid enrichment of stressed plants reached 19% to 75% of the enrichment detected in control plants, except for the branched-chain amino acids, which only reached 4% to 11%. On average, the enrichments were 2.7 times lower in imazapyr-treated plants than in control plants, with the exception of branched-chain amino acids, which exhibited 7 to 26 times less 13C enrichment. Observable phenotypic effects became evident 7 d after imazapyr application and comprised inhibition of growth, chlorosis of above-ground plant parts, and dieback of young leaves (Fig. 7C).

**DISCUSSION**

The quantitative analysis of pathway function and regulation is key to understanding and engineering plant physiology (Maliga and Graham, 2004). Here, we developed a cost-effective, high-throughput approach to assess whole-plant metabolic phenotypes in vivo. As shown, we coupled 13CO2 and 15NH4NO3 pulse studies under precisely controlled physiological conditions with ultra-precision GC-C-IRMS for parallel 13C and 15N labeling analysis in different plant tissues and even individual molecular compounds to elucidate plant metabolic traits. Different tube reactor layouts enabled 13CO2 isotope experiments with soil-grown seedlings and adult plants, up to a height of about 1 m (Fig. 1, A and B), whereas parallel 13CO2 and 15NH4NO3 labeling with hydroponic plant cultures could be conducted in a specific box reactor (Fig. 1C). In contrast to previous techniques (Tanaka and Osaki, 1983; Nouchi et al., 1995; Römisch-Margl et al., 2007), our methodology minimizes potential alterations of the studied plant during the experiment due to an atmosphere with ambient CO2 levels, controlled temperature, humidity, and illumination, and incubation times of only 10 min. The carefully experimental layout provided data with high precision and reproducibility among replicates, independent of the developmental stage of the analyzed plants. Assimilation and translocation of carbon in different tissues (Figs. 3 and 4) and even individual molecules (Fig. 5) could be quantified at deviations below 20% for adult plants. Given the fact that plants in advanced developmental stages are rather complex and subject to a certain biological variation, due to differentiation into specific organs and cell types, the precision and reproducibility can be regarded as excellent. For seedlings, they were partially even higher (Figs. 6 and 7). This allowed us to accurately discriminate metabolic properties differing only slightly, which seems a valuable characteristic of our approach.

**Assessment of Important Characteristics of Carbon Metabolism**

Beyond technical precision and accuracy, it was further important to validate the extent to which the method could assess key properties of plant physiology. A catalog of experiments addressed this issue. Grain yield, the most prominent characteristic of crop plants, is strongly dependent on the source-sink relationship between plant parts (Kato et al., 2004), whereby the operational mode of a distinct plant organ, as source or sink, changes throughout development (Meng et al., 2013). Pioneering studies with rice plants, using radioactive 14CO2 at high dosage, had revealed the translocation of assimilated carbon into the panicle during grain filling (Cock and Yoshida, 1972). This was quantitatively assessed here, however, by much safer and easier handling using stable 13CO2. Most of the assimilated carbon was recovered inside the rice panicle 24 h after the pulse (Figs. 4D and 5B). In line with...
this, a 90% decrease of $^{13}$C in the assimilating tissue of plants from the late grain-filling stage was detected during this chase period (Fig. 4D), which is in the range of values reported for other plants (Leake et al., 2006). During development from flowering to late grain filling, the relative contribution of different leaves to carbon assimilation probably corresponds to changing metabolic requirements (Meng et al., 2013). During late grain filling, the lower activity of carbon assimilation in mature leaves, relative to the flag leaf, indicated mechanisms of senescence (Fig. 4). In senescing leaves, the Rubisco content and, as a consequence thereof, the photosynthetic activity decrease rapidly. Therefore, mature leaves cannot assimilate as much $^{13}$CO$_2$ as younger leaves (Makino et al., 1984). At night, $^{13}$C was further lost by respiration. Additional carbon loss might have resulted from a combination of translocation to tissues not analyzed (e.g. the roots of plants grown on soil) and dilution of the label by overall plant biomass increase during the chase period (Leake et al., 2006).

**Integrated Analysis of Carbon and Nitrogen Metabolism**

The setup furthermore allowed combined labeling studies with $^{13}$CO$_2$ and $^{15}$NH$_4$NO$_3$, which are interesting due to the close connection of carbon and nitrogen metabolism in plants but have been conducted only rarely (Cliquet et al., 1990; Dyckmans et al., 2000). The studies highlighted fast interorgan distribution of carbon and nitrogen in seedlings within 2 h after assimilation (Fig. 2), whereby the shoot operated as a major sink and exhibited a 10- and 6-fold higher demand for carbon and nitrogen compounds, respectively, compared with the root (Fig. 3A), which reflects sufficient supply with water and nutrients, especially nitrogen (Wilson, 1988; Peuke et al., 1994). Obviously, the examined seedlings received all essential macronutrients and micronutrients and were exposed to optimal breeding conditions, concerning light, temperature, and humidity, so that carbon and nitrogen were used mainly for shoot growth and development. The relative flux maps for carbon and nitrogen, giving the relative distribution among the different tissues on the basis of isotope enrichment data, provide a straightforward snapshot of plant metabolism (Fig. 3). Given the available instrumentation, such insights can be easily provided within 1 d after an isotope experiment and thus allow for a fast evaluation (e.g. to study environmental stresses as demonstrated for salt-stressed rice; Figs. 2 and 3B). In light of breeding stress-tolerant plant lines, an important area of application is metabolic phenotyping in order to identify protective mechanisms (Cramer et al., 2011) and changes in sink-source relations (Roitsch, 1999; Albacete et al., 2014). Our data reflect these features for plants treated with salt, a major...
abiotic factor, particularly affecting the growth and productivity of salt-sensitive crops (Roy et al., 2014). Salt stress resulted in a strong perturbation of nitrogen uptake by the root and transport to the shoot, whereas carbon assimilation and translocation were not significantly affected. Stressed plants kept relatively more nitrogen in the root as compared with nonstressed plants (Fig. 3), probably to support the supply with nitrogen-containing compatible solutes (Wang et al., 2012). Furthermore, enhanced retention of nitrogen compounds in the root might be due to adaptive mechanisms to ensure adequate water and nutrient acquisition from the rhizosphere (Sharp et al., 2004).

Labeling Dynamics of Amino Acids Provide Qualitative Insight into Metabolic Pathways

The coupling of C-IRMS with GC separation, together with the development of suitable protocols for sampling and sample processing, provided time-resolved enrichment data for protein-derived amino acids across different tissues of rice. Amino acids are the analytes of choice for microbial pathway analysis, because they are much more abundant in cell extracts and protein than their precursors and provide extensive labeling information (Wittmann, 2007). On basis of the underlying biosynthetic precursor amino acid relationship, it is easy to deduce the labeling patterns of the precursor metabolites from labeling patterns of the corresponding amino acids.

The $^{13}$C labeling of amino acids from the same biosynthetic family was similar and obviously reflected the enrichment of their common precursor (Fig. 5A). In the seedling, amino acids stemming from precursors of the EMP pathway and the nonoxidative PP pathway exhibited fast and strong $^{13}$C enrichment (Fig. 5A, top), examples being Phe, Tyr, Ser, Gly, and His. In contrast, Glu, Pro, Asp, and Thr, originating from tricarboxylic acid cycle-based precursors, were labeled much slower and weaker. Although this does not directly provide quantitative flux rates, the $^{13}$C enrichment data reveal a fast and strong influx of $^{13}$C into the EMP pathway and the nonoxidative PP pathway, indicating high activities of these routes. The rather low enrichment of tricarboxylic acid cycle-related amino acids most likely reflects a down-regulation of the cycle in the light (Tcherkez et al., 2005). In addition, the slow dynamics could be an indication that Glu, Asp, and amino acids derived from them are not formed in the shoot but rather are synthesized in the root, followed by transport into the shoot. This is indeed taking place, which is discussed below on the basis of integrated $^{13}$C and $^{15}$N labeling data (Fig. 6). The $^{13}$C enrichment in the shoot of a seedling was about 8- to 10-fold higher than that in the leaf of an adult rice plant (Fig. 5A, bottom). Adult leaves assimilated the provided $^{13}$CO$_2$ to a high extent (Fig. 4) but incorporated only little of it into protein. This matches with the changing role of leaves from sink to source during reproduction, assimilating but not incorporating carbon dioxide (Thrower, 1962; Turgeon, 1989).
Labeling Dynamics of Amino Acids Indicate Tissue-Specific Metabolism

The $^{13}$C labeling of amino acids differed largely between individual tissues. For rice at late grain filling, leaf, flag leaf, and stem incorporated only little carbon into protein (Fig. 5B). The panicle showed the highest $^{13}$C enrichment among all tissues. Most assimilated carbon was hence translocated into the panicle, where it was incorporated into proteinogenic amino acids. The panicle exhibited an active de novo protein biosynthesis, reflecting that filling of the grains results in a strong demand for protein precursors (Cock and Yoshida, 1972). A compound-oriented view, based on the statistical significance of labeling patterns, groups amino acids into colored ellipses according to their labeling similarity (Fig. 5C). This immediately highlights specific metabolic fingerprints for all tissues of rice. Leaf and flag leaf differed only in a few amino acids, whereas stem and panicle revealed drastically altered patterns and evidence for a tissue-specific carbon metabolism. This type of visualization further highlighted strong differences between the carbon metabolism of an adult leaf and a young shoot.

Labeling Dynamics Provide Spatial Resolution of Amino Acid Metabolism

Combined $^{13}$C and $^{15}$N labeling of hydroponic rice seedlings showed an immediate occurrence of $^{15}$N-labeled Glu/Gln and Asp/Asn in the root, whereas the $^{13}$C-labeled forms appeared much later (Fig. 6C). Obviously, these amino acids were formed from carbon precursors in the root through $^{15}$NH$_4$ assimilation and transamination (Lam et al., 1995), which explains the exclusive enrichment with $^{15}$N (Fig. 6, A and C). Subsequently, they were transported into the shoot (Funayama et al., 2013) to serve as amino group donors for the biosynthesis of other amino acids (Kiyomiya et al., 2001). Glu, with its rather high $^{15}$N and low $^{13}$C enrichment, synthesized in the root and transported to the shoot (Fig. 6B, 2-h time point), is the precursor for the de novo synthesis of Pro, exhibiting a similar labeling pattern.

The immediate $^{13}$C enrichment (Fig. 6B, 0-h time point) indicates a fast biosynthesis of Ala, Gly, and Ser in the shoot. For Gly and Ser, this may be due to photorespiration, which typically leads to high turnover rates for these amino acids (Gauthier et al., 2010). Overall, this provides a spatially resolved picture of amino acid metabolism (Fig. 6), which explains the different dynamics of $^{13}$C and $^{15}$N enrichment. From similar effects after simultaneous labeling of rape (Brassica napus) plants with $^{13}$C and $^{15}$N, it has been hypothesized that the incorporation of assimilated $^{13}$C into amino acids is not tightly connected to nitrogen assimilation (Gauthier et al., 2010). This seems to also hold for rice.

The Response of Rice to the Herbicide Imazapyr Comprises Impaired Biosynthesis of Branched-Chain Amino Acids and Enhanced Protein Turnover

Upon treatment with imazapyr, the biosynthesis of Val, Leu, and Ile was strongly inhibited (Fig. 7B), observable already 6 h after treatment. The overall carbon assimilation of the treated plant, however, was unaffected (Fig. 7A). This distinct phenotype perfectly matches with the known mode of action of imazapyr. The herbicide is highly target specific. The enzyme acetohydroxyacid synthase, a key step in branched-chain amino acid biosynthesis, is the only site of action of this herbicide (Tan et al., 2005). In the longer term, imazapyr causes a dysfunction of cell growth as well as disruptions of DNA and protein biosynthesis and increased protein turnover to recycle branched-chain amino acids (Shaner and Reider, 1986; Scarpini et al., 1995; Royuela et al., 2000). As there was no more de novo synthesis, Val, Leu, and Ile exhibited lowest enrichment ($3\% \pm 3\%$ to $10\% \pm 5\%$) among all amino acids. In addition, our analysis demonstrates that metabolic changes at this early time point were not restricted to branched-chain amino acids as primary targets. In fact, metabolism was affected much more globally. Newly assimilated $^{13}$C was incorporated into protein to a lesser extent in herbicide-treated plants than in control plants (Fig. 7B). Growth defects were evident 7 d after imazapyr application (Fig. 7C), indicating a slow rate of plant death, which is related to the amount of intracellularly stored amino acids (Shaner and Singh, 1991). In this regard, our profiling toolbox appears valuable to study the mode-of-action of synthetic compounds at the initial level of plant phenotyping, the first level of a three-tiered approach for mode-of-action identification (Tresch, 2013).

CONCLUSION

Taken together, the developed approach is suitable to conduct stable isotope-labeling experiments for in vivo whole-plant metabolic profiling. The high technical data quality achievable under physiologically relevant conditions seems particularly promising to be applied to (1) stress-response studies, (2) phenotypic screening of plant lines, (3) mode-of-action studies, and (4) integrated analysis of carbon and nitrogen metabolism, among others. The underlying tracer studies require little time and low effort compared with model-based approaches and are potent as a screening tool (Schwender, 2008). Clearly, this type of analysis does not, per se, provide quantitative intracellular fluxes through individual reactions and pathways, but it can suggest metabolic activities (i.e. reallocation profiles or metabolic fingerprints). In this regard, our approach provides a useful complementation to the more demanding approaches of isotopically nonstationary metabolic flux analysis (Szcówka et al., 2013; Ma et al., 2014). The latter offers the greatest potential for quantitative resolution of metabolic fluxes in autotrophic plants but
involves extreme experimental and computational effort, high cost, and low throughput to derive flux information. In this regard, future combinations of both technologies for two-tiered complementary analysis could provide plant physiologists with a sophisticated toolbox for initial screening of several distinct phenotypes, thereby identifying the most promising ones for more comprehensive analysis. The high-precision GC-C-IRMS analysis, demonstrated here for amino acids, might be easily widened to fatty acids (Meier-Augenstein, 2002; Richter et al., 2010; Panetta and Jahnren, 2011), sterols (Jones et al., 1991), monosaccharides (Docherty et al., 2001; Derrien et al., 2003), and lignin (Göhi and Eglinton, 1996), which are all accessible via such an instrumentation. This promises to extend the approach to other pathways of primary metabolism (e.g. lipid, sugar, and cell wall biosynthesis) as well as to pathways of secondary metabolism (e.g. involving phenol, isoprenoid, and alkaloid biosynthesis).

MATERIALS AND METHODS

Plants
Rice (Oryza sativa ssp. japonica ‘Nipponbare’) plants were obtained from CropDesign.

Chemicals
As a tracer, $^{13}$CO$_2$ (greater than 99 atom % $^{13}$C) was purchased from Eurisotop. Labeled $^{15}$NH$_4$NO$_3$ (greater than 98 atom % $^{15}$N) was purchased from Sigma-Aldrich.

Media
Plants were grown either on soil (Einheitserde type GS90; 70% organic peat and 30% clay, pH 5.5–6; Einheitserde- und Humuswerke Gebr. Patzer) or on hydroponic medium (1.43 mM $^{15}$NH$_4$NO$_3$, 1 mM calcium chloride hexahydrate, 0.38 mM magnesium sulfate heptahydrate, 1.32 mM potassium sulfate, 0.32 mM monosodium phosphate, 1 mM ferric ethylenediaminetetraacetic acid, 8 mM manganese [II] chloride tetrahydrate, 0.15 µM zinc sulfate heptahydrate, 0.15 µM copper [II] sulfate pentahydrate, 0.075 µM ammonium heptamolybdate, and 1.39 µM boric acid; based on Yang et al., 1994). In isotope experiments, naturally labeled NH$_4$NO$_3$ was replaced by an equimolar amount of $^{15}$NH$_4$NO$_3$.

Plant Growth Conditions
Rice seeds were germinated on moist filter paper in a petri dish for 4 d at 26°C in the dark. The seeds were transferred into light (500 µmol m$^{-2}$ s$^{-1}$ photosynthetically active radiation) 1 d before they were transplanted either into 0.7-dm$^3$ pots used for further cultivation on soil or into hydroponic boxes. Prior to sowing, pots were soaked with deionized water containing 0.15% of the fungicide proplant (Stähler). Seeds were subjected to hot water treatment (60°C for 10 min) to prevent sheath rot. Rice plants were then grown under a 13/11-h day/night cycle at an average irradiance of 500 µmol m$^{-2}$ s$^{-1}$ photosynthetically active radiation during the light phase (Powerstar HQT-400W; Osram), a temperature cycle of 26°C/21°C, and a relative humidity of 60%. During the first 14 d of development, plants were irrigated two times per day with deionized water. During the first 3 weeks, plants were watered via top irrigation and later via subirrigation. Between weeks 3 and 10, plants were fertilized with Hakaphos-Blau solution (0.3% [v/v] in deionized water; Compo) twice per week, replacing one deionized water treatment, respectively. Plants in different developmental stages were used for $^{13}$CO$_2$ labeling experiments. Rice seedlings were generally used for experimental purposes after 12 d of cultivation. Flowering plants, as well as plants from the early and late grain-filling stages, were examined at 68, 75, and 88 d, respectively. For hydroponic cultures, plants were grown in hydroponic containers (27 × 17 × 12 cm) covered with a perforated Styrofoam plate. Plastic meshes were placed inside the holes (2 cm diameter) on which pregerminated seedlings were cultivated with their roots freely suspended in the hydroponic medium. Rice seedlings were generally used for experimental purposes after 12 d of cultivation. The hydroponic crops were incubated under the same conditions regarding light, temperature, and humidity as soil-grown plants.

High-Salt Treatment
In salt stress experiments, hydroponically growing seedlings were subjected to high-salt treatment for 6 d. For that purpose, 100 mM NaCl was added to the growth medium.

Imazapyr Treatment
Prior to the labeling experiment, soil-grown seedlings were subjected to imazapyr treatment, a nonselective herbicide of the imidazolinone group. The imazapyr solution (0.3 mM imazapyr [BASF], 0.1% [v/v] dimethyl sulfoxide, and 0.1% [v/v] Dash E.C. [BASF]) was applied with an air brush to reflect a dosage of 62.5 g ha$^{-1}$. Control plants were subjected to a solution containing only the solvents used (0.1% [v/v] dimethyl sulfoxide and 0.1% [v/v] Dash E.C.). Labeling experiments were conducted 4 h after herbicide application.

Isotopic Labeling Experiments with $^{13}$CO$_2$

The labeling experiments were conducted in specifically constructed labeling reactors (Fig. 1). A large tube reactor (0.5 m diameter, 1.3 m height, and 255 L volume) was designed for $^{13}$CO$_2$ isotope experiments with soil-grown adult plants (Fig. 1A), and a small tube reactor (0.5 m diameter, 0.5 m height, and 98 L volume) served for $^{13}$CO$_2$ isotope experiments at the seedling stage (Fig. 1B). The tube reactors were built from Plexiglas acrylic sheets (5 mm thickness; Hans Keim Kunststoffe). The material allowed full spectral transmission of sunlight at wavelengths between 400 and 900 nm, containing the essential spectral interval for plant growth (Supplemental Fig. S1). Ethylene propylene diene monomer rubber (Mercateo) was used as sealing material, because of its high flexibility and endurance. The rubber sealing was agglutinated to the Plexiglas or polycarbonate sheets with solvent-free glue (Loctite 406; Henkel). The tube was conglutinated to the lid with a two-component adhesive (Pattex Stabilit; Henkel). For the maintenance of a constant temperature, a cooling system with a water-cooled ventilator (Peltier cooler/heatex; 380 W; Uwe Electronic) connected to an external cryostat (Lauda RMT20; Lauda Dr. R. Wobser) was installed. The ventilator and the connectors were attached to the bottom plate of the reactor. To keep the humidity level at or above 60% of saturation during the experiments, water-soaked cloth was placed in a glass beaker inside the enclosure. Temperature and relative humidity were monitored online by a humidity and temperature logger (Voltcraft DL-120 TH; Conrad Electronic). In addition, the CO$_2$ concentration was measured (Voltcraft CM-10; Conrad Electronic). All reactors allowed a precise adjustment of the $^{13}$CO$_2$ level. For this purpose, they contained two perforated pipes, one in the upper back part (exhaust air) and one in the lower back part (supply air). These were connected to a CO$_2$ adsorption unit, consisting of a high-power pump (6,000 L min$^{-1}$; Scoprega), a fine dust filter (Filter Cartridge A; Kärcher), and a CO$_2$ adsorber (5 L; Drägersorb 800+; Drägerwerk). All power supplies, except for the external absorber pump, had 24-V D.C. to allow safe handling. All labeling reactors were installed inside the phyto chamber (Svalöf), directly beside the external absorber pump, had 24-V D.C. to allow safe handling. All labeling reactors were installed inside the phyto chamber (Svalöf), directly beside the enclosure. Hereby, the soil of the plant pots was covered with plastic wrap to avoid potential interference of CO$_2$ respired from soil with the defined gas atmosphere. The ambient CO$_2$ was removed (less than 20 µL L$^{-1}$) by adsorption for a short time period of about 30 s as described above. Immediately after purging, the desired amount of $^{13}$CO$_2$ was injected through a valve (screw cap with silicone diaphragm), positioned in the lid of each enclosure, and the plants were then incubated for a defined labeling period. Afterward, the plants or specific parts were harvested directly or cultivated further in the phyto chamber under ambient air prior to analysis. All harvested plants or plant tissues were immediately deep frozen in liquid nitrogen to stop metabolic activity. At each sampling time point, at least three biological replicates were obtained. In the case of seedling experiments, the whole shoot was harvested. Roots were
collected from hydroponically grown plantlets and treated equally. Of a full-grown plant, five tillers were harvested, whereby flag leaf, leaf, stem, and panicle were separated, followed by immediate quenching in liquid nitrogen. The individual tissues originating from the five tillers were pooled. The outermost, photosynthetically active leaves were removed from the stem prior to further treatment. Experiments were generally performed at 4 and 8 h after sunrise.

**Combined 13C and 15N Labeling Experiment**

A box reactor (carbon/nitrogen reactor; 0.5 × 0.5 × 0.5 m, 125 L volume) was developed for combined 13CO2 and 15NH4NO3 labeling with hydroponic rice cultures (Fig. 1C). Ammonium nitrate was selected because it generally serves as a nitrogen source and is taken up by the roots. Rice prefers ammonium over nitrate, as demonstrated by the higher uptake rate. Compared with nitrate, its uptake is less dependent on light intensity and oxygen concentration at the root site (Susakawa and Yamamoto, 1978). The reactor was built from polycarbonate (5 mm thickness; Hans Keim Kunststoffe), which allowed full spectral transmission of sunlight (Supplemental Fig. S1). Plants were pregrown in the phyto chamber as hydroponic cultures. Prior to the experiment, cultures were transferred from naturally labeled growth medium to a container with 15NH4NO3. The temperature of 120°C was kept for 2 min. Afterward, the temperature was increased at a rate of 8°C min−1 until 200°C. In a second gradient step, the temperature of 1000°C was transferred to an oxidizing combustion reactor (1,000°C). The initial oven temperature of 120°C was kept for 2 min. Afterward, the temperature was increased at a rate of 8°C min−1 until 200°C. In a second gradient step, the temperature of 1000°C was transferred to an oxidizing combustion reactor (1,000°C).

Amino acids were derivatized by the addition of 100 μL of internal standards ([1-13C]glycine, [1-15N]glycine, and [1-13C]labeled tyrosine) and evaporated under a nitrogen stream. A volume of 50 μL of internal standard (0.175 M Tris-HCl, pH 8.8, 5% [w/v] SDS, 15% [v/v] glycerol, and 0.3 M dithiothreitol) followed by centrifugation (13,000g, 10 min, and room temperature). The supernatant was mixed with 1.6 mL of ice-cold acetone and incubated for precipitation of cell protein for 1 h at −20°C. After centrifugation (13,000g, 10 min, and 4°C), the protein pellet was washed twice with 80% (v/v) acetone, air dried, and hydrolyzed into the amino acids for 12 h (125 μL, 6 M HCl, and 100°C). The hydrolysate was purified (Millipore centrifugal filter units, Ultrafree-MC, Durapore polyvinylidene difluoride, 0.22 μm Merck KGaA), followed by centrifugation (13,000g, 10 min, and room temperature). The supernatant was mixed with 1.6 mL of ice-cold acetone and incubated for precipitation of cell protein for 1 h at −20°C. After centrifugation (13,000g, 10 min, and 4°C), the protein pellet was washed twice with 80% (v/v) acetone, air dried, and hydrolyzed into the amino acids for 12 h (125 μL, 6 M HCl, and 100°C). The hydrolysate was purified (Millipore centrifugal filter units, Ultrafree-MC, Durapore polyvinylidene difluoride, 0.22 μm Merck KGaA), followed by centrifugation (13,000g, 10 min, and room temperature).

**Quantification of Isotopic 13C and 15N Enrichment**

Briefly, deep-frozen plant material was freeze dried (Christ Gamma 2-16 LSC; Martin Christ Gefriertrocknungsanlagen) and ground to a fine powder in a ball mill (3–5 min, 30 Hz; Retsch MM300; Retsch). A sample (0.8–0.9 mg dry weight) was then transferred into a tin capsule (3.5 × 5 mm; HEKAtech). The isotopic enrichment was determined using an elemental analyzer (FLASH 2000; Thermo Scientific) coupled to an isotope-ratio mass spectrometer (Delta V Plus; Thermo Scientific). The gas flow was set to 300 mL min−1, and the column temperature was kept at 41°C.

**Quantification of Isotopic 13C and 15N Enrichment of Proteinogenic Amino Acids**

Deep-frozen plant material was freeze dried (Christ Gamma 2-16 LSC; Martin Christ Gefriertrocknungsanlagen) and ground to a fine powder in a ball mill (3–5 min, 30 Hz; Retsch MM300; Retsch). A sample (0.8–0.9 mg dry weight) was then transferred into a tin capsule (3.5 × 5 mm; HEKAtech). The isotopic enrichment was determined using an elemental analyzer (FLASH 2000; Thermo Scientific) coupled to an isotope-ratio mass spectrometer (Delta V Plus; Thermo Scientific). The gas flow was set to 300 mL min−1, and the column temperature was kept at 41°C.

Combined 13C and 15N labeling was performed for combined 13CO2 and 15NH4NO3 labeling with hydroponic rice cultures (Fig. 1C). Ammonium nitrate was selected because it generally serves as a nitrogen source and is taken up by the roots. Rice prefers ammonium over nitrate, as demonstrated by the higher uptake rate. Compared with nitrate, its uptake is less dependent on light intensity and oxygen concentration at the root site (Susakawa and Yamamoto, 1978). The reactor was built from polycarbonate (5 mm thickness; Hans Keim Kunststoffe), which allowed full spectral transmission of sunlight (Supplemental Fig. S1). Plants were pregrown in the phyto chamber as hydroponic cultures. Prior to the experiment, cultures were transferred from naturally labeled growth medium to a container with 15NH4NO3. The temperature of 120°C was kept for 2 min. Afterward, the temperature was increased at a rate of 8°C min−1 until 200°C. In a second gradient step, the temperature was increased at a rate of 8°C min−1 until 200°C. In a second gradient step, the temperature of 1000°C was transferred to an oxidizing combustion reactor (1,000°C). The initial oven temperature of 120°C was kept for 2 min. Afterward, the temperature was increased at a rate of 8°C min−1 until 200°C. In a second gradient step, the temperature was increased at a rate of 8°C min−1 until 200°C. In a second gradient step, the temperature of 1000°C was transferred to an oxidizing combustion reactor (1,000°C).

**Calculation of 13C and 15N Enrichment**

The δ values (%o) are expressed relative to international standards as:

\[
\delta = \left( \frac{R_{\text{sample}} - R_{\text{standard}}} {R_{\text{standard}}} \right) \times 1000
\]

where \(R\) is the ratio of 13C/12C or 15N/14N. The 13C and 15N measurements were calibrated against Vienna Pee Dee Belemnite and atmospheric nitrogen, respectively, using the international reference materials cellulose (IAEA-CH-3) and ammonium sulfate (IAEA-N-1) as secondary standards. Typical values for δ15N of unlabeled C3 plant material are between −34‰ and −22‰ (Meier-Augenstein, 1999a; Richter et al., 2010). Enrichment values also were expressed as atomic percent (AP), which is the percentage of 13C or 15N atoms relative to the total amount of carbon or nitrogen atoms in the sample. Atomic percent enrichment was derived via Equation 2:

\[
AP = 100 \times \left( \frac{R_{\text{sample}} - 1} {R_{\text{standard}} - 1} \right)
\]

Atomic percent excess (APE), the absolute value for isotopic enrichment, was calculated by subtracting the enrichment of the control from the enrichment of the sample:

\[
\text{APE} = AP_{\text{sample}} - AP_{\text{control}}
\]

**Data Correction for Natural Isotopes**

Naturally occurring isotopes of the analyte and of derivative groups were considered (Wittmann, 2007), and data correction following GC-C-IRMS measurement was done as described previously (Menges and Daenzer, 2000; Docherty et al., 2001; Heinzle et al., 2008). Briefly, δ values were normalized to their respective unlabeled equivalents by subtracting the values of the unlabeled control from the corresponding value of the labeled sample:

\[
\delta_{\text{corr}} = \delta_{\text{sample}} - \delta_{\text{control}}
\]

where \(\delta_{\text{corr}}\) is the labeled compound, \(\delta_{\text{sample}}\) is the derivatized labeled compound, and \(\delta_{\text{control}}\) is the derivatized unlabeled compound. For each experiment, unlabeled control plants that were harvested (at least three biological replicates) were exposed to the exact same breeding conditions as the labeled plants (i.e. they were grown on the same batch of soil or hydroponic medium, respectively). Furthermore, unlabeled controls were subjected to the same sample-processing steps as labeled samples.

For the amino acid pathway illustrations (Fig. 5, A and B), the corrected δ values were normalized to 100%. Therefore, the highest δcorr value of the respective data set was set to 100%. The normalized value of any number \(X_{\text{norm}}\) of the original data set was calculated by the following equation:

\[
X_{\text{norm}} = \frac{X_{\text{corr}} \times 100} {\delta_{\text{corr}}}
\]

where \(\delta_{\text{corr}}\) was the highest δcorr value.

**Determination of Root-to-Shoot Ratio and Translocation Flux**

To set up a flux map for a rice seedling, it was important to derive the root-to-shoot ratio of the analyzed plantlets. It was assumed that the decrease of

were divided by 6. Shoot and root enrichment values of 13C as well as 15N enrichment of individual plant parts after 2 h of tracing. Considering that shoot obtained for 12-d-old seedlings. This value was used to estimate the percentage where

\[ M_{\text{shoot}} = 6 \times \left( \delta^{13}C_{\text{shoot}} - \delta^{13}C_{\text{root}} \right) \]

\[ = M_{\text{root}} = 6 \times \left( \delta^{15}N_{\text{root}} - \delta^{15}N_{\text{root}} \right) \]

where \( M \) is the mass of shoot and root as indicated. A root-to-shoot ratio of 1:6 was obtained for 12-d-old seedlings. This value was used to estimate the percentage enrichment of individual plant parts after 2 h of tracing. Considering that shoot biomass was 6 times as much as root biomass, it follows that 13N enrichment of the shoot was diluted by a factor of 6 upon translocation to the shoot, whereas 13C enrichment accumulated 6-fold upon translocation to the root. Accordingly, 15N values of the shoot were multiplied by 6, whereas 13C values of the root were divided by 6. Shoot and root enrichment values of 13C as well as 15N labeling, at time point zero, were summed, displaying 100% uptake. On this basis, 13C and 15N percentage enrichments were estimated for root and shoot. The amount of 13C labeling that could not be recovered in plant tissue was assumed to be lost by respiration.

Statistical Analysis

The statistical significance of differences between mean values was determined using Student’s t test or one-way ANOVA followed by a posthoc Tukey’s test. Differences were considered significant when the \( P \) value was below 0.05.

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Absorption spectra of material used for the construction of labeling reactors.

Supplemental Figure S2. Profiles of temperature and relative humidity inside the different reactor housings.

Supplemental Figure S3. Influence of magnitude and duration of 13CO2 labeling pulse, number of plants in the enclosure, and daytime on the enrichment of seedlings.

Supplemental Figure S4. Simplified pathway illustration of shoot amino acid 13C enrichment of rice seedlings raised on soil compared with hydroponic culture.

Supplemental Table S1. Raw data of Figure 2 and Figure 3, comparing 13C with 15N labeling of seedlings.

Supplemental Table S2. Raw data of Figure 4B, comparing 13C labeling of plants in the flowering stage with plants in grain filling early and grain filling late.

Supplemental Table S3. Raw data of Figure 4D, comparing 13C labeling of the different tissue types of plants in the late grain filling stage.

Supplemental Table S4. Raw data of Figure 5A, comparing 13C labeling of a seedling shoot with the leaf of a plant in the late grain filling stage.

Supplemental Table S5. Raw data of Figure 5B, comparing 13C labeling of the different tissue types of a plant in the late grain filling stage.

Supplemental Table S6. Raw data of Figure 5C, statistical significance between measured amino acid enrichments.

Supplemental Table S7. Raw data of Figure 6, comparing shoot and root 13C and 15N labeling.

Supplemental Table S8. Raw data of Figure 7A, displaying 13C enrichment of imazapyr-treated rice seedlings, immediately after the labeling pulse.

Supplemental Table S9. Raw data of Figure 7B, displaying 13C enrichment of imazapyr-treated rice seedlings after a two-hour chase period.

Supplemental Table S10. Raw data of Figure S2, displaying means (\( n = 3 \)) of temperature and humidity during the experiment inside the different labeling reactors.

Supplemental Table S11. Raw data of Figure S3, validation of labeling system.

Supplemental Table S12. Raw data of Figure S4, comparing 13C labeling of seedlings grown on soil with seedlings grown on hydroponic medium.

Screening Technology for Quantitative Isotope Experiments

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