DISS. ETH No. 22109

Carbon isotopic composition of leaf dark-respired CO_2: Influence of different substrates and environmental drivers

A dissertation submitted to

ETH Zurich

For the degree of

Doctor of Sciences

presented by

Marco Lehmann

Diplom - Biologe, Saarland University, Saarbruecken

born 28th February 1983

in Landau i.d. Pfalz

Citizen of Germany

Prof. Dr. Nina Buchmann, examiner

Dr. Roland Werner, co-examiner

Dr. Rolf Siegwolf, co-examiner

2014

"You will die but the carbon will not; its career does not end with you. It will return to the soil and there a plant may take it up again in time, sending it once more on a cycle of plant and animal life."

(Jacob Bronowski, 1908 -1974)

“For myself.

For my friends.

For my family.

FOREVER”

(Throwdown)

Table of Content

Abstract

The investigation of plant respiration as a major process in plant biochemistry has expanded our understanding of the carbon cycle in autotrophic organisms. Plants dissimilate respiratory substrates to gain energy for maintenance of various metabolic processes and for production of intermediates and reducing equivalents, thereby continuously releasing CO_2 via leaf respiration. Leaf respired CO_2 derives mainly from oxidative decarboxylation reactions from the Krebs cycle and from interacting anabolic and catabolic reactions. With the help of carbon stable isotopes, the fate of carbon through the plant metabolic network can be followed by measuring the carbon isotopic composition of respiratory substrates and of leaf dark-respired CO_2 ($\delta^{13}C_R$). Compared to putative respiratory substrates, $\delta^{13}C_R$ can be up to 16‰ less negative and shows high variations during a daily cycle. However, the factors explaining changes in $\delta^{13}C_R$ are not fully understood so far.

This thesis aims to disentangle the respiratory substrates and the environmental drivers leading to changes in $\delta^{13}C_R$. Therefore, we exposed various plant species of different functional groups, exhibiting low and high variations in $\delta^{13}C_R$, to different environmental conditions (temperature, soil moisture, and light). We measured $\delta^{13}C_R$ with an in-tube incubation technique and $\delta^{13}C$ of different respiratory substrates (e.g., carbohydrates, starch, organic acids) with compound-specific isotope analysis during a daily cycle. Moreover, we fed different plant species, including an *Arabidopsis thaliana* mutant, with position-specific ^{13}C labelled malate and pyruvate and measured label-derived leaf $^{13}CO_2$ respiration on-line with laser spectroscopy during light-dark transition courses, including the period of light enhanced dark respiration shortly upon darkening.

We found that $\delta^{13}C_R$ and $\delta^{13}C$ of different leaf respiratory substrates show similar variations under different temperature and soil moisture conditions during a daily cycle. Interestingly, $\delta^{13}C$ of malate was less negative compared to other respiratory substrates and often less negative than $\delta^{13}C_R$ across different environmental conditions. Moreover, we found strong relationships between $\delta^{13}C$ of malate and $\delta^{13}C_R$ during darkness and illumination, indicating that malate is an important substrate of $\delta^{13}C_R$ in potato plants. However, further experiments showed clear species-specific variations in $\delta^{13}C$ of malate, which could not fully explain the high daily variations in $\delta^{13}C_R$ in those species. Thus, malate can be excluded as the sole respiratory substrate in all species, indicating that additional respiratory processes and substrates must play a role. Furthermore, leaf feeding studies with position-specific ^{13}C labelled malate and pyruvate clearly confirmed that the intramolecular isotopic pattern in respiratory substrates has a strong influence on $\delta^{13}C_R$. Thereby, respiration of C-1 position of pyruvate was found to be stronger than respiration of C-1 and C-4 position of malate, while respiration of C-2 position of pyruvate was weakest. Thus, we can show that individual atom positions in respiratory substrates can take different metabolic pathways and, depending on the species, that they either are respired or incorporated into new biomolecules.

Overall, this work demonstrates that changes in $\delta^{13}C_R$ generally reflect changes in $\delta^{13}C$ of different respiratory substrates under different environmental conditions and shows the high variability and species-specific differences in $\delta^{13}C_R$ and $\delta^{13}C$ of different respiratory substrates. Hence, this work improves our understanding of processes influencing $\delta^{13}C_R$, providing a new fundament for further studies on plant biochemistry or plant respiration at leaf or plant scale.

Zusammenfassung

Untersuchungen an der Respiration von Pflanzen, ein Hauptprozess der Pflanzen-Biochemie, haben das Verständnis über den Kohlenstoffkreislauf innerhalb photoautotropher Organismen erweitert. Während Pflanzen respiratorische Substrate abbauen, um Energie für verschiedene metabolische Prozesse und für die Produktion von Zwischenprodukten und Reduktionsäquivalenten zu gewinnen, respirieren sie kontinuierlich CO_2. Das abgegebene CO_2 stammt hauptsächlich von oxidativ decarboxylierenden Reaktionen des Krebs-Zyklus und von assoziierten anabolischen und katabolischen Reaktionen. Mit der Hilfe von stabilen Kohlenstoffisotopen kann der Weg des Kohlenstoffs durch das Netzwerk des pflanzlichen Metabolismus verfolgt werden, indem man die Kohlenstoffisotopenzusammensetzung von respiratorischen Substraten und des im Dunklen abgegebenen CO_2 ($\delta^{13}C_R$) in Blättern misst. Dabei wurde festgestellt, dass $\delta^{13}C_R$ bis zu 16‰ weniger negativ ist als potentielle respiratorische Substrate und während eines Tagesverlaufes stark variiert. Nichtsdestotrotz sind die Faktoren, welche die Unterschiede in $\delta^{13}C_R$ verursachen, nicht vollständig verstanden.

Diese Doktorarbeit zielt darauf ab, die respiratorischen Substrate und Umweltbedingungen zu bestimmen, die zu Veränderungen in $\delta^{13}C_R$ führen. Hierfür wurden Pflanzen verschiedener funktioneller Gruppen, welche niedrige und hohe Variationen in $\delta^{13}C_R$ zeigen, unterschiedlichen Umweltbedingungen (Temperatur, Bodenfeuchte, und Licht) ausgesetzt. Wir bestimmten $\delta^{13}C_R$ während eines Tagesverlaufs mit einer „in-tube incubation“ Methode und $\delta^{13}C$ von verschiedenen potentiellen respiratorischen Substraten mit komponentenspezifischen Isotopenuntersuchungen. In weiteren Experimenten wurden neben einer *Arabidopsis*

thaliana Mutante noch weitere Arten mit positionsspezifisch ^{13}C markiertem Malat und Pyruvat gefüttert und die erhöhte $^{13}CO_2$ Blattrespiration während Licht-Dunkel Übergangsverläufen on-line laserspektroskopisch gemessen.

Die Veränderung in $\delta^{13}C_R$ und in $\delta^{13}C$ von verschiedenen respiratorischen Substraten auf Grund verschiedener Temperatur- oder Bodenfeuchtigkeitsbedingungen waren während eines Tagesverlaufs sehr ähnlich. Interessanterweise war vor allem $\delta^{13}C$ von Malat weniger negativ als andere respiratorische Substrate und oft auch negativer als $\delta^{13}C_R$ über alle Umweltbedingungen hinweg. Zudem fanden wir enge Zusammenhänge zwischen $\delta^{13}C$ von Malat und $\delta^{13}C_R$ während des Tages und der Nacht. Malat könnte somit ein wichtiges Substrat von $\delta^{13}C_R$ in der Kartoffelpflanze sein. In weiteren Versuchen fanden wir deutliche artabhängige Unterschiede in $\delta^{13}C$ von Malat, die hohen Variationen in $\delta^{13}C_R$ während eines Tagesverlaufs konnten jedoch in diesen Arten nicht vollständig erklärt werden. Malat als wichtigstes respiratorisches Substrat in allen Arten kann somit ausgeschlossen werden und es lässt sich deshalb vermuten, dass noch weitere respiratorische Prozesse und Substrate eine Rolle spielen müssen. Dies wurde klar bestätigt in Blattfütterungsstudien mit positionsspezifisch ^{13}C markiertem Malat und Pyruvat, die zeigten, dass die intramolekulare Isotopenverteilung einen wichtigen Einfluss auf $\delta^{13}C_R$ hat. Dabei wurde beobachtet, dass die C-1 Position von Pyruvat am stärksten respiriert wurde, verglichen zu der C-1 und C-4 Position von Malat, während die C-2 Position von Pyruvat am schwächsten respiriert wurde. Somit konnten wir zeigen, dass einzelne Atompositionen von respiratorischen Substraten unterschiedliche metabolische Wege einschlagen können und je nach Pflanze in unterschiedlichen

Ausmaßen respiriert oder zur Biosynthese verschiedener Biomoleküle verwendet werden.

Insgesamt zeigt diese Arbeit, dass Veränderungen in $\delta^{13}C_R$ generell die Veränderungen in $\delta^{13}C$ von verschiedenen respiratorischen Substraten auf Grund verschiedener Umweltbedingungen wiederspiegeln und macht vor allem die hohe Variabilität und artspezifischen Unterschiede in $\delta^{13}C_R$ und $\delta^{13}C$ von verschiedenen respiratorischen Substraten deutlich. Somit trägt diese Arbeit zu einem besseren Verständnis der Prozesse bei, die $\delta^{13}C_R$ beeinflussen, so dass zukünftige Studien der Pflanzenbiochemie und Respiration auf Blatt- oder Pflanzenebene davon profitieren werden.

Chapter 1

1. General Introduction

On the metabolic origin of dark respiration

Plants as photoautotrophic organisms use the energy of light to assimilate atmospheric CO_2. Responsible for the carbon incorporation is the most abundant protein in leaves, the ribulose-1,5-bisphosphate-carboxylase/oxygenase (RuBisCO). The enzyme catalyzes the reaction of ribulose-1,5-bisphosphate with CO_2, producing an unstable six-carbon intermediate that instantaneously falls apart into two 3-phosphoglycerate molecules, a three-carbon molecule. Furthermore, 3-phosphoglycerate enters the plant metabolic network and is used to build up sugars and starch for carbon storage. Subsequently, the stored substrates are broken down during darkness and undergo complex reaction sequences, summarized as dark respiration, or are used for biosynthesis of compounds (Hopkins, 2006). According to Lambers *et al.* (2005), 25 to 70% of all assimilated carbohydrates are distributed to respiration, depending strongly on external growth conditions, like nutrient supply, temperature, and water availability. Under suboptimal conditions the maximum relative growth rate of plants will decrease as costs for maintenance of respiration will increase (higher costs for nutrient acquisition; temperature optimum of enzyme and more; Lambers *et al.*, 2005).

The classical biochemical starting point of respiration begins with the breakdown of freshly assimilated sugars or sugars derived from transitory starch (functioning as plant internal carbon storage) during glycolysis, which results in pyruvate formation (Fig. 1).

Afterwards, throughout the first CO_2 producing reaction during respiration, pyruvate is converted into acetyl-CoA and CO_2 (from the C-1 position of pyruvate) by the pyruvate dehydrogenase complex (PDH) via oxidative decarboxylation in the mitochondrion.

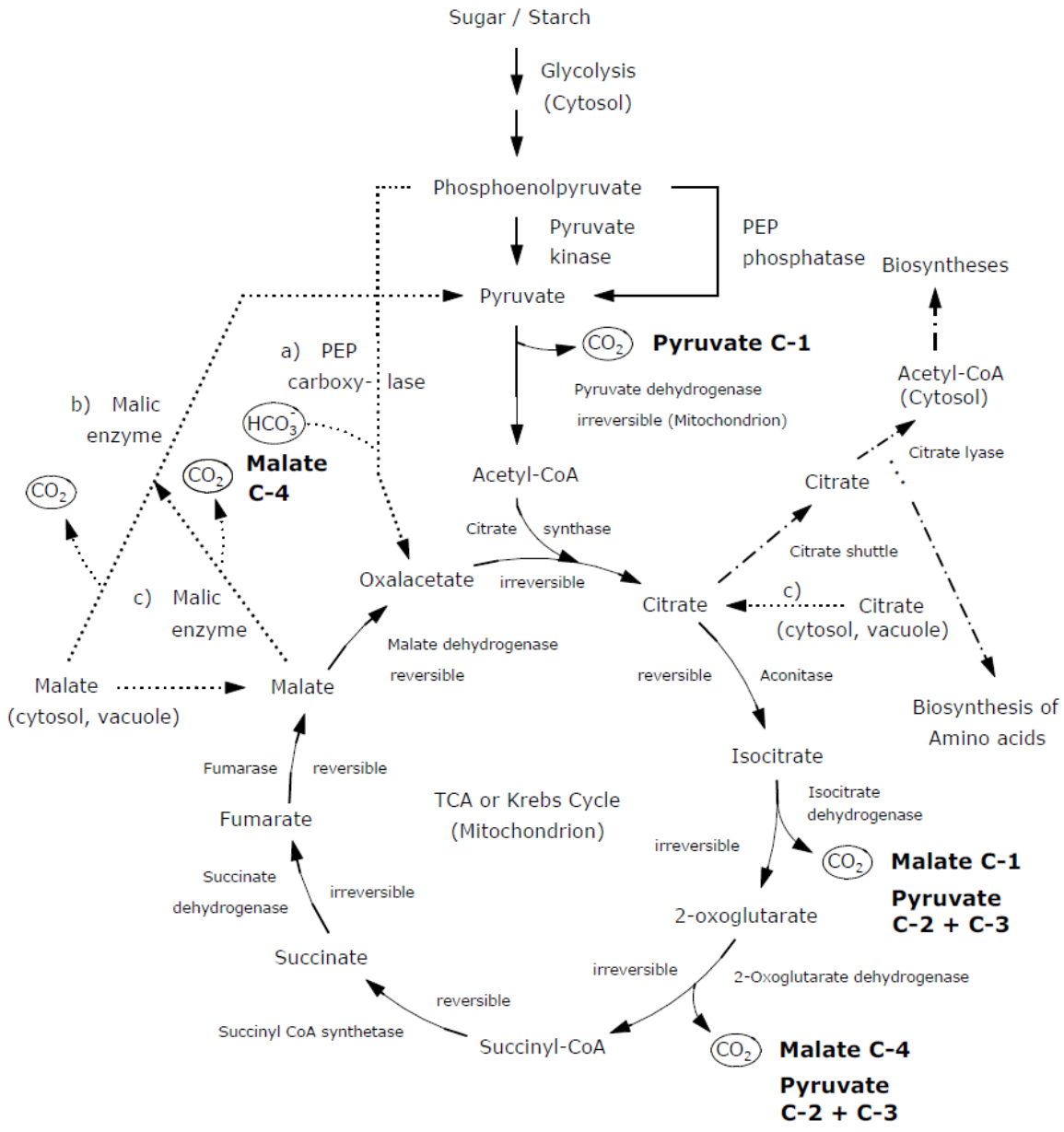

Figure 1: Scheme of metabolites and enzymatic reactions in and around the Krebs cycle (Bowsher *et al.*, 2008; Werner *et al.*, 2011). Circles mark CO_2 releasing reactions during dark respiration. The two potential key substrates of dark respiration, pyruvate and malate, and their molecule positions most likely contributing to respiration are emphasized.

The acetyl-CoA residue (former C-2 & C-3 positions of pyruvate) functions as an educt for the citrate synthase reaction, together with oxalacetate derived from malate or from the phosphoenolpyruvate carboxylase reaction. The tricarboxylic acid (citrate) is the initial intermediate of the Krebs cycle (KC or tricarboxylic acid cycle; TCA cycle), which is the central metabolic pathway of plant respiration. The KC produces reducing equivalents ($FADH_2$, $NADH/H^+$) and carbon skeletons for further biochemical reactions (e.g. biosynthesis of amino acids and lipids) and CO_2, which is respired by the plants (Bowsher *et al.*, 2008). The two CO_2 releasing steps in the KC are catalyzed by isocitrate dehydrogenase and 2-oxoglutarate dehydrogenase, using pyruvate and malate as potential respiratory substrates. In addition, the cycle intermediate malate can be converted into pyruvate and CO_2 (from the C-4 position of malate) by the mitochondrial malic enzyme reaction (ME). Overall, PDH, Krebs cycle reactions, and ME are important origins of total emitted CO_2 during dark respiration and affect its isotopic signature.

Stable isotopes

New instrumental developments and methodical improvements in the last century have established stable isotopes as an important and versatile scientific tool. With the help of stable isotopes it is possible to trace element cycles over a wide range of scales and to investigate complex biological and environmental processes even in living organism such as plants and animals. For instance, in the plant biosphere, stable isotopes of the elements carbon, hydrogen, oxygen, and nitrogen have been mainly used to investigate basic processes of element uptake, allocation, and release. This can be done since the elements have more than one stable isotope, which differ in their number of neutrons. The ones with fewer neutrons are called the "lighter" stable isotopes and are mostly

more abundant, while the ones with more neutrons are called "heavier" stable isotopes and are less abundant, with a natural isotope abundance of about 1% or less relative to the other isotopes of the same element (Werner, 1998). However, they have identical chemical properties, only minor physical differences, and show no radioactive decay (which is reason for the attribute “stable”). Stable isotope results in environmental sciences are mostly expressed as delta (δ) values using the δ-notation, which was introduced by McKinney *et al.* (1950). The δ-notation for carbon stable isotopes is defined as deviation of the $^{13}C/^{12}C$ ratio of the sample material relative to the $^{13}C/^{12}C$ ratio of the international standard VPDB (Vienna PeeDee Belemnite) and given in ‰ (here slightly modified after Coplen, 2011):

$$\delta^{13}C\ (‰) = \frac{^{13}C/^{12}C_{sample}}{^{13}C/^{12}C_{VPDB}} - 1$$

The fact that carbon isotope ratios can change under natural conditions is mainly attributed to two isotope effects, involving “lighter” and “heavier” isotopes: (1) Equilibrium isotope effects during phase transitions, e.g., dissolution of CO_2 in H_2O to ^{13}C-enriched HCO_3^- (Deuser & Degens, 1967; Werner, 1998) and (2) kinetic isotope effects defined as the ratio of rate constants for (bio)chemical reactions (Werner, 1998; Tcherkez *et al.*, 2011). Most enzymatically catalyzed reactions in plants discriminate against the “heavier” stable carbon isotope (^{13}C) due to kinetic isotope effects, which can cause isotope fractionation. This can be explained with differences in the zero-point energy of C-C bonds (Simon & Palm, 1966; Werner, 1998), causing that more energy is being required to break or to create C-C bonds with ^{13}C than with ^{12}C. For instance, C_3 plant material is about 20‰ more negative compared to atmospheric CO_2 due to the

strong discrimination against ^{13}C during photosynthetic carbon fixation (Farquhar *et al.*, 1989). Such biochemical processes causing carbon isotope fractionation can be determined by measuring $\delta^{13}C$ differences of various substrates. Therefore, precise instruments such as isotope ratio mass spectrometers (IRMS) or nuclear magnetic resonance spectrometers (NMR) are necessary. For IRMS analysis, samples are converted by combustion, oxidation, or pyrolysis into simple gases before measurement (Werner, 1998). Nowadays, IRMS can also be coupled with gaseous or liquid chromatographic devices. For instance, complex mixtures of plant substrates can be separated on HPLC-columns (high performance liquid chromatography) into single substrates, and $\delta^{13}C$ and concentration of each single substrate can be determined with IRMS (HPLC-IRMS; Krummen *et al.*, 2004). Subsequently, the isotopic analysis of the substrates gives us new insights into unknown biochemical processes in plants.

The isotopic composition of leaf dark-respired CO_2 and environmental drivers

Studies investigating $\delta^{13}C$ of leaf dark-respired CO_2 ($\delta^{13}C_R$) have increased in recent years. The main focus was on daily chances in $\delta^{13}C_R$, which was investigated in a wide range of species and under various environmental, controlled conditions (Barbour *et al.*, 2007; Werner *et al.*, 2007; Gessler *et al.*, 2009; Werner *et al.*, 2009; Wegener *et al.*, 2010), but also under natural conditions in the field (Hymus *et al.*, 2005; Prater *et al.*, 2006; Sun *et al.*, 2009; Rascher *et al.*, 2010; Barbour *et al.*, 2011; Dubbert *et al.*, 2012). To determine the carbon isotope ratio of leaf dark-respired CO_2 many methods have been developed. A common and an easy-to-use method is the in-tube incubation technique, where leaf material is transferred into gas-tight reaction tubes and flushed with CO_2-free air. Subsequently, the tube is incubated in the dark for a few minutes and

$\delta^{13}C_R$ measured with an IRMS (Werner *et al.*, 2007). Alternatively, $\delta^{13}C_R$ and respiration rates can be measured with higher resolution using a cavity ring-down laser spectrometer (see chapter 3 for more details). Several experimental observations showed that $\delta^{13}C_R$ was up to 16‰ less negative compared to putative respiratory substrates and that it highly varied during a daily cycle, depending on the species (Duranceau *et al.*, 1999; Ghashghaie *et al.*, 2001; Werner *et al.*, 2009; Wegener *et al.*, 2010). Besides, environmental conditions such as temperature and soil moisture can influence $\delta^{13}C_R$, with less negative values under drought compared to well-watered conditions (Duranceau *et al.*, 1999; Ghashghaie *et al.*, 2001) and more negative values under high temperatures compared to low temperatures (Tcherkez *et al.*, 2003). Nevertheless, it is still not fully understood what causes the ^{13}C enrichment and the variations in leaf dark-respired CO_2. Several mechanisms and possibilities such as the use of different respiratory substrates, fragmentation fractionation, and isotope effects on CO_2 releasing reactions have been discussed in latest reviews (Werner & Gessler, 2011; Ghashghaie & Badeck, 2014).

Substrates and mechanisms causing changes in $\delta^{13}C_R$

$\delta^{13}C_R$ *per se* is linked to the carbon isotopic composition of potential respiratory substrates ($\delta^{13}C_{RS}$) such as carbohydrates and organic acids. Measurements of daily and long-term cycles of different $\delta^{13}C_{RS}$ have shown significant temporal changes (Goettlicher *et al.*, 2006; Gessler *et al.*, 2007; Wild *et al.*, 2010). However, daily changes in $\delta^{13}C_{RS}$ under different environmental conditions are still not well studied compared to those in $\delta^{13}C_R$, especially isotope studies of organic acids are rare in spite of their supposed significance for respiratory processes (Plaxton & Podesta, 2006).

Moreover, the ^{13}C enrichment in leaf dark-respired CO_2 was often discussed to be related to fragmentation fractionation, due to heterogeneous intramolecular ^{13}C distribution within respiratory substrates (Tcherkez *et al.*, 2004). For instance, Rossmann *et al.* (1991) found a ^{13}C enrichment in the C-3 and C-4 position of glucose compared to the other molecule positions. Consequently, pyruvate derived from glucose is enriched in ^{13}C at the C-1 position, which can be released as CO_2 by PDH in the dark. In contrast, the acetyl-CoA residue of pyruvate is depleted in ^{13}C, but also contributes to respiration or to the production of biomolecules such as amino acids or lipids. In addition to fragmentation processes, PDH includes additional isotope effects on the C-1 and C-2 positions of pyruvate (former C-3/C-4 and C-2/C-5 positions of glucose), which will lead to ^{13}C depletion in lipids in case of an incomplete conversion of pyruvate (Melzer & Schmidt, 1987).

$\delta^{13}C_R$ during light enhanced dark respiration (LEDR)

While measuring leaf dark respired CO_2 during a daily cycle, research focused on a period called light enhanced dark respiration (LEDR). LEDR happens roughly during the first 20 to 30 min after experimental sudden darkening of leaves and leads to a short increase in the amount of respired CO_2 (Atkin *et al.*, 1998; Griffin & Turnbull, 2012). Recent studies could show an enrichment of respired CO_2 of up to 14.8‰ during LEDR compared to respiratory substrates (Werner *et al.*, 2009; Wegener *et al.*, 2010). Observations after sunset in field experiments were comparable (Barbour *et al.*, 2011), showing that changes in $\delta^{13}C_R$ at the plant scale could also be observed at larger scales. Under both circumstances, the ^{13}C enrichment is followed by a strong ^{13}C depletion in leaf dark-respired CO_2. It was suggested that this course might be driven by

decarboxylation of an accumulated malate pool in the light (Gessler *et al.*, 2009). Malate was shown to be enriched in ^{13}C compared to other respiratory substrates (Gleixner *et al.*, 1998; Ghashghaie *et al.*, 2001). Several studies suggested that this is caused by a strong ^{13}C enrichment in the C-4 position compared to the other molecule positions in malate due to an anapleurotic flux via PEPC (a flux replenishing KC intermediates used for respiratory or biosynthetic processes; Melzer & O'Leary, 1987; Melzer & O'Leary, 1991; Savidge & Blair, 2004). The C-4 position might be released by ME, contributing to the ^{13}C enrichment in leaf dark-respired CO_2 during LEDR (Barbour *et al.*, 2007; Gessler *et al.*, 2009; Barbour *et al.*, 2011). Nevertheless, up to now no experimental evidence is available to prove this hypothesis.

Objectives

This PhD thesis aimed to disentangle substrates and environmental drivers leading to changes in the carbon isotope ratio of leaf dark-respired CO_2. The main focus was therefore on daily courses and light-dark transitions, including LEDR. Our main objectives were:

- to identify substrates of leaf dark-respired CO_2 (chapters 2, 3, 4),
- to analyze relationships between $\delta^{13}C_R$ and $\delta^{13}C_{RS}$ (chapters 2, 3),
- to determine daily changes and changes during LEDR in $\delta^{13}C_R$, $\delta^{13}C_{RS}$, and their concentrations under different temperature and soil moisture treatments (chapters 2, 3),
- to investigate the relationship between $\delta^{13}C_R$ and respiration rates (chapter 3),

- to determine temporal and species-specific variations in respiration of position-specific ^{13}C labelled malate and pyruvate and to understand the associated mechanisms during light-dark transitions, including LEDR (chapter 4), and
- to evaluate the non-respiratory fate of position-specific ^{13}C labelled malate and pyruvate during light-dark transitions, including LEDR (chapter 4).

Approaches and plant material

For the first study (chapter 2), we performed an experiment with potato plants (*Solanum tuberosum*). Potato is generally adapted to moderate climatic conditions of about 20°C (Levy & Veilleux, 2007), so that treatments with high temperature or dry conditions should easily facilitate changes in photosynthetic and post-photosynthetic carbon isotope fractionation. Besides, potato is a fast and simple to grow C_3 plant and its robust properties make it an ideal candidate for climate chamber experiments. Thus, we exposed potato plants to different temperature and soil moisture treatments under controlled climatic conditions. We analyzed soluble carbohydrates (Boschker *et al.*, 2008; Rinne *et al.*, 2012) and organic acids (Hettmann *et al.*, 2005; Wild *et al.*, 2010) from potato leaves (Gleixner *et al.*, 1998) with compound-specific isotope analysis (CSIA), using HPLC-IRMS.

For the second study (chapter 3), we carried out several experiments with species of different plant functional groups, comprising differences in $\delta^{13}C_R$ (Priault *et al.*, 2009; Wegener *et al.*, 2010). Especially, species with high and low diurnal variations in $\delta^{13}C_R$ such as the Mediterranean woody species *Halimium halimifolium* and the non-aromatic herb *Oxalis triangularis* were investigated. Respiratory isotope fractionation and respiration rates are known to highly differ between those species. Hence, differences in

the isotopic composition of respiratory substrates are likely to occur. Thus, we determined the relationship of $\delta^{13}C_R$ and respiration rates under different environmental conditions with an in-tube incubation technique and measured $\delta^{13}C$ of putative respiratory substrates (sugars, amino acids, and malate) with CSIA in several species during a daily cycle.

For the 3rd study (chapter 4), we performed feeding experiments with position-specific ^{13}C labelled malate and pyruvate, mainly with *Halimium halimifolium* and *Oxalis triangularis* plants. In addition, we used wild type and *nadme1x2* plants of *Arabidopsis thaliana*, the model plant of molecular plant biology. The double knockout mutant *nadme1x2* lacks any functional mitochondrial malic enzyme (Tronconi *et al.*, 2008). Hence, differences in respiration of ^{13}C labelled malate can be expected between mutant and wild type plants. Thus, leaf material of the different species was fed with position-specific ^{13}C labelled malate (^{13}C-1, ^{13}C-4) and pyruvate (^{13}C-1, ^{13}C-2) via the xylem stream. $\delta^{13}C$ of leaf metabolic fractions were determined and label-derived leaf $^{13}CO_2$ respiration measured on-line with laser spectroscopy during light-dark transitions.

Thesis outline

The work is organized in five chapters. Chapter 1 introduces the basic principles of dark respiration, stable isotopes, substrates and drivers of $\delta^{13}C_R$, and objectives and approaches of this study. Chapter 2 is a study about substrates and environmental drivers of leaf dark-respired CO_2 in potato plants. Chapter 3 presents a manuscript about the relationship of $\delta^{13}C_R$ with LEDR and with $\delta^{13}C$ of malate in several species. Chapter 4 is a study about substrates and mechanisms leading to changes in $\delta^{13}C_R$ during light-dark

transitions, especially during LEDR. Finally, main results are discussed in chapter 5, with an outlook on future work.

References

Atkin OK, Evans JR, Siebke K. 1998. Relationship between the inhibition of leaf respiration by light and enhancement of leaf dark respiration following light treatment. *Australian Journal of Plant Physiology* **25**: 437-443.

Barbour MM, Hunt JE, Kodama N, Laubach J, McSeveny TM, Rogers GN, Tcherkez G, Wingate L. 2011. Rapid changes in $\delta^{13}C$ of ecosystem-respired CO_2 after sunset are consistent with transient ^{13}C enrichment of leaf respired CO_2. *New Phytologist* **190**: 990-1002.

Barbour MM, McDowell NG, Tcherkez G, Bickford CP, Hanson DT. 2007. A new measurement technique reveals rapid post-illumination changes in the carbon isotope composition of leaf-respired CO_2. *Plant, Cell and Environment* **30**: 469-482.

Boschker HTS, Moerdijk-Poortvliet TCW, van Breugel P, Houtekamer M, Middelburg JJ. 2008. A versatile method for stable carbon isotope analysis of carbohydrates by high-performance liquid chromatography/isotope ratio mass spectrometry. *Rapid Communications in Mass Spectrometry* **22**: 3902-3908.

Bowsher C, Steer M, Tobin A 2008. Plant Biochemistry. New York, NY, USA: Garland Science, pp. 143-193.

Coplen TB. 2011. Guidelines and recommended terms for expression of stable-isotope-ratio and gas-ratio measurement results. *Rapid Communications in Mass Spectrometry* **25**: 2538-2560.

Deuser WG, Degens ET. 1967. Carbon isotope fractionation in system CO_2 (gas)-CO_2(aqueous)-HCO_3^-(aqueous). *Nature* **215**: 1033-1035.

Dubbert M, Rascher KG, Werner C. 2012. Species-specific differences in temporal and spatial variation in $\delta^{13}C$ of plant carbon pools and dark-respired CO_2 under changing environmental conditions. *Photosynthesis Research* **113**: 297-309.

Duranceau M, Ghashghaie J, Badeck F, Deleens E, Cornic G. 1999. $\delta^{13}C$ of CO_2 respired in the dark in relation to $\delta^{13}C$ of leaf carbohydrates in *Phaseolus vulgaris* L. under progressive drought. *Plant, Cell and Environment* **22**: 515-523.

Farquhar GD, Ehleringer JR, Hubick KT. 1989. Carbon isotope discrimination and photosynthesis. *Annual Review of Plant Physiology and Plant Molecular Biology* **40**: 503-537.

Gessler A, Tcherkez G, Karyanto O, Keitel C, Ferrio JP, Ghashghaie J, Kreuzwieser J, Farquhar GD. 2009. On the metabolic origin of the carbon isotope composition of CO_2 evolved from darkened light-acclimated leaves in *Ricinus communis*. *New Phytologist* **181**: 374-386.

Gessler A, Keitel C, Kodama N, Weston C, Winters AJ, Keith H, Grice K, Leuning R, Farquhar GD. 2007. $\delta^{13}C$ of organic matter transported from the leaves to the roots in *Eucalyptus delegatensis*: short-term variations and relation to respired CO_2. *Functional Plant Biology* **34**: 692-706.

Ghashghaie J, Badeck FW. 2014. Opposite carbon isotope discrimination during dark respiration in leaves versus roots - a review. *New Phytologist* **201**: 751-769.

Ghashghaie J, Duranceau M, Badeck FW, Cornic G, Adeline MT, Deleens E. 2001. $\delta^{13}C$ of CO_2 respired in the dark in relation to $\delta^{13}C$ of leaf metabolites: comparison between *Nicotiana sylvestris* and *Helianthus annuus* under drought. *Plant, Cell and Environment* **24**: 505-515.

Gleixner G, Scrimgeour C, Schmidt H-L, Viola R. 1998. Stable isotope distribution in the major metabolites of source and sink organs of *Solanum tuberosum* L.: a powerful tool in the study of metabolic partitioning in intact plants. *Planta* **207**: 241-245.

Goettlicher S, Knohl A, Wanek W, Buchmann N, Richter A. 2006. Short-term changes in carbon isotope composition of soluble carbohydrates and starch: from canopy leaves to the root system. *Rapid Communications in Mass Spectrometry* **20**: 653-660.

Griffin KL, Turnbull MH. 2012. Out of the light and into the dark: post-illumination respiratory metabolism. *New Phytologist* **195**: 4-7.

Hettmann E, Gleixner G, Juchelka D. 2005. IRM-LC/MS: $\delta^{13}C$ analysis of organic acids in plants. *Application note 30075, Thermo Fisher Scientific*.

Hopkins WG 2006. Photosynthesis and respiration. New York, NY, USA: Chelsea House, pp. 88-109.

Hymus GJ, Maseyk K, Valentini R, Yakir D. 2005. Large daily variation in ^{13}C-enrichment of leaf-respired CO_2 in two *Quercus* forest canopies. *New Phytologist* **167**: 377-384.

Krummen M, Hilkert AW, Juchelka D, Duhr A, Schluter HJ, Pesch R. 2004. A new concept for isotope ratio monitoring liquid chromatography/mass spectrometry. *Rapid Communications in Mass Spectrometry* **18**: 2260-2266.

Lambers H, Robinson S, Ribas-Carbo M 2005. Regulation of Respiration In Vivo. In: Lambers H, Ribas-Carbo M eds. *Plant Respiration*: Springer Netherlands, pp. 1-15.

Levy D, Veilleux RE. 2007. Adaptation of potato to high temperatures and salinity - A review. *American Journal of Potato Research* **84**: 487-506.

McKinney CR, McCrea JM, Epstein S, Allen HA, Urey HC. 1950. Improvements in mass spectrometers for the measurement of small differences in isotope abundance ratios. *Review of Scientific Instruments* **21**: 724-730.

Melzer E, O'Leary MH. 1991. Aspartic-acid synthesis in C_3 plants. *Planta* **185**: 368-371.

Melzer E, Schmidt H-L. 1987. Carbon isotope effects on the pyruvate dehydrogenase reaction and their importance for relative carbon-13 depletion in lipids. *Journal of Biological Chemistry* **262**: 8159-8164.

Melzer E, O'Leary MH. 1987. Anapleurotic CO_2 fixation by phosphoenolpyruvate carboxylase in C_3 plants. *Plant Physiology* **84**: 58-60.

Plaxton WC, Podesta FE. 2006. The functional organization and control of plant respiration. *Critical Reviews in Plant Sciences* **25**: 159-198.

Prater JL, Mortazavi B, Chanton JP. 2006. Diurnal variation of the $\delta^{13}C$ of pine needle respired CO_2 evolved in darkness. *Plant, Cell and Environment* **29**: 202-211.

Priault P, Wegener F, Werner C. 2009. Pronounced differences in diurnal variation of carbon isotope composition of leaf respired CO_2 among functional groups. *New Phytologist* **181**: 400-412.

Rascher KG, Maguas C, Werner C. 2010. On the use of phloem sap $\delta^{13}C$ as an indicator of canopy carbon discrimination. *Tree Physiology* **30**: 1499-1514.

Rinne KT, Saurer M, Streit K, Siegwolf RTW. 2012. Evaluation of a liquid chromatography method for compound-specific $\delta^{13}C$ analysis of plant carbohydrates in alkaline media. *Rapid Communications in Mass Spectrometry* **26**: 2173-2185.

Rossmann A, Butzenlechner M, Schmidt H-L. 1991. Evidence for a nonstatistical carbon isotope distribution in natural glucose. *Plant Physiology* **96**: 609-614.

Savidge WB, Blair NE. 2004. Patterns of intramolecular carbon isotopic heterogeneity within amino acids of autotrophs and heterotrophs. *Oecologia* **139**: 178-189.

Simon H, Palm D. 1966. Isotope effects in organic chemistry and biochemistry. *Angewandte Chemie-International Edition* **5**: 920-933.

Sun W, Resco V, Williams DG. 2009. Diurnal and seasonal variation in the carbon isotope composition of leaf dark-respired CO_2 in velvet mesquite (*Prosopis velutina*). *Plant, Cell and Environment* **32**: 1390-1400.

Tcherkez G, Mahe A, Hodges M. 2011. $^{12}C/^{13}C$ fractionations in plant primary metabolism. *Trends in Plant Science* **16**: 499-506.

Tcherkez G, Farquhar G, Badeck F, Ghashghaie J. 2004. Theoretical considerations about carbon isotope distribution in glucose of C_3 plants. *Functional Plant Biology* **31**: 857-877.

Tcherkez G, Nogues S, Bleton J, Cornic G, Badeck F, Ghashghaie J. 2003. Metabolic origin of carbon isotope composition of leaf dark-respired CO_2 in French bean. *Plant Physiology* **131**: 237-244.

Tronconi MA, Fahnenstich H, Weehler MCG, Andreo CS, Flugge UI, Drincovich MF, Maurino VG. 2008. *Arabidopsis* NAD-malic enzyme functions as a homodimer and heterodimer and has a major impact on nocturnal metabolism. *Plant Physiology* **146**: 1540-1552.

Wegener F, Beyschlag W, Werner C. 2010. The magnitude of diurnal variation in carbon isotopic composition of leaf dark respired CO_2 correlates with the difference between $\delta^{13}C$ of leaf and root material. *Functional Plant Biology* **37**: 849-858.

Werner C, Gessler A. 2011. Diel variations in the carbon isotope composition of respired CO_2 and associated carbon sources: a review of dynamics and mechanisms. *Biogeosciences* **8**: 2437-2459.

Werner C, Wegener F, Unger S, Nogues S, Priault P. 2009. Short-term dynamics of isotopic composition of leaf-respired CO_2 upon darkening: measurements and implications. *Rapid Communications in Mass Spectrometry* **23**: 2428-2438.

Werner C, Hasenbein N, Maia R, Beyschlag W, Maguas C. 2007. Evaluating high time-resolved changes in carbon isotope ratio of respired CO_2 by a rapid in-tube incubation technique. *Rapid Communications in Mass Spectrometry* **21**: 1352-1360.

Werner RA, Buchmann N, Siegwolf RTW, Kornexl BE, Gessler A. 2011. Metabolic fluxes, carbon isotope fractionation and respiration - lessons to be learned from plant biochemistry. *New Phytologist* **191**: 10-15.

Werner RA 1998. Entwicklung neuer Verfahren für die on-line $^{18}O/^{16}O$- und $^{2}H/^{1}H$-Isotopenverhältnismessung und ihre Anwendung zur Authentizitaets- und Herkunftsbestimmung bei Naturstoffen. Muenchen, Germany: Hieronymus, pp. 4-9.

Wild B, Wanek W, Postl W, Richter A. 2010. Contribution of carbon fixed by Rubisco and PEPC to phloem export in the Crassulacean acid metabolism plant *Kalanchoe daigremontiana*. *Journal of Experimental Botany* **61**: 1375-1383.

Chapter 2

2. Malate as main carbon source of leaf dark-respired CO_2 across different environmental conditions in potato plants

Marco M. Lehmann[1], Katja T. Rinne[2], Carola Blessing[1], Rolf T. W. Siegwolf[2], Nina Buchmann[1], Roland A. Werner[1]

[1]Institute of Agricultural Sciences, ETH Zurich, Universitätsstr. 2, CH-8092 Zurich, Switzerland;

[2]Laboratory of Atmospheric Chemistry, Paul Scherrer Institute (PSI), CH-5232 Villigen, Switzerland

Manuscript was submitted to *Journal of Experimental Botany*.

Abstract

Dissimilation of carbon sources during plant respiration in support of metabolic processes results in the continuous release of CO_2. The carbon isotopic composition of leaf dark-respired CO_2 (*i.e.,* $\delta^{13}C_R$) shows daily enrichments up to 14.8‰ under different environmental conditions. However, the reasons for this ^{13}C enrichment in leaf dark-respired CO_2 are not fully understood, since daily changes in $\delta^{13}C$ of putative leaf respiratory carbon sources ($\delta^{13}C_{RS}$) are not yet clear. Thus, we exposed potato plants (*Solanum tuberosum*) to different temperature and soil moisture treatments. We determined $\delta^{13}C_R$ with an in-tube incubation technique and $\delta^{13}C_{RS}$ with compound-specific isotope analysis during a daily cycle. The highest $\delta^{13}C_{RS}$ values were found in the organic acid malate under different environmental conditions, showing less negative values compared to $\delta^{13}C_R$ (up to 5.2‰) and compared to $\delta^{13}C_{RS}$ of soluble carbohydrates, citrate, and starch (up to 8.8‰). Moreover, linear relationships between $\delta^{13}C_R$ and $\delta^{13}C_{RS}$ among different putative carbon sources were strongest for malate during daytime ($r^2 = 0.69$, $p \leq 0.001$) and nighttime ($r^2 = 0.36$, $p \leq 0.001$) under all environmental conditions. A multiple linear regression analysis revealed $\delta^{13}C_{RS}$ of malate as the most important carbon source influencing $\delta^{13}C_R$. Thus, our results strongly indicate malate as main carbon source of ^{13}C enriched dark-respired CO_2 in potato plants, probably driven by an anapleurotic flux replenishing intermediates of the Krebs cycle.

Key words: compound-specific isotope analysis (CSIA), drought, high temperature, organic acids, plant respiration, stable carbon isotopes, sugars, tricarboxylic acid cycle (TCA cycle)

Abbreviations: $\delta^{13}C_R$, carbon isotopic composition of leaf dark-respired CO_2; $\delta^{13}C_{RS}$, carbon isotopic composition of putative leaf respiratory carbon sources; A_n, net assimilation rate; C_i, intercellular CO_2 concentration; CSIA, compound-specific isotope analysis; g_s, stomatal conductance; HPLC, high performance liquid chromatography; KC, Krebs cycle; LEDR, light enhanced dark respiration; ME, malic enzyme; OAA, oxaloacetate; PDH, pyruvate dehydrogenase; PEPC, phosphoenolpyruvate carboxylase; SPS, sucrose phosphate synthase; *SWC*, volumetric soil water content

Introduction

The investigation of plant respiration as a major process in plant biochemistry has expanded our understanding of the carbon cycling in autotrophic organisms. Plants dissimilate carbon sources for the production of intermediates and reducing equivalents in support of metabolic processes, thereby continuously releasing CO_2 via plant respiration (Hopkins, 2006). Leaf respired CO_2 is mainly derived from oxidative decarboxylation reactions catalyzed by enzymes from the Krebs cycle (KC) and from interacting anabolic and catabolic reactions (Voet & Voet, 2011).

Using stable isotopes, the pathway of carbon can be traced from photosynthetic carbon fixation to respiratory carbon loss. On the one hand, C_3 plants discriminate heavily against ^{13}C due to photosynthetic isotope fractionation, leading to general ^{13}C depletion in plant biomass of about 20‰ in comparison to atmospheric CO_2 (Farquhar *et al.*, 1989). The exact magnitude of photosynthetic carbon isotope discrimination depends on the intercellular CO_2 concentration (C_i) in the substomatal cavity, which is regulated by other physiological parameters such as net assimilation rate (A_n) and stomatal conductance (g_s). Thereby, environmental conditions such as light, temperature, soil moisture, and air humidity will influence these parameters and with them the photosynthetic carbon isotope discrimination. On the other hand, the carbon isotopic composition of leaf dark-respired CO_2 (*i.e.,* $\delta^{13}C_R$) has clearly been shown to be less negative than leaf metabolites in several plant species (Ghashghaie *et al.*, 2003; Bowling *et al.*, 2008; Werner & Gessler, 2011; Ghashghaie & Badeck, 2014). In a daily cycle, leaf dark-respired CO_2 follows a progressive ^{13}C enrichment during the day and a gradual ^{13}C depletion during the course of the night (Hymus *et al.*, 2005; Prater *et al.*, 2006),

resulting in a strong temporal variability of up to 14.8‰ (Barbour *et al.*, 2007; Werner *et al.*, 2009; Wegener *et al.*, 2010), which differs among functional groups (Priault *et al.*, 2009; Werner *et al.*, 2009).

$\delta^{13}C_R$ is thereby linked to the carbon isotopic composition of putative leaf respiratory carbon sources (*i.e.,* $\delta^{13}C_{RS}$) such as carbohydrates (soluble mono- and di-saccharides, and starch) and organic acids. Previous studies showed that environmental drivers such as temperature and soil moisture influence $\delta^{13}C_R$ and $\delta^{13}C_{RS}$. More negative $\delta^{13}C_R$ values with increasing temperature have been observed with short-term changes in leaf temperature during darkness in *Phaseolus vulgaris* (Tcherkez et al., 2003), while long-term effects of higher temperatures on $\delta^{13}C_R$ and $\delta^{13}C_{RS}$ has not yet been conducted under controlled conditions. Other studies have demonstrated less negative $\delta^{13}C_R$ and $\delta^{13}C_{RS}$ values under dry conditions compared to those under wet conditions (Duranceau *et al.*, 1999; Ghashghaie *et al.*, 2001). Similar observations were made in field experiments (Sun *et al.*, 2009; Dubbert *et al.*, 2012). Conversely, more negative $\delta^{13}C_R$ values have been found under dry conditions for Mediterranean trees and herbs such as *Quercus ilex* and *Tuberaria guttata* compared to those under wet conditions (Unger *et al.*, 2010), which have been explained with accompanied increases in temperatures and vapour pressure deficit. Nevertheless, the combined effects of temperature and soil moisture on $\delta^{13}C_R$ and $\delta^{13}C_{RS}$ under controlled conditions have not yet been tested.

Moreover, $\delta^{13}C_R$ is determined by various post-photosynthetic carbon isotope fractionation processes at pivotal branching points in respiratory pathways, carbon isotope effects on enzymatic reactions, and changes in respiratory substrates (for a detailed review see Werner & Gessler, 2011). The ^{13}C enrichment in leaf dark-respired

CO_2 itself is thought to be a result of fragmentation fractionation processes based on heterogeneous intramolecular carbon isotope distribution in respiratory carbon sources (Tcherkez *et al.*, 2004). For instance, C-3 and C-4 positions of glucose are known to be enriched in ^{13}C compared to the other molecule positions due to an isotope effect of the aldolase reaction (Rossmann *et al.*, 1991; Gleixner & Schmidt, 1997). Breakdown of glucose during glycolysis produces pyruvate with a ^{13}C enriched C-1 position (former C-3 and C-4 positions of glucose). Thereafter, the pyruvate dehydrogenase reaction (PDH) releases the C-1 position as ^{13}C enriched CO_2, whereas the more ^{13}C depleted acetyl-CoA residue is used in the KC (Priault *et al.*, 2009; Werner & Gessler, 2011). Thus, a PDH dominated respiratory pathway may lead to ^{13}C enrichment in leaf dark-respired CO_2.

However, the knowledge about $\delta^{13}C_R$ is often based on light-acclimated leaves, which have been transferred into darkness to allow respiratory measurements. This approach holds an unpreventable bias known as “light enhanced dark respiration” (LEDR), which need to be taken into account if interpreting daytime $\delta^{13}C_R$ values. LEDR is a short-term light-dark transition period, describing an increase in the amount of leaf dark-respired CO_2 shortly upon darkening for about 20 min, which depends on light intensity (Atkin *et al.*, 1998). On the one hand, LEDR may be influenced by reassembly of the KC, which is discussed to be only partially active under light conditions (Tcherkez *et al.*, 2005; Sweetlove *et al.*, 2010; Werner & Gessler, 2011; Werner *et al.*, 2011). On the other hand, LEDR may be driven by a breakdown of a light-accumulated malate pool, causing ^{13}C enriched leaf dark-respired CO_2 (Barbour *et al.*, 2007; Gessler *et al.*, 2009; Werner *et al.*, 2009; Barbour *et al.*, 2011; Werner & Gessler, 2011). Malate itself is also known to be ^{13}C enriched compared to other carbon sources (Gleixner *et al.*, 1998; Ghashghaie *et al.*,

2001). The ^{13}C enrichment in malate was attributed to an anapleurotic flux via the phosphoenolpyruvate carboxylase reaction (PEPC), which fixes ^{13}C enriched hydrogen carbonate and replenishes KC intermediates (Melzer & O'Leary, 1987; Savidge & Blair, 2004). Thus, a possible breakdown of malate by the mitochondrial malic enzyme reaction, or within the KC, may influence $\delta^{13}C_R$ (Barbour *et al.*, 2007; Werner *et al.*, 2011). In addition, plants may also use to a certain extent more complex carbon sources such as lipids and proteins under severe environmental conditions or under prolonged darkness (Tcherkez *et al.*, 2003; Usadel *et al.*, 2008). However, the driving processes, the respiratory carbon sources, and the mechanisms causing changes in $\delta^{13}C_R$ during day and night are not fully resolved thus far.

Hence, with this study we intend to assess two major research questions: What causes the high daily variations in $\delta^{13}C_R$? How are $\delta^{13}C_R$ and $\delta^{13}C_{RS}$ influenced by temperature and soil moisture conditions? Our main objectives were (1) to analyze the relationship between $\delta^{13}C_R$ and $\delta^{13}C_{RS}$ values and (2) to determine changes in $\delta^{13}C_R$ and $\delta^{13}C_{RS}$ values, as well as in concentrations of the putative carbon sources under different environmental conditions. Therefore, we exposed potato plants to different controlled temperature and soil moisture conditions and measured $\delta^{13}C_R$ with an in-tube incubation technique, as well as $\delta^{13}C_{RS}$ and concentrations of soluble carbohydrates, organic acids, and starch from leaves with compound specific isotope analysis (CSIA) on a daily basis.

Materials & Methods

Plant Material

Potato plants (*Solanum tuberosum* L. cv. Annabell) were grown from tubers of the same size in 5 L pots filled with bark humus soil (Ökohum, Herrenhof, Switzerland) in a greenhouse, with average temperatures of 20°C/16°C and vapour pressure deficits (*VPD*) of about 0.9 kPa/0.4 kPa (day/night). The plants were exposed to a 16 h daylight period supplemented by 400 W sodium-lamps (Powertone Son-T Plus, Philips, Amsterdam, Netherlands). 40 days after planting, plants were transferred into walk-in climate chambers for acclimatization for two weeks. The 16 h daylight in the climate chambers had an averaged photosynthetic photon flux density of about 400 µmol m^{-2} s^{-1} at leaf level, thus plants were not fully light-saturated. Before the treatment period, soil water status was optimal for at least three days after watering, while an individual plant consumed about 300 ml water per day. 50 ml of a 0.4% fertilizer solution (v/v, Gesal, Zürich, Switzerland) was applied two times to all plants during the whole experiment of 70 days.

Treatments were applied during the last 15 days of the experiment. Plants were exposed to high temperature (T_{high}) of 28°C/23°C (day/night) and low temperature conditions (T_{low}) of 22°C/17°C, at a *VPD* of about 0.9 kPa/0.35 kPa for both temperature treatments. Three climate chambers were used for replication of each temperature treatment. Within each climate chamber there were two soil-moisture treatments with nine plants each. Thereby, dry soil moisture conditions were kept constantly at 50-60% of the daily water consumption of each individual plant, determined by weighing the entire pots. Plants under wet conditions were kept at 100%.

The final sampling period lasted 32 h during the last two days of the experiment, when dry soil conditions were established for both temperature treatments. Sampling was done on a daily basis every two (nighttime) or four hours (daytime). During sampling, individual plants had between 3-6 ranks, with about four fully developed leaves per rank. Always the third last fully developed leaf per rank was sampled at all points in time, but within 24 h only one sample was taken from each individual plant to avoid any stress response induced by sampling. Sampled leaf material was immediately frozen in liquid nitrogen and stored at -80°C. Subsequently, the leaf material was freeze-dried and milled to powder by a steel ball mill (MM200, Retsch, Haan, Germany) for all further isotopic and biochemical analyses. Additionally to leaf sampling, air CO_2 samples from all six climate chambers were collected at the same points in time during the sampling period, showing a mean $\delta^{13}C$ value of -12.2‰ and typical daily variations of SD ≤ 1.4‰; no differences between temperature treatments ($p \geq 0.05$) and points in time ($p \geq 0.05$; linear mixed effects model) were observed during the daily cycle.

Physiological measurements and biomass determination

Several leaf physiological parameters were determined with an infrared gas analyzer (LI-6400, LI-COR, Lincoln, Nebraska, USA), including net assimilation rate (A_n), intercellular CO_2 concentration (C_i), and stomatal conductance (g_s). All measurements were taken in the last four hours of the daylight phase. To monitor volumetric soil water content (*SWC*), up to three soil moisture sensors (EC-5 and logger Em5b, Decagon Devices, Pullman, USA) were installed for each treatment. Shortly after the sampling period, total plant biomass was harvested, oven-dried (60°C), and weighed. The fresh tuber weight and tuber count (number of potatoes) were determined.

Carbon isotope and concentration analyses

$\delta^{13}C$ values are expressed as described by Craig (1957) and modified by Coplen (2011):

$\delta^{13}C$ (‰) = $R_{sample}/R_{standard}$ -1

where R_{sample} is the $^{13}C/^{12}C$ ratio of the sample material and $R_{standard}$ that of the international standard VPDB (Vienna PeeDee Belemnite).

Determination of $\delta^{13}C_R$

The in-tube incubation technique was used for the collection of leaf dark-respired CO_2 during daytime and nighttime (Werner *et al.*, 2007). A leaf was placed in a 12 ml gas-tight exetainer (Labco, Lampeter, UK), which was immediately darkened by a lightproof casing to trigger leaf dark respiration. The tube was then flushed for 1 min with synthetic air until a CO_2 free atmosphere was established, which was monitored with an infrared gas analyzer (LI-6262, LI-COR, Lincoln, Nebraska, USA). After an incubation time of 3 min in darkness, an aliquot of dark-respired CO_2 was transferred with a gas-tight syringe into a new exetainer filled with dry N_2. $\delta^{13}C_R$ values were determined with an IRMS, using a modified Gasbench II (Thermo Fisher, Bremen, Germany) connected to a Delta^{plus}XP-IRMS, similar to Zeeman *et al.* (2008). The transfer of the CO_2 sample into a new exetainer, as well as the IRMS measuring procedure, was tested with air of known $\delta^{13}C$ of CO_2 to ensure no isotope fractionation had occurred. Measurement precision of a quality control standard (three standards per 24 samples) was SD < 0.1‰.

Determination of $\delta^{13}C$ in bulk leaves and leaf starch

Extraction of leaf starch was performed as described in previous studies (Wanek *et al.*, 2001; Goettlicher *et al.*, 2006; Richter *et al.*, 2009). Leaf starch was isolated from 50 mg leaf material with methanol/chloroform/water (MCW, 12:5:3, v/v/v) at 70 °C for 30 min. Samples were centrifuged (10000 g, 2 min) and supernatants removed, while the leaf starch containing pellets were washed with MCW and deionized water and dried at room temperature (RT). Pellets were then re-suspended in water and boiled at 99°C for 15 min to facilitate starch gelatinization. Subsequently, leaf starch was enzymatically digested with α-amylase (EC 3.2.1.1, Sigma-Aldrich, Buchs, Switzerland) at 85°C for 2 h, and cleaned with centrifugation filters to remove enzymes (Vivaspin, Sartorius, Göttingen, Germany). To determine $\delta^{13}C$ of bulk leaves ($\delta^{13}C_{leaf}$) and starch, an elemental analyzer (Flash EA 1112 Series) coupled to a Delta^{plus}XP-IRMS was used (both Thermo Fisher, Bremen, Germany; Werner *et al.*, 1999). Measurements of samples, blanks, and reference material followed the identical treatment principle described by Werner & Brand (2001). The long-term precision of a quality control standard for all sequences was SD < 0.12‰.

Isotopic and concentration analysis of soluble carbohydrates and organic acids

Water soluble compounds were extracted with water at 85°C for 30 min from 100 mg leaf material similar to Streit *et al.* (2013). Subsequently, soluble carbohydrates and organic acids were separated by ion-exchange chromatography (Wanek *et al.*, 2001; Goettlicher *et al.*, 2006; Richter *et al.*, 2009), using Dowex 50WX8 in H^+-form and Dowex 1X8 in $NaCOO^-$-form (both 100-200 mesh, Sigma-Aldrich, Buchs, Switzerland). To avoid clogging of the HPLC column by polyphenols, all samples designated for carbohydrate

analyses were filtered with 100 mg Sep-Pak C18 Vac RC Cartridges (Waters AG, Milford, Massachusetts, USA). Finally, all carbohydrate and organic acid samples were cleaned with 0.45 µm PTFE syringe filter (Infochroma AG, Zug, Switzerland) prior to HPLC measurements.

To determine $\delta^{13}C_{RS}$ values and the concentrations of soluble carbohydrates and organic acids, a HPLC-IRMS system consisting of a high performance liquid chromatograph coupled to a Delta V Advantage IRMS by a LC IsoLink (all Thermo Fisher, Bremen, Germany) was used according to Krummen *et al.* (2004). Carbohydrates were separated on a 3 x 150 mm anion-exchange column CarboPac PA20 (Dionex, Olten, Switzerland) using 2 mM NaOH as the mobile phase and a flow speed of 250 µl min^{-1} (Boschker *et al.*, 2008; Rinne *et al.*, 2012). Low column temperature of 20°C was used to prevent isomerization of hexoses (Rinne *et al.*, 2012). This enabled chromatographic separation for sucrose and glucose, but fructose $\delta^{13}C_{RS}$ and concentrations measurements were affected by partial co-elution of fructose with other compounds. To correct $\delta^{13}C_{RS}$ values and to calculate concentrations from the peak areas, interspersed standard solutions in a concentration range of 20-180 ng C μl^{-1} were measured within each sequence. The measurement precision of $\delta^{13}C_{RS}$ values in all carbohydrate standards was SD < 0.5‰. Below a concentration of 60 ng C μl^{-1}, the precision of fructose standards was lower for certain batches, and therefore these results were excluded.

Organic acids were separated on a 4.6 x 300 mm Allure Organic Acids column (Restek, Bellefonte, USA) at 5-10°C. The mobile phase was a 100 mM monopotassium phosphate buffer (pH = 3) with a flow speed of 500 µL min^{-1} (Hettmann *et al.*, 2005). The measurement precision of $\delta^{13}C$ in organic acid standards was SD < 0.4‰. Low citrate

concentrations from T_{low} samples (< 45 ng C μl^{-1}) impeded the analytical accuracy of the $\delta^{13}C_{RS}$ values, therefore these samples were not taken into account.

All purification steps were tested for each analytical batch of 24 samples with 2.5 mg standard solutions of all measured carbohydrates and organic acids in this study, with known $\delta^{13}C$ by EA-IRMS. Differences between $\delta^{13}C$ values before and after purification were generally ≤ 0.2‰, indicating no significant isotope fractionation for any standard. Mean recovery was 101±6% for fructose, 96±6% for glucose, 89±3% for sucrose, 91±3% for malate, and 86±3% for citrate.

Determination of starch concentration

For the extraction of leaf starch for concentration analyses we used a modified method of Critchley *et al.* (2001). Leaf starch was isolated with 1.12 M perchloric acid from 50 mg leaf material at RT for 15 min and centrifuged (10 min, 3000 g, 4 °C). The supernatant was removed and the leaf starch containing pellet was washed free from pigments with deionized water and ethanol. Pellets were then dried at RT, re-suspended in water, and gelatinized. Subsequently, starch samples were enzymatically hydrolyzed to glucose for 2 h at 37°C with a solution mix of α-amylase (EC 3.2.1.1, Sigma-Aldrich, Buchs, Switzerland) and α-amyloglucosidase (EC 3.2.1.3, Roche, Rotkreuz, Switzerland) in 220 mM sodium acetate buffer (pH = 4.8). The glucose concentration was determined at 340 nm with a 96-well microplate reader (ELx800, BioTek, Luzern, Switzerland) using a coupled enzymatic reaction (Hoch *et al.*, 2002). Potato starch was used as a standard. Glucose concentrations are expressed in molarity of starch monomers.

Data analysis

R version 3.0.2 (R Core Team, 2013) was used for (multiple) linear regression analyses and linear mixed effects models (R package nlme). Models included fixed effects (temperature, soil moisture, sampling time) and random effects (climate chambers, individual plants). If applicable, $\delta^{13}C$ values and concentrations were logarithmically transformed to ensure normal distribution. For the best-fit combination of the multiple linear regression analysis, variables were excluded if $p \geq 0.05$.

Results

Physiological parameters and biomass

Physiological parameters (A_n, C_i, g_s and *SWC*) of potato plants exposed to four different treatments were monitored during the treatment period of 15 days (Fig. 1). The net assimilation rate declined during the treatment period under all four treatments (Fig. 1A). During the sampling period (Fig. 1A, day 15), A_n was significantly influenced by soil moisture ($p = 0.02$, Tab. 1), with lowest values (1.9 µmol m^{-2} s^{-1}) under T_{high} and dry conditions and highest values (5.4 µmol m^{-2} s^{-1}) under T_{low} and wet conditions, whereas the temperature influence caused by trend lower A_n values under T_{high} than under T_{low}. The intercellular CO_2 concentration increased during the treatment period for all four treatments (Fig. 1B). During the sampling period (Fig. 1B, day 15), C_i was independently influenced by temperature ($p = 0.012$, Tab. 1) and soil moisture ($p = 0.01$, Tab.1), with lowest C_i (247.5 µmol mol^{-1}) under T_{low} and dry conditions and highest C_i (332.8 µmol mol^{-1}) under T_{high} and wet conditions. Stomatal conductance during the treatment period was lower under dry treatments compared to those under wet treatments (Fig. 1C).

During the sampling period (Fig. 1C, day 15), g_s was significantly influenced by soil moisture ($p \leq 0.001$, Tab. 1), with lowest g_s (about 0.06 mol m^{-2} s^{-1}) in plants of both dry treatments and highest g_s (0.22 mol m^{-2} s^{-1}) in plants under T_{high} and wet conditions, whereas the temperature influence under wet conditions caused by trend higher g_s values under T_{high} than under T_{low}. The volumetric soil water content was lower under dry conditions (about 7-14%) compared to wet conditions (23-27.5%) for the last 9 days of the treatment period (Fig. 1D), including the sampling period (Fig. 1D, day 15), where *SWC* was significantly affected only by soil moisture treatments ($p = 0.002$, Tab. 1). Generally, no significant interactions between temperature and soil moisture were observed for any parameter (Tab. 1). In addition, only soil moisture treatments affected plant biomass ($p = 0.008$, Tab. 1) and tuber weight ($p = 0.023$, Tab. 1) taken shortly after the sampling period, independent of temperature treatments. Thereby, highest values were measured by trend under T_{low} and wet conditions and lowest values under T_{high} and dry conditions (Tabs. 1, 2), indicating different stress levels created by the four treatments.

Carbon isotopes in potato leaves

Daily cycles of $\delta^{13}C_R$ and $\delta^{13}C_{leaf}$

$\delta^{13}C$ values of leaf dark-respired CO_2 ($\delta^{13}C_R$) varied significantly over time ($p \leq 0.001$, Tab. 3) with values in the range of -21.9‰ and -32‰, declining strongly during nighttime and increasing again during the daytime for all four treatments (Fig. 2A). An interaction between temperature and time showed that the influence of temperature differed with time ($p = 0.014$, Tab. 3). Daytime $\delta^{13}C_R$ values under T_{high} were up to 4.7‰ more negative compared to those under T_{low}, independent of soil moisture conditions,

whereas nighttime $\delta^{13}C_R$ values of both temperature treatments were particularly in the second night very similar. Dry soil moisture conditions caused less negative $\delta^{13}C_R$ values compared to those under wet conditions during the daily cycle ($p = 0.013$, Tab. 3), with a maximum difference of 2.7‰, independent of temperature treatments. On average, the difference between daytime and nighttime $\delta^{13}C_R$ values was highest under T_{low} and wet conditions, with 5.7‰, and lowest under T_{high} and dry conditions, with 2.5‰.

The bulk leaf material reflects all environmental conditions experienced during the whole growth period. $\delta^{13}C_{leaf}$ of all treatments showed no changes during the sampling period and no interactions between treatments and time (Fig. 2B; Tab. 3). Under T_{high}, $\delta^{13}C_{leaf}$ values were up to 2.2‰ more negative compared to those under T_{low}, resulting in a significant temperature effect independent of soil moisture conditions ($p = 0.022$, Tab. 3). Similarly, soil moisture showed a significant effect on $\delta^{13}C_{leaf}$ ($p = 0.005$, Tab. 3), independent of temperature treatments, with values up to 1.1‰ less negative under dry than under wet conditions.

$\delta^{13}C_{RS}$ of soluble carbohydrates, organic acids, and starch

Highest $\delta^{13}C$ values in putative leaf respiratory carbon sources ($\delta^{13}C_{RS}$) were found in the organic acid malate, while soluble carbohydrates (fructose, glucose and sucrose) exhibited generally lowest $\delta^{13}C_{RS}$ values (Fig. 3). Thereby, $\delta^{13}C_{RS}$ of soluble carbohydrates of all treatments were in the range of -27.2‰ and -36.6‰. More negative $\delta^{13}C_{RS}$ values of glucose and sucrose under T_{high} compared to those under T_{low} were found, independent of soil moisture conditions, while less negative $\delta^{13}C_{RS}$ values under dry conditions compared to those under wet conditions were observed, independent of temperature treatments (Figs. 3B, 3C; Tab. 3). Significant interactions between

temperature and time for $\delta^{13}C_{RS}$ of glucose (p = 0.008, Tab. 3) and sucrose (p = 0.003, Tab. 3) showed that daily cycles differed between temperatures. Additionally, soil moisture conditions caused significant temporal variations during the daily cycle in $\delta^{13}C_{RS}$ of sucrose (p = 0.002, Tab. 3).

We observed significant linear relationships between fructose and glucose for $\delta^{13}C_{RS}$ (r^2 = 0.74, p ≤ 0.001) and concentration values (r^2 = 0.8, p ≤ 0.001), while relationships between the other $\delta^{13}C_{RS}$ values and concentrations of different carbon sources were weaker (data not shown). However, the deviant results for $\delta^{13}C_{RS}$ of fructose in comparison to the other sugars are assumed to reflect peak overlap issues of this sugar (Tabs. 3, 4). This is clearly reflected also in the concentration results (Fig. 4A). Consequently, the fructose results will not be discussed further in detail.

$\delta^{13}C_{RS}$ of malate (Fig. 3D) in the range of -24‰ and -29.3‰ and $\delta^{13}C_{RS}$ of citrate (Fig. 3E) in the range of -29.6‰ and -32.1‰ showed no temporal variations (p = 0.198 and p = 0.052 for malate and citrate, respectively, Tab. 3). Significant interactions between temperature and soil moisture treatments were observed for $\delta^{13}C_{RS}$ of malate (p = 0.017; Tab. 3), resulting in larger differences between $\delta^{13}C_{RS}$ values of soil moisture conditions under T_{high} than under T_{low} (Fig. 3D). Citrate showed less negative $\delta^{13}C_{RS}$ values under dry conditions than under wet conditions (p = 0.009; Tab.3).

$\delta^{13}C_{RS}$ of starch of all treatments (Fig. 3F), ranging from -25.2‰ and -32.1‰, was influenced by soil moisture conditions (p = 0.046, Tab. 3), independent of temperature treatments, while temperature showed no significant effect (p = 0.107, Tab. 3). In addition, soil moisture conditions caused significant temporal variations during the daily cycle in $\delta^{13}C_{RS}$ of starch (p = 0.032, Tab. 3).

Concentrations of soluble carbohydrates, organic acids, and starch

Concentrations of glucose of all treatments (Fig. 4B), ranging from 5 to 17 µg mg^{-1}, showed no temporal variations ($p = 0.927$, Tab. 3). In contrast, concentrations of sucrose (Fig. 4C) in the range of 4 to 27 µg mg^{-1} showed clear daily variations ($p \leq 0.001$, Tab. 3), with highest concentrations for all treatments by the end of the day, except for T_{high} and dry conditions. Glucose concentrations were significantly higher under dry than under wet conditions ($p \leq 0.001$, Tab. 3), while converse results were observed for sucrose ($p = 0.031$, Tab. 3). Generally, no effect of temperature on the concentration of any soluble carbohydrate was observed.

Malate concentrations of all treatments (Fig. 4D), ranging from 3 to 22 µg mg^{-1}, showed a daily pattern with declining concentrations in the beginning of the night and an increase after 2-4 h in the dark ($p = 0.035$, Tab. 3). In contrast to soluble carbohydrates, malate concentrations were significantly higher under T_{high} than under T_{low} ($p = 0.011$, Tab. 3), but were not affected by soil moisture treatments ($p = 0.999$, Tab. 3). Citrate concentrations under T_{high} of about 3 µg mg^{-1} were the lowest of all measured putative carbon sources available for leaf dark respiration and showed no changes due to soil moisture treatments and time (Fig. 4E; Tab. 3).

Starch concentrations (Fig. 4F), ranging from 12 to 50 µg mg^{-1}, showed significant temporal variations ($p \leq 0.001$, Tab. 3), independent of any treatment. The average starch concentration of 44 µg mg^{-1} under T_{low} and wet conditions was clearly higher (about 2.5 times) compared to those under other treatments. In addition, interactions between temperature and soil moisture treatments led to smaller differences between

the values of wet and dry conditions under T_{high} compared to those under T_{low} ($p \leq 0.001$, Tab. 3).

Linear relationships between $\delta^{13}C_R$ and $\delta^{13}C_{RS}$

Linear regression analyses were performed to understand the biochemical link between $\delta^{13}C_R$ *and* $\delta^{13}C_{RS}$ across all treatments (Fig. S1; Tab. 4). $\delta^{13}C_{RS}$ of malate explained most of the daily variation of $\delta^{13}C_R$ ($r^2 = 0.26$, $p \leq 0.001$), while the explanatory power of fructose, glucose, and citrate was lower. The lowest linear relationships during the daily cycle were found between $\delta^{13}C_R$ and $\delta^{13}C_{RS}$ of sucrose and starch. Due to the high daily variations in $\delta^{13}C_R$ we carried out the same analysis separately for daytime and nighttime. Daytime linear relationships were generally stronger than during nighttime, with $\delta^{13}C_R$ strongly related to $\delta^{13}C_{RS}$ of malate, citrate, and $\delta^{13}C_{leaf}$ ($r^2 > 0.6$, $p \leq 0.001$), but lower related to $\delta^{13}C_{RS}$ of soluble carbohydrates and starch. During nighttime, $\delta^{13}C_{RS}$ of malate explained 36% of the variation in $\delta^{13}C_R$, but $\delta^{13}C_{RS}$ of fructose and glucose, as well as $\delta^{13}C_{leaf}$, showed similarly high explanatory power.

Influence of environmental drivers and carbon sources on $\delta^{13}C_R$

Furthermore, a stepwise (backward) multiple linear regression analysis was performed to identify environmental drivers and carbon sources influencing $\delta^{13}C_R$ (Tab. 5). Daytime/nighttime showed the strongest and positive effect on $\delta^{13}C_R$ ($\beta = 0.73$, $p \leq 0.001$), while $\delta^{13}C_{RS}$ of malate was the carbon source that affected $\delta^{13}C_R$ most ($\beta = 0.4$, $p \leq 0.001$). By comparison, the influence of $\delta^{13}C_{RS}$ of starch and soil moisture conditions on $\delta^{13}C_R$ values was minor.

Discussion

This study clearly demonstrates that different temperature and soil moisture conditions influence $\delta^{13}C$ of leaf dark respired CO_2 ($\delta^{13}C_R$), $\delta^{13}C$ of different putative leaf respiratory carbon sources ($\delta^{13}C_{RS}$), and concentrations of carbon sources during a daily cycle in potato leaves. Furthermore, our findings strongly indicate malate as main carbon source of daytime and nighttime $\delta^{13}C_R$ across different environmental conditions.

Impact of temperature and soil moisture on isotopic compositions

After two weeks of treatment, we already found a clear temperature effect on $\delta^{13}C_{leaf}$, with up to 2.2‰ more negative $\delta^{13}C_{leaf}$ values under T_{high} conditions compared to those under T_{low} conditions (Fig. 2B). This is in agreement with a study showing more negative $\delta^{13}C$ values with increasing temperature for bulk leaves of *Xanthium* species (Smith *et al.*, 1976). Similar to Tcherkez *et al.* (2003) under short-term temperature treatments, we observed more negative $\delta^{13}C_R$ value with increasing temperature (Fig. 2A), but due to our long-term treatment we found also more negative $\delta^{13}C_{RS}$ values (Fig. 3). On the other hand, dry conditions in both of the temperature treatments caused less negative $\delta^{13}C_{leaf}$, $\delta^{13}C_R$, and $\delta^{13}C_{RS}$ values compared to those under wet conditions, which is consistent with previous studies under controlled conditions (Duranceau *et al.*, 1999; Ghashghaie *et al.*, 2001).

The isotopic results under the different environmental conditions can be directly linked to the leaf gas exchange observed during the 32 h sampling period (day 15 of the treatment period). Increasing temperature caused lower A_n values under both soil moisture conditions (Fig. 1A, Tab. 1), indicating that plants under T_{high} were beyond the photosynthetic optimum. This result is in agreement with earlier studies, showing that

cold-adapted potato plants have reduced rates of photosynthesis with temperatures above 20°C (Levy & Veilleux, 2007). Additionally, A_n might be also influenced by leaf ageing, since A_n decreased under all treatments during the treatment period. On the other hand, g_s tended to higher values with increasing temperature, but only under wet conditions (Fig. 1C, Tab. 1). An increase of g_s under T_{high} might be triggered by increasing transpiration rates, which could be a physiological response to compensate reduced rates of A_n by cooling the leaf temperature under T_{high} conditions. However, this was only observed in plants under T_{high} and well-watered conditions, when *SWC* was high. Subsequently, lower carbon fixation and higher CO_2 diffusion into the stomatal cavities under T_{high}, in comparison to T_{low}, caused an increase of C_i (Fig. 1B) and more negative $\delta^{13}C_R$ and $\delta^{13}C_{RS}$ values (Tab. 6). Furthermore, dry soil moisture conditions caused reduced rates of A_n and g_s compared to those under wet conditions (Figs. 1A, 1C, Tab. 1), independent of temperature treatments. This can be explained with the severe drought stress, reflecting low *SWC* values (Fig. 1D). Consequently, plants under dry conditions experienced reduced CO_2 diffusion into the stomatal cavities, leading to lower C_i and less negative $\delta^{13}C$ values (Tab. 6).

Plants under T_{high} and dry conditions showed the lowest performance during the sampling period compared to plants under other treatments, which is reflected in low A_n values (Fig. 1A), plant biomass, tuber weight, and tuber count (Tab. 2). $\delta^{13}C_R$ and $\delta^{13}C_{RS}$ in these plants were expected to be the most positive compared to other treatments due to a severe drought caused by the double impact of high temperature and dry soil moisture. Instead, $\delta^{13}C_R$ and $\delta^{13}C_{RS}$ of the plants under the highest stress level (T_{high} and dry conditions) were rather similar to those under lowest stress level (T_{low} and wet

conditions). This was particularly observed for $\delta^{13}C_{RS}$ of soluble carbohydrates and starch (Fig. 3). Again, this is an indicator of low A_n under T_{high} and dry conditions, resulting in a moderate reduction of C_i, while at the same time g_s strongly reduces CO_2 diffusion into the stomatal cavities, causing an increase of C_i. Consequently, this led to intermediate $\delta^{13}C_R$ and $\delta^{13}C_{RS}$ values under T_{high} and dry conditions (Tab. 6). In summary our findings indicate that combined effects of temperature and soil moisture conditions on $\delta^{13}C_R$ and $\delta^{13}C_{RS}$ could cancel out the individual impacts of each driver.

Environmental impacts on concentrations of putative carbon sources

Soil moisture and temperature affected concentrations of putative leaf respiratory carbon sources differently. Sucrose concentration decreased under dry conditions (Fig. 4c; Tab. 3), which is in contrast to a recent study (Lemoine *et al.*, 2013). This may be explained by reduced rates of sucrose synthesis due to lowering of the sucrose phosphate synthase reaction (SPS; Vu *et al.*, 1998). The decrease of the enzyme activity is probably triggered by limited rates of phloem sugar transport observed under drought (Ruehr *et al.*, 2009). This in turn could be an explanation for lower plant biomass and tuber weight/count in response to higher temperatures and dry conditions (Tabs. 1, 2). Subsequently, the increase of fructose and glucose concentrations under drought may also be a consequence of lower SPS activity (Figs. 4A, 4B; Tab. 3), since the demand for both hexoses for sucrose synthesis was reduced. Additionally, increasing fructose and glucose concentrations under drought might have osmotic functionality, maintaining metabolic activity (Lemoine *et al.*, 2013).

On the other hand, malate concentrations increased with temperature (Fig. 4; Tab. 3), which is most likely a consequence of higher PEPC activity (Chinthapalli *et al.*, 2003).

Higher malate concentrations may also support respiratory processes in the KC or regulation of stomatal opening (Finkemeier & Sweetlove, 2009). Moreover, decreased starch concentrations in leaves under treatments with higher environmental stress than T_{low} and wet conditions (Fig. 4F) were similar to previous findings (Lemoine *et al.*, 2013). The result also supports the assumption that reduced amounts of assimilated carbon due to lower A_n under T_{high} or dry conditions were used for maintenance of biochemical processes rather than for carbon storage. Additionally, this indicates that plants under T_{high} or dry conditions were under severe environmental stress.

Malate as main respiratory carbon source of daytime and nighttime $\delta^{13}C_R$

The daily cycle of $\delta^{13}C_R$ was highly variable, showing less negative daytime and more negative nighttime values, while $\delta^{13}C_{RS}$ values generally showed lower changes during the same period (Figs. 2A, 3; Tab. 3). $\delta^{13}C_{RS}$ values of all treatments compared to $\delta^{13}C_R$ values were more negative for soluble carbohydrates (up to 9.3‰) and citrate (up to 4.1‰), but also less negative for starch (up to 4‰) and malate (up to 5.2‰) during the daily cycle (Figs. 2A, 3). In particular, malate was strongly enriched in ^{13}C, by up to 8.8‰, compared to all other putative carbon sources (Fig. 3). This was similar to a previous study investigating metabolites in potato leaves (Gleixner *et al.*, 1998) and indicates a possible biochemical link between ^{13}C enriched leaf dark-respired CO_2 and ^{13}C enriched malate.

For a better understanding of the overall biochemical connections between $\delta^{13}C_R$ and different putative carbon sources, we carried out linear regression analyses, independent of environmental conditions (Tab. 4). The daily linear relationship between $\delta^{13}C_R$ and $\delta^{13}C_{RS}$ of malate was stronger compared to all other putative carbon sources

(r^2 = 0.26, p ≤ 0.001). The strength of this relationship increased for $\delta^{13}C_R$ and $\delta^{13}C_{RS}$ of malate when considering daytime (r^2 = 0.69, p ≤ 0.001) and nighttime (r^2 = 0.36, p ≤ 0.001) separately. Moreover, relationships of $\delta^{13}C_R$ with $\delta^{13}C_{RS}$ of malate were stronger than with $\delta^{13}C$ of bulk leaves, which reflects the average $\delta^{13}C$ value of all respiratory substrates. Thus, relationships of $\delta^{13}C_R$ with $\delta^{13}C_{RS}$ of malate were above this average, while this was not the case for most of the carbon sources.

However, comparisons between daytime and nighttime relationships must be done carefully (Tab. 4), due to the bias in daytime $\delta^{13}C_R$ measured during LEDR. Nevertheless, the influence of LEDR on $\delta^{13}C_R$ is assumed to be constant during the light period and should not vary with environmental conditions due to the constant and reproducible sampling procedure, implying that daytime $\delta^{13}C_R$ values under all environmental conditions are generally comparable with each other. *Vice versa*, the influence of temperature and soil moisture conditions on $\delta^{13}C_R$ should also not be significantly affected by the LEDR process during the daily cycle (Tab. 3).

LEDR is discussed to be fuelled by malate (Atkin *et al.*, 1998; Barbour *et al.*, 2007; Gessler *et al.*, 2009; Werner & Gessler, 2011). Consequently, the strong daytime relationship between $\delta^{13}C_R$ and $\delta^{13}C_{RS}$ of malate might be explained by a higher respiratory consumption of malate during the LEDR period, provoking less negative daytime $\delta^{13}C_R$ values (Fig. 2A). Furthermore, transferring light-acclimated leaves into darkness is suggested to lead to reassembly of the KC by activation of light-inhibited enzymatic reactions of the cycle (Tcherkez *et al.*, 2005; Sweetlove *et al.*, 2010; Werner & Gessler, 2011). During LEDR the KC might not be fully active, leading to changes in metabolic fluxes and isotope fractionations, which may not occur during nighttime when KC is fully

reassembled (Werner *et al.*, 2011). This could be an important factor, explaining light-dark differences in the relationships between $\delta^{13}C_R$ and $\delta^{13}C_{RS}$ of different carbon sources in this study (Tab. 4).

In contrast to malate, $\delta^{13}C_{RS}$ of carbon storage compounds, such as starch and sucrose, were less related to $\delta^{13}C_R$ during daytime and nighttime (Tab. 4). This can particularly be explained for starch due to the fact that its isotopic composition is always a mix of fresh and old assimilates, constraining good relationships with the isotopic composition of recently respired CO_2. Moreover, the high daytime relationship between $\delta^{13}C_R$ and $\delta^{13}C_{RS}$ of citrate might be explained by the close biochemical relationship of citrate with malate via the mitochondrial malate dehydrogenase and citrate synthase (Voet & Voet, 2011). However, citrate was ^{13}C depleted and showed very low concentrations compared to other carbon sources (Figs. 3E, 4E), contradicting the role of citrate as an important carbon source of $\delta^{13}C_R$.

We also observed regular decreases in malate concentrations in the beginning of the night across all environmental conditions (Fig. 4D), as observed in previous studies (Urbanczyk-Wochniak *et al.*, 2005; Gessler *et al.*, 2009), which may reflect the use of malate for respiratory processes shortly upon darkening, e.g., LEDR. It has also been suggested that malate accumulates during daytime (Barbour *et al.*, 2007; Gessler *et al.*, 2009; Werner & Gessler, 2011). However, low temporal variations in malate concentrations during daytime do not support this hypothesis.

Furthermore, the hypothesis that $\delta^{13}C_R$ is mainly influenced by the putative carbon source malate across all treatments was also indicated by a stepwise multiple linear regression analysis (Tab. 5). The findings are in line with our other observations showing

(1) that daytime and nighttime periods have a clear influence on $\delta^{13}C_R$ (Fig. 2A); (2) that $\delta^{13}C_{RS}$ of malate has the strongest influence on $\delta^{13}C_R$ compared to all other putative carbon sources; and (3) that influences of other environmental drivers and of different putative carbon sources are weaker and less significant compared to daytime/nighttime and malate. Overall, the findings strongly indicate $\delta^{13}C_{RS}$ of malate as main carbon source of $\delta^{13}C_R$ during the daily cycle across all environmental conditions within this study.

A mechanistic explanation for the respiratory use of malate can be found within the amphibole functionality of the KC and associated reactions (malic enzyme, PDH; Fig. 5). Generally, the breakdown of glucose during glycolysis produces pyruvate. Leaf feeding experiments using position-specific ^{13}C labelled pyruvate have shown in different species that respiration of the C-1 position of pyruvate is higher compared to respiration of the C-2 and C-3 position of pyruvate during daytime (Priault *et al.*, 2009; Wegener *et al.*, 2010), as well as during nighttime (Werner *et al.*, 2009). This clearly indicates that acetyl-CoA (C-2 and C-3 position of pyruvate) from the PDH reaction, which enters the KC, is used for biosynthesis of diverse metabolic compounds (e.g. amino acids or lipids), rather than for respiration (Fig. 5). If this is true, withdrawn KC intermediates must be refilled due to stoichiometric reasons to maintain the functionality of the KC. This could be achieved by an anapleurotic flux via PEPC, which has often been described as replenishing KC intermediates (Melzer & O'Leary, 1987; Savidge & Blair, 2004). The PEPC reaction produces ^{13}C enriched oxaloacetate, of which the greatest proportion is directly converted into malate via malate dehydrogenase reaction. A breakdown of this malate pool within the KC or associated reactions (malic enzyme, PDH) would then produce ^{13}C

enriched leaf dark-respired CO_2 (Fig. 5), explaining the close relationship between $\delta^{13}C_R$ and $\delta^{13}C_{RS}$ of malate found in this study. Moreover, malate is supposed to be ^{13}C enriched at the C-4 position via PEPC, while other positions of the molecule are ^{13}C depleted via glycolysis (Melzer & O'Leary, 1987; Savidge & Blair, 2004), causing dampening of the ^{13}C enrichment at the C-4 position when measuring $\delta^{13}C$ of the whole malate molecule (Fig. 3D). Therefore, slight changes in $\delta^{13}C$ of malate may indicate higher changes at the C-4 position, which can be decarboxylated by the malic enzyme reaction or within the KC and thus be highly relevant for variations in $\delta^{13}C_R$. In brief, our findings strongly suggest that $\delta^{13}C_{RS}$ of malate has a strong influence on $\delta^{13}C_R$ during daytime, as well as nighttime, across different environmental conditions in this study and that their biochemical link is driven by an anapleurotic flux via PEPC, replenishing KC intermediates.

Conclusions

Here we showed for the first time results of $\delta^{13}C$ of leaf dark-respired CO_2 and $\delta^{13}C$ of putative respiratory carbon sources under the combined influence of controlled temperature and soil moisture conditions on a daily basis in a C_3 plant. Overall, we found that $\delta^{13}C_R$ values generally reflect changes in $\delta^{13}C_{RS}$ values in putative respiratory carbon sources due to the influence of different temperature and soil moisture treatments on leaf physiological parameters. It is worth noting that the temperature in this study exceeded the photosynthetic optimum of the potato plants under T_{high}, unexpectedly leading to more negative $\delta^{13}C$ values under T_{high} and dry conditions than those observed under T_{low} and dry conditions. This demonstrates that conclusions about the individual influence of an environmental driver on $\delta^{13}C$ values should be drawn carefully and that

verification of the isotopic results by gas exchange measurements is mandatory. Moreover, our findings indicate malate as main respiratory carbon source of leaf dark-respired CO_2 in potato plants. This could also be the case in plant species comparable with potato plants, but should not be generalized and transferred to respiratory processes in species of different functional groups such as trees or shrubs without verification. Finally, we recommend for subsequent studies on this topic the inclusion of isotopic measurements of malate or of the organic acid pool, given the strong indications observed herein for a biochemical link between $\delta^{13}C$ of malate and $\delta^{13}C$ of leaf dark-respired CO_2.

Acknowledgements

We thank Christiane Werner (University of Bayreuth) and Romain Barnard (INRA Dijon) for their experimental recommendations, Theodor Ballmer (Agroscope Reckenholz-Tänikon) for greenhouse organization, Dieter Juchelka (Thermo Fisher) for data evaluation advice, and Barbara Kornexl and Sebastian Zielis (both ETH Zurich) for setting up the starch concentration measurements. Technical support at PSI Villigen was given by Kathrin Streit, Lola Schmid, Yves Letz, and Sweety; and at ETH Zurich by Annika Ackermann, Thomas Baur, Fridolin Stocker, Fiona Cimei, and EA Burns. The research project CIFRes (205321_132768) was financed by SNF. RTWS acknowledges the support by the SNF via R'equip (206021_128761).

References

Atkin OK, Evans JR, Siebke K. 1998. Relationship between the inhibition of leaf respiration by light and enhancement of leaf dark respiration following light treatment. *Australian Journal of Plant Physiology* **25**: 437-443.

Barbour MM, Hunt JE, Kodama N, Laubach J, McSeveny TM, Rogers GN, Tcherkez G, Wingate L. 2011. Rapid changes in $\delta^{13}C$ of ecosystem-respired CO_2 after sunset are consistent with transient ^{13}C enrichment of leaf respired CO_2. *New Phytologist* **190**: 990-1002.

Barbour MM, McDowell NG, Tcherkez G, Bickford CP, Hanson DT. 2007. A new measurement technique reveals rapid post-illumination changes in the carbon isotope composition of leaf-respired CO_2. *Plant, Cell and Environment* **30**: 469-482.

Boschker HTS, Moerdijk-Poortvliet TCW, van Breugel P, Houtekamer M, Middelburg JJ. 2008. A versatile method for stable carbon isotope analysis of carbohydrates by high-performance liquid chromatography/isotope ratio mass spectrometry. *Rapid Communications in Mass Spectrometry* **22**: 3902-3908.

Bowling DR, Pataki DE, Randerson JT. 2008. Carbon isotopes in terrestrial ecosystem pools and CO_2 fluxes. *New Phytologist* **178**: 24-40.

Chinthapalli B, Murmu J, Raghavendra AS. 2003. Dramatic difference in the responses of phosphoenolpyruvate carboxylase to temperature in leaves of C_3 and C_4 plants. *Journal of Experimental Botany* **54**: 707-714.

Coplen TB. 2011. Guidelines and recommended terms for expression of stable-isotope-ratio and gas-ratio measurement results. *Rapid Communications in Mass Spectrometry* **25**: 2538-2560.

Craig H. 1957. Isotopic standards for carbon and oxygen and correction factors for mass-spectrometric analysis of carbon dioxide. *Geochimica Et Cosmochimica Acta* **12**: 133-149.

Critchley JH, Zeeman SC, Takaha T, Smith AM, Smith SM. 2001. A critical role for disproportionating enzyme in starch breakdown is revealed by a knock-out mutation in *Arabidopsis*. *Plant Journal* **26**: 89-100.

Dubbert M, Rascher KG, Werner C. 2012. Species-specific differences in temporal and spatial variation in $\delta^{13}C$ of plant carbon pools and dark-respired CO_2 under changing environmental conditions. *Photosynthesis Research* **113**: 297-309.

Duranceau M, Ghashghaie J, Badeck F, Deleens E, Cornic G. 1999. $\delta^{13}C$ of CO_2 respired in the dark in relation to $\delta^{13}C$ of leaf carbohydrates in *Phaseolus vulgaris* L. under progressive drought. *Plant, Cell and Environment* **22**: 515-523.

Farquhar GD, Ehleringer JR, Hubick KT. 1989. Carbon isotope discrimination and photosynthesis. *Annual Review of Plant Physiology and Plant Molecular Biology* **40**: 503-537.

Finkemeier I, Sweetlove LJ. 2009. The role of malate in plant homeostasis. *F1000 Biology Reports* **1**: Art. 47.

Gessler A, Tcherkez G, Karyanto O, Keitel C, Ferrio JP, Ghashghaie J, Kreuzwieser J, Farquhar GD. 2009. On the metabolic origin of the carbon isotope composition of CO_2 evolved from darkened light-acclimated leaves in *Ricinus communis*. *New Phytologist* **181**: 374-386.

Ghashghaie J, Badeck FW. 2014. Opposite carbon isotope discrimination during dark respiration in leaves versus roots - a review. *New Phytologist* **201**: 751-769.

Ghashghaie J, Badeck FW, Lanigan G, Nogues S, Tcherkez G, Deleens E, Cornic G, Griffiths H. 2003. Carbon isotope fractionation during dark respiration and photorespiration in C3 plants. *Phytochemistry Reviews* **2**: 145-161.

Ghashghaie J, Duranceau M, Badeck FW, Cornic G, Adeline MT, Deleens E. 2001. $\delta^{13}C$ of CO_2 respired in the dark in relation to $\delta^{13}C$ of leaf metabolites: comparison between *Nicotiana sylvestris* and *Helianthus annuus* under drought. *Plant, Cell and Environment* **24**: 505-515.

Gleixner G, Scrimgeour C, Schmidt H-L, Viola R. 1998. Stable isotope distribution in the major metabolites of source and sink organs of *Solanum tuberosum* L.: a powerful tool in the study of metabolic partitioning in intact plants. *Planta* **207**: 241-245.

Gleixner G, Schmidt H-L. 1997. Carbon isotope effects on the fructose-1,6-bisphosphate aldolase reaction, origin for non-statistical ^{13}C distributions in carbohydrates. *Journal of Biological Chemistry* **272**: 5382-5387.

Goettlicher S, Knohl A, Wanek W, Buchmann N, Richter A. 2006. Short-term changes in carbon isotope composition of soluble carbohydrates and starch: from canopy leaves to the root system. *Rapid Communications in Mass Spectrometry* **20**: 653-660.

Hettmann E, Gleixner G, Juchelka D. 2005. IRM-LC/MS: $\delta^{13}C$ analysis of organic acids in plants. *Application note 30075, Thermo Fisher Scientific*.

Hoch G, Popp M, Koerner C. 2002. Altitudinal increase of mobile carbon pools in *Pinus cembra* suggests sink limitation of growth at the Swiss treeline. *Oikos* **98**: 361-374.

Hopkins WG 2006. Photosynthesis and respiration. New York, NY, USA: Chelsea House, pp. 88-109.

Hymus GJ, Maseyk K, Valentini R, Yakir D. 2005. Large daily variation in ^{13}C-enrichment of leaf-respired CO_2 in two *Quercus* forest canopies. *New Phytologist* **167**: 377-384.

Krummen M, Hilkert AW, Juchelka D, Duhr A, Schluter HJ, Pesch R. 2004. A new concept for isotope ratio monitoring liquid chromatography/mass spectrometry. *Rapid Communications in Mass Spectrometry* **18**: 2260-2266.

Lemoine R, La Camera S, Atanassova R, Deedaldeechamp F, Allario T, Pourtau N, Bonnemain JL, Laloi M, Coutos-Theevenot P, Maurousset L, et al. 2013. Source-to-sink transport of sugar and regulation by environmental factors. *Frontiers in Plant Science* **4**: Art. 272.

Levy D, Veilleux RE. 2007. Adaptation of potato to high temperatures and salinity - A review. *American Journal of Potato Research* **84**: 487-506.

Melzer E, O'Leary MH. 1987. Anapleurotic CO_2 fixation by phosphoenolpyruvate carboxylase in C_3 plants. *Plant Physiology* **84**: 58-60.

Prater JL, Mortazavi B, Chanton JP. 2006. Diurnal variation of the $\delta^{13}C$ of pine needle respired CO_2 evolved in darkness. *Plant, Cell and Environment* **29**: 202-211.

Priault P, Wegener F, Werner C. 2009. Pronounced differences in diurnal variation of carbon isotope composition of leaf respired CO_2 among functional groups. *New Phytologist* **181**: 400-412.

R Core Team. 2013. R: A language and environment for statistical computing. *R foundation for statistical computing, Vienna, Austria*.

Richter A, Wanek W, Werner RA, Ghashghaie J, Jaeggi M, Gessler A, Brugnoli E, Hettmann E, Gottlicher SG, Salmon Y, et al. 2009. Preparation of starch and soluble sugars of plant material for the analysis of carbon isotope composition: a

comparison of methods. *Rapid Communications in Mass Spectrometry* **23**: 2476-2488.

Rinne KT, Saurer M, Streit K, Siegwolf RTW. 2012. Evaluation of a liquid chromatography method for compound-specific $\delta^{13}C$ analysis of plant carbohydrates in alkaline media. *Rapid Communications in Mass Spectrometry* **26**: 2173-2185.

Rossmann A, Butzenlechner M, Schmidt H-L. 1991. Evidence for a nonstatistical carbon isotope distribution in natural glucose. *Plant Physiology* **96**: 609-614.

Ruehr NK, Offermann CA, Gessler A, Winkler JB, Ferrio JP, Buchmann N, Barnard RL. 2009. Drought effects on allocation of recent carbon: from beech leaves to soil CO_2 efflux. *New Phytologist* **184**: 950-961.

Savidge WB, Blair NE. 2004. Patterns of intramolecular carbon isotopic heterogeneity within amino acids of autotrophs and heterotrophs. *Oecologia* **139**: 178-189.

Smith BN, Oliver J, Millan CM. 1976. Influence of carbon source, oxygen concentration, light intensity, and temperature on $^{13}C/^{12}C$ ratios in plant tissues. *Botanical Gazette* **137**: 99-104.

Streit K, Rinne KT, Hagedorn F, Dawes MA, Saurer M, Hoch G, Werner RA, Buchmann N, Siegwolf RTW. 2013. Tracing fresh assimilates through *Larix decidua* exposed to elevated CO_2 and soil warming at the alpine treeline using compound-specific stable isotope analysis. *New Phytologist* **197**: 838-849.

Sun W, Resco V, Williams DG. 2009. Diurnal and seasonal variation in the carbon isotope composition of leaf dark-respired CO_2 in velvet mesquite (*Prosopis velutina*). *Plant, Cell and Environment* **32**: 1390-1400.

Sweetlove LJ, Beard KFM, Nunes-Nesi A, Fernie AR, Ratcliffe RG. 2010. Not just a circle: flux modes in the plant TCA cycle. *Trends in Plant Science* **15**: 462-470.

Tcherkez G, Cornic G, Bligny R, Gout E, Ghashghaie J. 2005. In vivo respiratory metabolism of illuminated leaves. *Plant Physiology* **138**: 1596-1606.

Tcherkez G, Farquhar G, Badeck F, Ghashghaie J. 2004. Theoretical considerations about carbon isotope distribution in glucose of C_3 plants. *Functional Plant Biology* **31**: 857-877.

Tcherkez G, Nogues S, Bleton J, Cornic G, Badeck F, Ghashghaie J. 2003. Metabolic origin of carbon isotope composition of leaf dark-respired CO_2 in French bean. *Plant Physiology* **131**: 237-244.

Unger S, Maguas C, Pereira JS, Aires LM, David TS, Werner C. 2010. Disentangling drought-induced variation in ecosystem and soil respiration using stable carbon isotopes. *Oecologia* **163**: 1043-1057.

Urbanczyk-Wochniak E, Baxter C, Kolbe A, Kopka J, Sweetlove LJ, Fernie AR. 2005. Profiling of diurnal patterns of metabolite and transcript abundance in potato (*Solanum tuberosum*) leaves. *Planta* **221**: 891-903.

Usadel B, Blasing OE, Gibon Y, Retzlaff K, Hoehne M, Gunther M, Stitt M. 2008. Global transcript levels respond to small changes of the carbon status during progressive exhaustion of carbohydrates in *Arabidopsis* rosettes. *Plant Physiology* **146**: 1834-1861.

Voet D, Voet JG 2011. Biochemistry, 4th edition. New York, NY, USA: Wiley, pp. 789-822.

Vu JCV, Baker JT, Pennanen AH, Allen LH, Bowes G, Boote KJ. 1998. Elevated CO_2 and water deficit effects on photosynthesis, ribulose bisphosphate carboxylase-

oxygenase, and carbohydrate metabolism in rice. *Physiologia Plantarum* **103**: 327-339.

Wanek W, Heintel S, Richter A. 2001. Preparation of starch and other carbon fractions from higher plant leaves for stable carbon isotope analysis. *Rapid Communications in Mass Spectrometry* **15**: 1136-1140.

Wegener F, Beyschlag W, Werner C. 2010. The magnitude of diurnal variation in carbon isotopic composition of leaf dark respired CO_2 correlates with the difference between $\delta^{13}C$ of leaf and root material. *Functional Plant Biology* **37**: 849-858.

Werner C, Gessler A. 2011. Diel variations in the carbon isotope composition of respired CO_2 and associated carbon sources: a review of dynamics and mechanisms. *Biogeosciences* **8**: 2437-2459.

Werner C, Wegener F, Unger S, Nogues S, Priault P. 2009. Short-term dynamics of isotopic composition of leaf-respired CO_2 upon darkening: measurements and implications. *Rapid Communications in Mass Spectrometry* **23**: 2428-2438.

Werner C, Hasenbein N, Maia R, Beyschlag W, Maguas C. 2007. Evaluating high time-resolved changes in carbon isotope ratio of respired CO_2 by a rapid in-tube incubation technique. *Rapid Communications in Mass Spectrometry* **21**: 1352-1360.

Werner RA, Buchmann N, Siegwolf RTW, Kornexl BE, Gessler A. 2011. Metabolic fluxes, carbon isotope fractionation and respiration - lessons to be learned from plant biochemistry. *New Phytologist* **191**: 10-15.

Werner RA, Brand WA. 2001. Referencing strategies and techniques in stable isotope ratio analysis. *Rapid Communications in Mass Spectrometry* **15**: 501-519.

Werner RA, Bruch BA, Brand WA. 1999. ConFlo III - an interface for high precision $\delta^{13}C$ and $\delta^{15}N$ analysis with an extended dynamic range. *Rapid Communications in Mass Spectrometry* **13**: 1237-1241.

Zeeman MJ, Werner RA, Eugster W, Siegwolf RTW, Wehrle G, Mohn J, Buchmann N. 2008. Optimization of automated gas sample collection and isotope ratio mass spectrometric analysis of $\delta^{13}C$ of CO_2 in air. *Rapid Communications in Mass Spectrometry* **22**: 3883-3892.

Figures & Tables

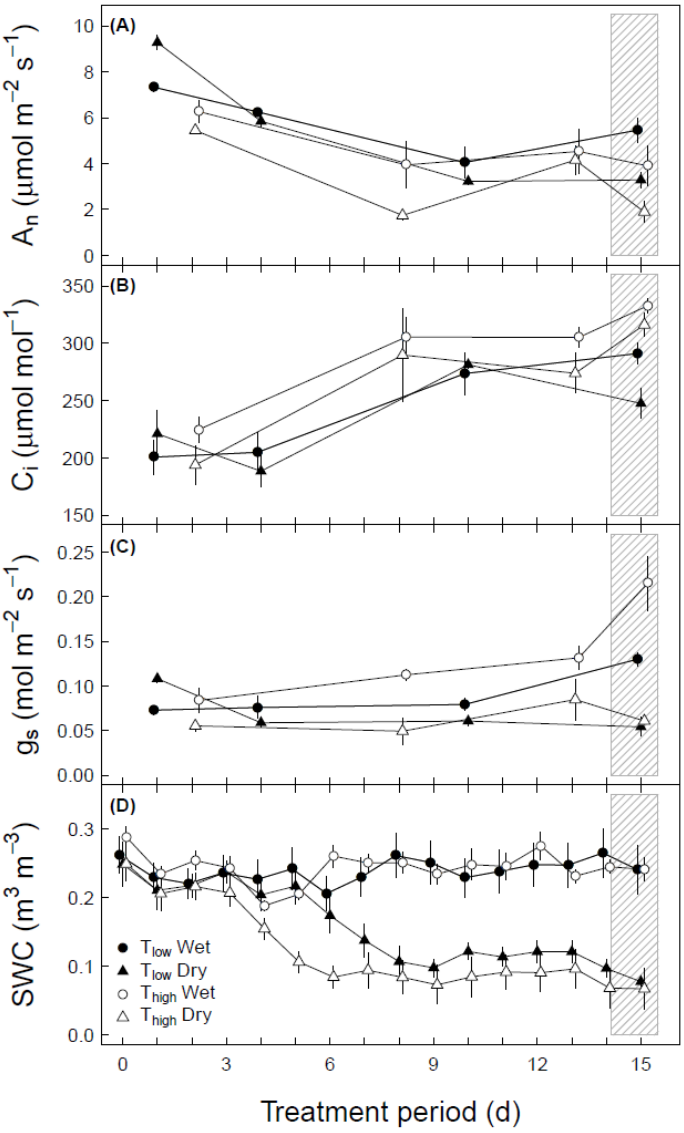

Figure 1: Physiological parameters under different environmental conditions during the treatment period: (A) net assimilation rate (A_n, µmol m^{-2} s^{-1}), (B) intercellular CO_2 concentration (C_i, µmol mol^{-1}), (C) stomatal conductance (g_s, mol m^{-2} s^{-1}), (D) volumetric soil water content (*SWC*, m^3/m^{-3}). Potato plants were treated with a combination of T_{low} (low temperature; closed symbols), T_{high} (high temperature; open symbols), and wet (circles) or dry (triangles) conditions. Boxed areas indicate the sampling period. Means ± SE are given (n = 3).

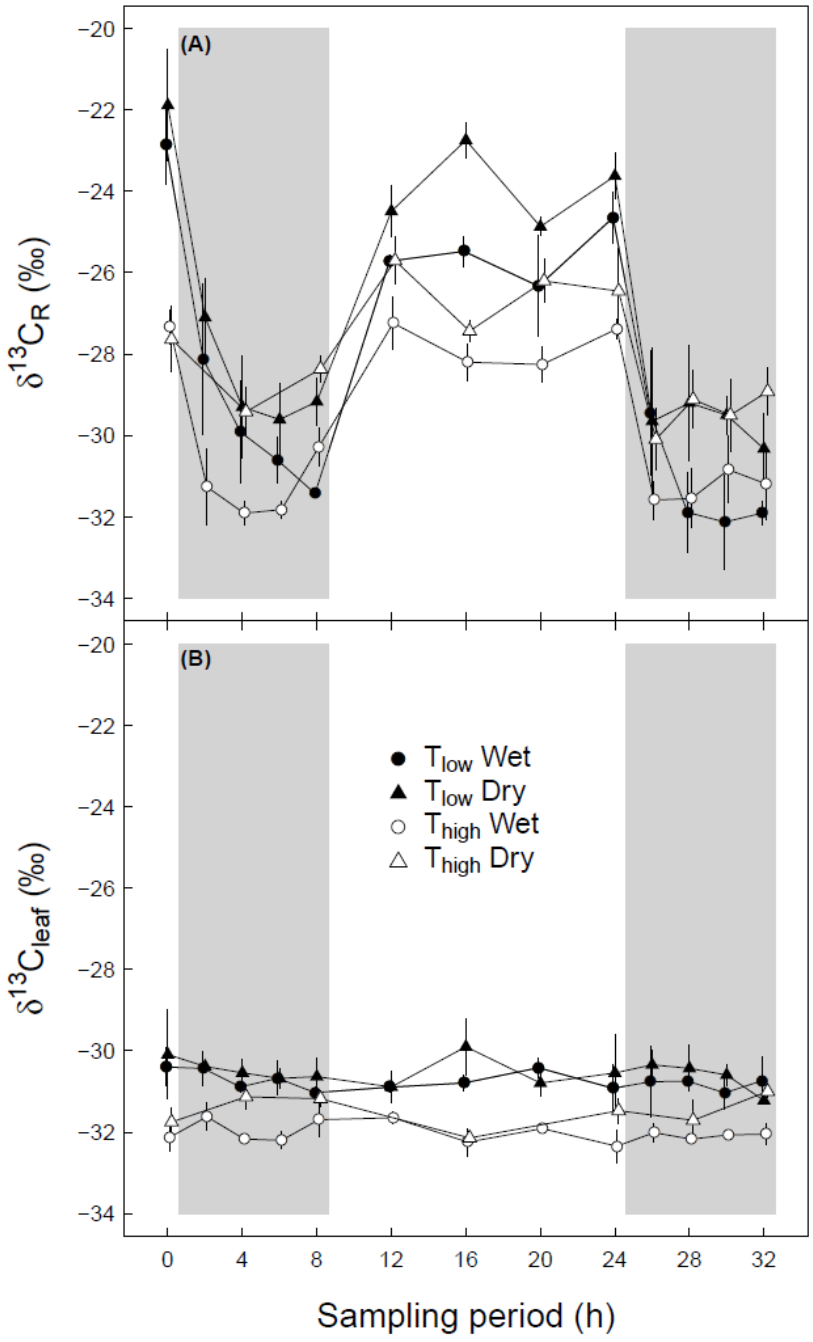

Figure 2: Daily cycles of the carbon isotopic composition of (A) leaf dark-respired CO_2 ($\delta^{13}C_R$) and (B) bulk leaves ($\delta^{13}C_{leaf}$) under different environmental conditions during the sampling period. Potato plants were treated with a combination of T_{low} (low temperature; closed symbols), T_{high} (high temperature; open symbols), and wet (circles) or dry (triangles) conditions. Grey areas indicate nighttime. Means ± SE are given (n = 3).

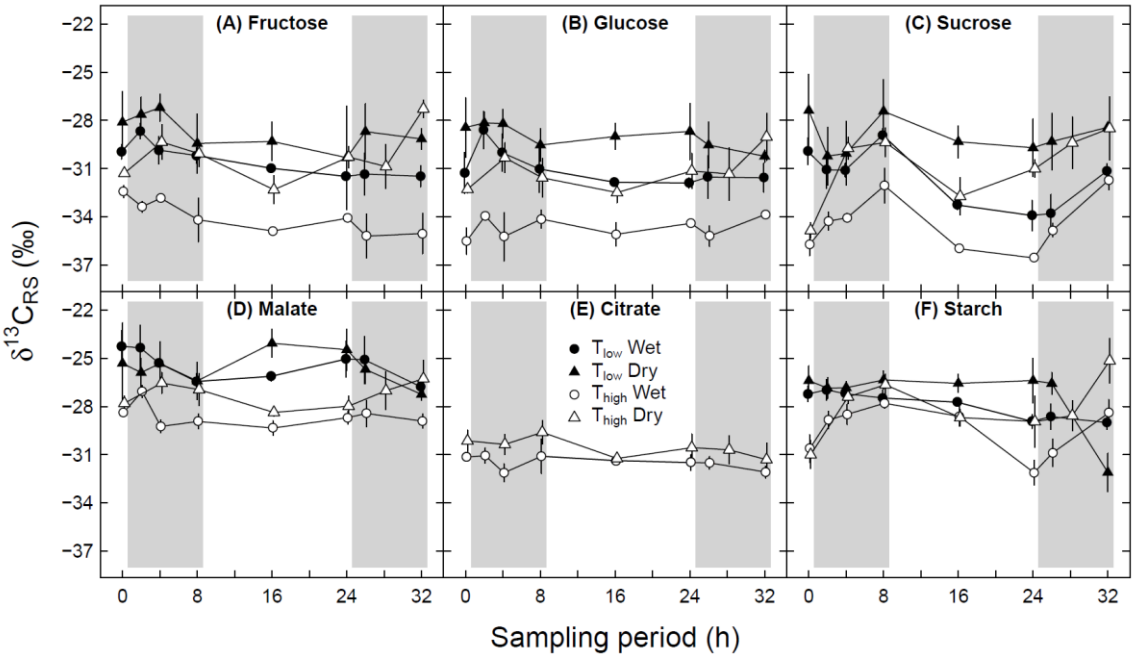

Figure 3: Daily cycles of the carbon isotopic composition of different leaf respiratory carbon sources ($\delta^{13}C_{RS}$) under different environmental conditions during the sampling period: (A) fructose, (B) glucose, (C) sucrose, (D) malate, (E) citrate, and (F) starch. Potato plants were treated with a combination of T_{low} (low temperature; closed symbols), T_{high} (high temperature; open symbols), and wet (circles) or dry (triangles) conditions. Results for fructose are affected by co-elution with other compounds. Grey areas indicate nighttime. Means ± SE are given (n = 2 to 3).

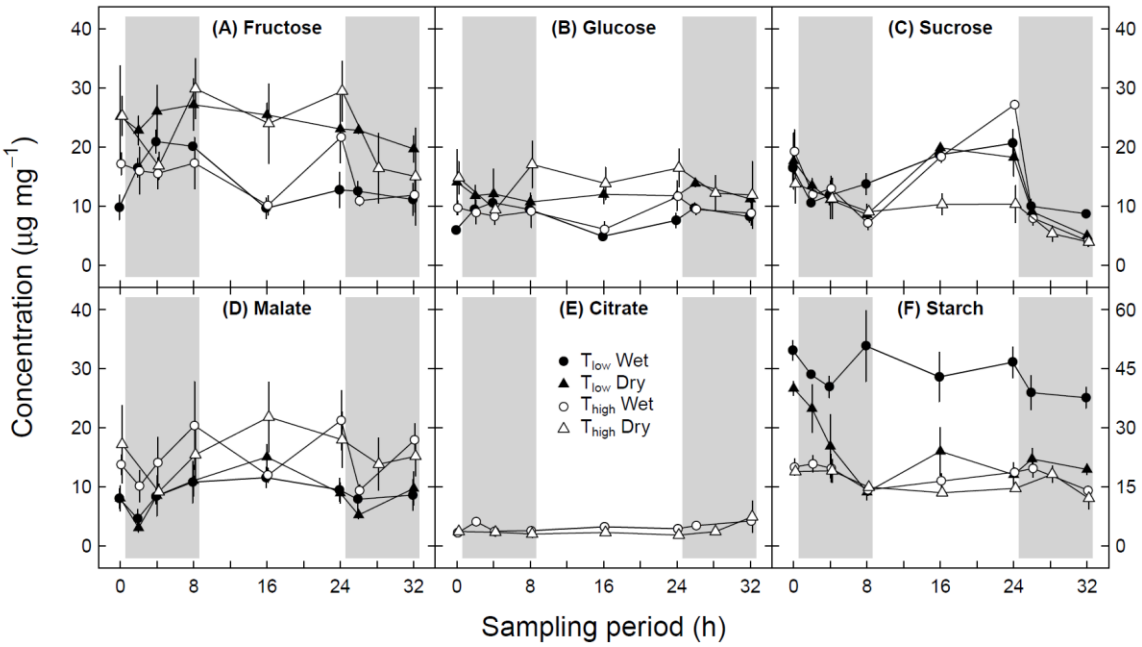

Figure 4: Daily cycles of the concentration of different leaf respiratory carbon sources under different environmental conditions during the sampling period: (A) fructose, (B) glucose, (C) sucrose, (D) malate, (E) citrate, and (F) starch. Potato plants were treated with a combination of T_{low} (low temperature; closed symbols), T_{high} (high temperature; open symbols), and wet (circles) or dry (triangles) conditions. Grey areas indicate nighttime. Results for fructose are affected by co-elution with other compounds. Please note different y-axis scale in (F) starch. Means ± SE are given (n = 2 to 3).

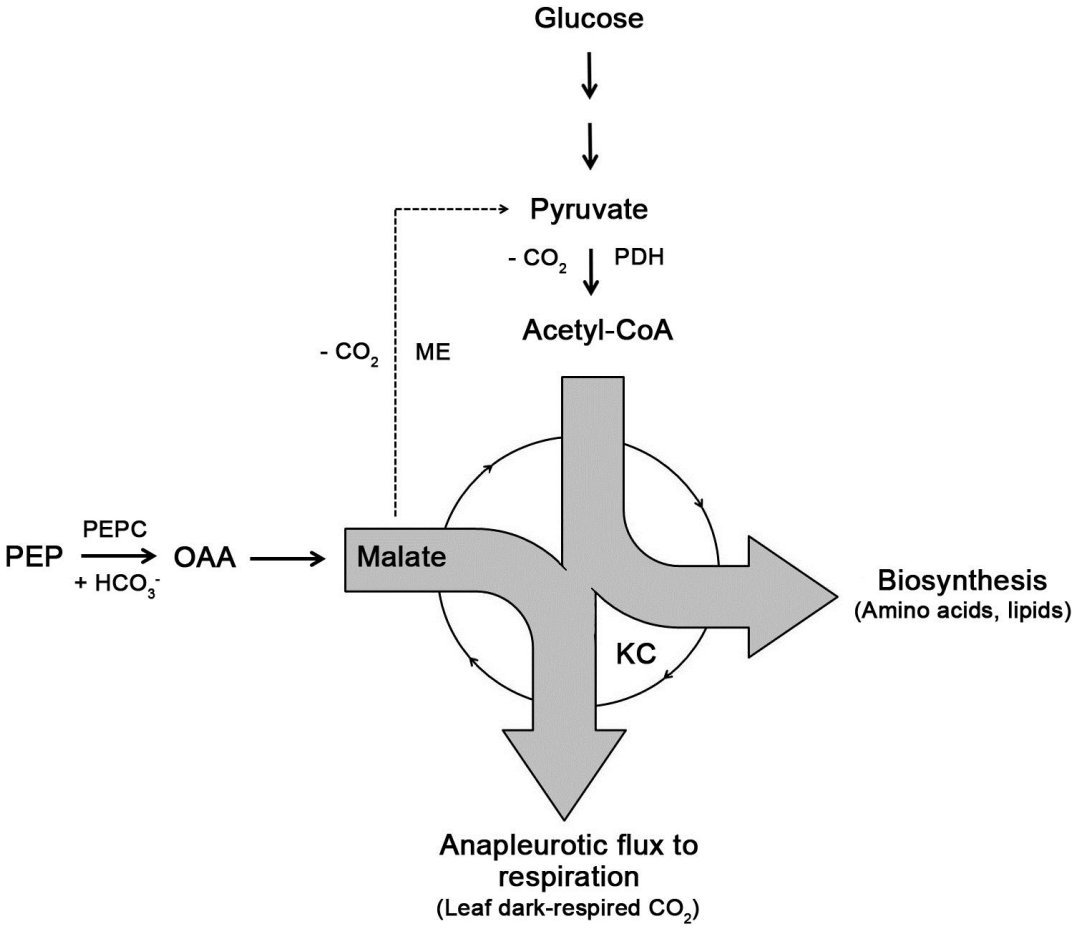

Figure 5

Figure 5: Simplified schematic of respiratory processes in potato leaves. Breakdown of glucose during glycolysis produces pyruvate, which is converted into CO_2 and acetyl-CoA by the pyruvate dehydrogenase reaction (PDH). Acetyl-CoA is used for biosynthesis rather than for respiration, causing a drain of Krebs cycle (KC) intermediates. An anapleurotic flux via phosphoenolpyruvate carboxylase (PEPC) replenishes KC intermediates to maintain the functionality of the KC. Thereby, malate enters the KC and is used as carbon source for leaf dark-respired CO_2. Dashed line indicates an alternative CO_2 producing reaction via malic enzyme (ME). PEP = Phosphoenolpyruvate, OAA = Oxaloacetate.

Table 1: Results of linear mixed effects models testing the effects of temperature (low, high) and soil moisture (wet, dry) on physiological parameters (A_n, net assimilation rate; C_i, intercellular CO_2 concentration; g_s, stomatal conductance; *SWC*, volumetric soil water content), total plant biomass, tuber weight, and tuber count during the sampling period. p-values are given for treatments and their interaction. Significant differences are given in bold ($p \leq 0.05$).

Parameter	A_n	C_i	g_s	*SWC*	Plant biomass	Tuber weight	Tuber count
Temperature	0.070	**0.012**	0.127	0.863	0.978	0.359	0.400
Soil moisture	**0.020**	**0.010**	**0.001**	**0.002**	**0.008**	**0.023**	0.233
Temp : Moisture	0.522	0.110	0.174	0.845	0.565	0.892	0.486

Table 2: Total plant biomass (dry weight), tuber weight (fresh weight), and tuber count (number of potatoes) after the sampling period. Potato plants were treated with a combination of T_{low} (low temperature), T_{high} (high temperature), and wet or dry conditions. Means ± SE are given (n = 3). Please refer to Table 1 for statistical analysis.

	Treatments			
Parameter	T_{low} Wet	T_{low} Dry	T_{high} Wet	T_{high} Dry
Total biomass (g)	10.6 ± 1.4	7.8 ± 1.2	10.3 ± 1	8.1 ± 0.5
Tuber weight (g)	513.9 ± 18.4	458.3 ± 15	481.4 ± 15.9	430 ± 24.2
Tuber count (No.)	21.3 ± 2.7	20.2 ± 1.7	19.3 ± 1.2	19 ± 2.3

Table 3: Results of linear mixed effects models testing the effects of temperature (low, high) and soil moisture (wet, dry) on $\delta^{13}C$ values in different putative leaf respiratory carbon sources, bulk leaves ($\delta^{13}C_{leaf}$), and in leaf dark-respired CO_2 ($\delta^{13}C_R$), as well as on concentrations of different carbon sources during the sampling period. Results for fructose are affected by co-elution with other compounds. p-values are given for treatments, time, and their interactions. Significant differences are given in bold ($p \leq 0.05$).

	$\delta^{13}C$							
Parameter	Fructose	Glucose	Sucrose	Malate	Citrate	Starch	$\delta^{13}C_{Leaf}$	$\delta^{13}C_R$
Temperature	**0.019**	**0.004**	**0.028**	**0.015**	n.a.	0.107	**0.022**	**0.044**
Soil moisture	**0.001**	**0.001**	**0.001**	**0.049**	**0.009**	**0.046**	**0.005**	**0.013**
Time	**0.001**	0.195	0.081	0.198	0.052	**0.001**	0.066	**0.001**
Temp : Moisture	**0.035**	0.063	0.543	**0.017**	n.a.	0.270	0.165	0.875
Temp : Time	0.256	**0.008**	**0.003**	0.807	n.a.	0.113	0.812	**0.014**
Moisture : Time	0.061	0.291	**0.002**	0.060	0.411	**0.032**	0.596	0.883

	Concentration					
Parameter	Fructose	Glucose	Sucrose	Malate	Citrate	Starch
Temperature	0.663	0.352	0.142	**0.011**	n.a.	**0.002**
Soil moisture	**0.001**	**0.001**	**0.031**	0.999	0.052	**0.001**
Time	**0.016**	0.927	**0.001**	**0.035**	0.110	**0.001**
Temp : Moisture	0.475	0.705	0.462	0.796	n.a.	**0.001**
Temp : Time	0.901	0.847	0.113	0.387	n.a.	0.324
Moisture : Time	0.831	0.629	0.063	0.889	0.895	0.071

n.a. = not available

Table 4: Linear regression analyses relating $\delta^{13}C$ of leaf dark-respired CO_2 to $\delta^{13}C$ of putative respiratory carbon sources and to $\delta^{13}C$ of bulk leaves ($\delta^{13}C_{leaf}$) across all environmental conditions for daytime (0 h, 16 h, 24 h), for nighttime (2 h, 4 h, 8 h, 26 h, 28 h, 32 h), and for the total daily cycle (sampling period over 32 h). Results for fructose are affected by co-elution with other compounds. Generic regression equation y = mx + b was used. r^2 values are given, stars indicate p-values. All correlation coefficients were positive.

	r^2		
Putative carbon sources	Daytime	Nighttime	Daily
Fructose	0.35***	0.34***	0.12***
Glucose	0.54***	0.34***	0.13***
Sucrose	0.59***	0.20***	0.04*
Malate	0.69***	0.36***	0.26***
Citrate	0.67***	0.28**	0.17**
Starch	0.48***	0.16**	0.06*
$\delta^{13}C_{Leaf}$	0.63***	0.33***	0.20***

* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$

Table 5: Result of stepwise (backward) multiple linear regression analysis showing the best-fit combination of independent environmental drivers (temperature, soil moisture, daytime/nighttime), time, and $\delta^{13}C$ of glucose, sucrose, malate and starch as variables influencing $\delta^{13}C$ of leaf dark-respired CO_2 ($\delta^{13}C_R$) during the sampling period in potato leaves. Standardized β-coefficients and p-values are given.

Drivers and carbon sources influencing $\delta^{13}C_R$	Standardized β-coefficient	p-value
Daytime/Nighttime	0.73	<0.001
Malate	0.40	<0.001
Starch	0.11	0.019
Soil moisture	0.14	0.013

Table 6: Leaf physiological parameters and $\delta^{13}C$ values during the sampling period in potato plants under different treatments compared to those in potato plants growing under T_{low} and wet conditions. The following variables were considered: A_n, net assimilation rate; g_s, stomatal conductance; C_i, intercellular CO_2 concentration; $\delta^{13}C_R$, $\delta^{13}C$ of leaf dark-respired CO_2; $\delta^{13}C_{RS}$, $\delta^{13}C$ of different putative respiratory carbon sources (fructose, glucose, sucrose, starch, and malate). Arrows indicate strong (↑, ↓), intermediate (↗, ↘), or no changes (→) due to the influence of treatment combinations (T_{low}, low temperature; T_{high}, high temperature; and wet or dry conditions).

Treatments	A_n	g_s	C_i	$\delta^{13}C_R$	$\delta^{13}C_{RS}$
T_{low} Dry	↘	↓	↓	↑	↑
T_{high} Wet	↘	↑	↑	↓	↓
T_{high} Dry	↓	↓	↗	→	→

Chapter 3

3. Strong differences in ^{13}C of respiratory CO_2 and malate indicate species-specific metabolic isotope fractionation

Marco M. Lehmann[1], Frederik Wegener[2], Roland A. Werner[1], Christiane Werner[2]

[1]Institute of Agricultural Sciences, ETH Zurich, Universitätsstr. 2, CH-8092 Zurich, Switzerland;

[2]Agro-Ecosystem Research, BAYCEER, University of Bayreuth, Universitätsstr. 30, D-95447 Bayreuth, Germany

Manuscript was submitted to *Plant Biology*.

Abstract

Plant respiration and its isotopic composition contain potential information about plant internal processes and prevalent climatic conditions, although we still lack detailed knowledge on underlying metabolic processes. An increase in isotopic composition of leaf dark-respired CO_2 ($\delta^{13}C_R$) shortly after illumination has previously been related to light enhanced dark respiration (*LEDR*), and ^{13}C-enriched malate was suggested as main respiratory substrate of this phenomenon. We determined $\delta^{13}C_R$ and *LEDR*, as well as $\delta^{13}C$ and concentrations of potential respiratory substrates during the diurnal cycle in several plant species, exposing them to different environmental conditions. An interspecific comparison between $\delta^{13}C_R$ and *LEDR* revealed no straight-forward relationship between both parameters (i.e. high *LEDR* was not always related to high ^{13}C enrichment of CO_2), showing that the extent of changes in $\delta^{13}C_R$ and *LEDR* are highly species-specific. Interestingly, extraordinarily high $\delta^{13}C$ values of malate (-10.9‰) in the oxalate accumulating C_3 plant *Oxalis triangularis* were observed, demonstrating so far unknown isotope fractionations. However, neither diurnal changes in $\delta^{13}C$ of malate nor in malate concentrations could fully explain diurnal changes in $\delta^{13}C_R$. We conclude that malate is not the main carbon source of high ^{13}C enriched respiratory CO_2 and *LEDR* in all species, indicating that metabolic origins and associated isotope fractionation are highly species-specific.

Keywords: compound-specific isotope analysis (CSIA), temperature, interrupted light, organic acids, respiration

Introduction

Recent biogeochemical studies investigate the biosphere carbon exchange at different scales from C assimilation during photosynthesis to C release during respiration with stable carbon isotope approaches (e.g. Brueggemann *et al.*, 2011; Werner *et al.*, 2012). However, substrates and environmental drivers causing changes in respiration and in associated carbon isotope fractionation are still not fully understood. This lack of knowledge restrains the information gained from carbon isotopic composition of leaf dark-respired CO_2 (i.e., $\delta^{13}C_R$), which could be a useful indicator of plant physiological parameters, prevalent environmental conditions, and current respiratory substrates.

Already in 1999, Duranceau *et al*. reported a ^{13}C enrichment in leaf dark-respired CO_2 up to 6‰ relative to the potential respiratory substrate (sucrose) in different plant species. Indeed, $\delta^{13}C_R$ was shown to be generally less negative compared to potential respiratory substrates in different species, environmental conditions, and ecotypes (Werner & Gessler, 2011; Ghashghaie & Badeck, 2014). Leaf dark-respired CO_2 progressively increases in ^{13}C during daytime and rapidly decreases in ^{13}C during nighttime (Hymus *et al.*, 2005; Prater *et al.*, 2006), showing species-specific variations up to 14.8‰ during the diurnal cycle (Priault *et al.*, 2009; Rascher *et al.*, 2010; Wegener *et al.*, 2010). Environmental factors are known to influence $\delta^{13}C_R$: for instance less negative $\delta^{13}C_R$ values were found under dry conditions in comparison to wet conditions (Duranceau *et al.*, 1999; Ghashghaie *et al.*, 2001), whereas more negative $\delta^{13}C_R$ values were shown as short-term response to high temperatures compared to low temperatures under prolonged continuous darkening (Tcherkez *et al.*, 2003). $\delta^{13}C_R$ values were further correlated with cumulative CO_2 uptake and shown to increase with light intensity

(Hymus *et al.*, 2005; Priault *et al.*, 2009; Werner *et al.*, 2009). Moreover, diurnal changes in $\delta^{13}C_R$ may depend on changes in $\delta^{13}C$ of different respiratory substrates, isotope fractionation of CO_2 releasing reactions, fragmentation fractionation (Tcherkez *et al.*, 2004), branching points in biochemical pathways, and the turnover rate of a reaction (for a review, see Werner & Gessler, 2011).

Furthermore, a distinct period shortly upon darkening called light enhanced dark respiration (*LEDR*) has been discussed to be at the origin of the release of ^{13}C enriched leaf dark-respired CO_2. LEDR is defined as enhanced CO_2 release after prolonged periods of illumination during light-dark transitions, with a duration of approximately 20 min into the dark and highest respiration rates within the first 5 min (Atkin *et al.*, 1998). The amount of CO_2 released during *LEDR* increases with light intensity and length of the preceding light period (Atkin *et al.*, 2000). Controlled experiments have shown that the course of leaf dark-respired CO_2 during *LEDR* follows a characteristic pattern from highly ^{13}C enriched to more ^{13}C depleted CO_2 after darkening (Barbour *et al.*, 2007; Gessler *et al.*, 2009; Werner *et al.*, 2009), pointing towards a tight relationship between high variations in $\delta^{13}C_R$ and *LEDR*. In addition to laboratory studies, Barbour *et al.* (2011) provided evidence for the natural occurrence of LEDR in a grassland ecosystem after sunset. However, biochemical mechanisms and associated isotope fractionations causing changes in $\delta^{13}C_R$ during *LEDR* are still not fully understood (Werner *et al.*, 2011).

Changes in $\delta^{13}C_R$ and *LEDR* are probably associated with the activity of the Krebs cycle (KC), which is suggested to function as a uncompleted cycle during the light period (Hanning & Heldt, 1993; Tcherkez *et al.*, 2005; Bowsher *et al.*, 2008; Sweetlove *et al.*, 2010). Under such metabolic conditions the intermediates in the KC are fueled by so

called anapleurotic fluxes, which rapidly replenish withdrawn molecules (used, e.g., for biosynthesis of amino acids or secondary metabolites). The non-photosynthetic phosphoenolpyruvate carboxylase (PEPC) reaction fuels the KC with ^{13}C enriched intermediates, particularly under anabolic conditions in C_3 plants (Werner *et al.*, 2011). PEPC itself fractionates against ^{13}C in bicarbonate by about 2.2‰, but due to overlapping isotope effects of equilibrium hydration of CO_2 (about -9‰) and equilibrium CO_2 dissolution into water (1.1‰), the PEPC reaction fractionates against ^{13}C in CO_2 by -5.7‰ in total (Farquhar *et al.*, 1989), causing relatively ^{13}C enriched substrates in metabolic pathways. For instance, leaf PEPC in C_3 plants produces ^{13}C enriched oxaloacetate in the light. An associated malate dehydrogenase reaction converts oxaloacetate mainly into malate, which may accumulate under noncyclic KC conditions during illumination (Barbour *et al.*, 2007; Gessler *et al.*, 2009; Werner *et al.*, 2011). Moreover, the anapleurotic flux via PEPC is supposed to cause a substantial ^{13}C enrichment in the C-4 position of malate (Melzer & O'Leary, 1987; Melzer & O'Leary, 1991; Savidge & Blair, 2004), and therefore an increase in mean $\delta^{13}C$ of the whole malate molecule. Subsequently, decarboxylation of the C-4 position of malate in the dark either by the NAD-depending malic enzyme or at least partially within the “closed” KC has been hypothesized to lead to an increase in *LEDR* (Hill & Bryce, 1992; Igamberdiev *et al.*, 2001) and $\delta^{13}C_R$ (Barbour *et al.*, 2007; Gessler *et al.*, 2009; Werner & Gessler, 2011; Werner *et al.*, 2011). However, up to now, we still lack experimental evidence on $\delta^{13}C$ of malate ($\delta^{13}C_{malate}$) and malate concentrations in different species along the diurnal cycle, which are required to identify the causes of the observed changes in $\delta^{13}C_R$ and *LEDR.*

Thus, we hypothesized that the ^{13}C enrichment in leaf dark-respired CO_2 increases with amounts of CO_2 released during *LEDR* and that malate is the main substrate of this phenomenon. Therefore, we experimentally changed environmental conditions, which are known to impact $\delta^{13}C_R$ and *LEDR*: first, we applied different light treatments, since the extent of the *LEDR* reaction has been reported to depend on incident illumination; second, we measured $\delta^{13}C_R$ and *LEDR* in different species under different temperature treatments and third, we analyzed $\delta^{13}C$ and concentrations of different potential respiratory substrates, in particular malate, along the diurnal cycle. The experiments were performed with different plant functional types, comprising species with known low or high temporal variations in $\delta^{13}C_R$, to identify potential species-specific differences in metabolic pathways and associated isotope fractionation.

Material & Methods

Plant material and growth conditions

Plant species from different functional groups (Priault *et al.*, 2009) were used for the experiments: a small aromatic shrub (*Salvia officinalis* L.), a non-aromatic herb (*Oxalis triangularis* A.St.-Hil.), and two Mediterranean woody species (*Halimium halimifolium* L. and *Arbutus unedo* L.). All plants were grown under controlled climatic conditions in growth chambers. Artificial light was provided during a 12 h light period. Air temperature was 23°C and 15°C during the light and dark periods, respectively. Relative air humidity was 60% and photosynthetic photon flux density at substrate surface height was 400 µmol m^{-2} s^{-1}. $\delta^{13}C$ of ambient air CO_2 in the climate chamber was -11.7 ± 0.2‰, measured with an IRMS as described below for $\delta^{13}C_R$.

Experimental design

Interrupted light experiment

H. halimifolium plants were held under constant temperature of 20°C for one month before starting the experiment. $\delta^{13}C_R$ and amounts of leaf dark respired CO_2 were measured in two hour intervals during the light period and consecutively during the following dark period (4, 10, 42, 75, 120, 240, 480 and 660 min after darkening). In contrast to control plants, which were illuminated continuously for 12 hours, treated plants where fully shaded every two hours for 10 min immediately after the leaf sampling for $\delta^{13}C_R$ measurements in the light. To assure the same light duration in both treatments, the light period of the treated plants was increased (30 min in the morning and 20 min in the evening).

Temperature experiment with different species

H. halimifolium, *O. triangularis*, and *S. officinalis* plants were treated with (i) high temperature (constantly 30°C) and (ii) low temperature (23°C day, 15°C night) for three weeks. Thereafter, plants were darkened at three points in time for two hours during the light period (10:00, 15:00 and 19:00). $\delta^{13}C_R$ and *LEDR* were measured at six points in time during each dark period (4, 10, 28, 50, 100 and 120 min).

Diurnal courses of $\delta^{13}C_R$ and $\delta^{13}C$ of potential respiratory substrates

A. unedo, *H. halimifolium* and *O. triangularis* plants were held under growth conditions outlined above. $\delta^{13}C_R$ was measured at five points in time during a diurnal cycle (08:00, 10:00, 14:30, 19:30, 22:00). Leaf material from the same plants was harvested at the

same points in time, immediately frozen in liquid N_2, and stored at -80°C until further analysis.

Determination of $\delta^{13}C_R$ and R

$\delta^{13}C$ values are expressed as

$\delta^{13}C$ (‰) = $R_{sample}/R_{standard}$ -1

, where R_{sample} is the $^{13}C/^{12}C$ ratio of the sample and $R_{standard}$ that of the international standard (Vienna PeeDee Belemnite, VPDB).

$\delta^{13}C_R$ values were measured with an in-tube incubation technique as described in Werner *et al.* (2007). In brief, leaves were placed in septum-capped glass vials (Exetainer, 12 ml, LABCO, High Wycombe, UK), darkened with a light-impermeable casing, and immediately flushed with CO_2 free air. For a reasonable signal on the IRMS ($[CO_2]$ > 350 ppm), the incubation time in the dark (4 or 5 min) was adjusted for each experiment and kept constant thereafter. Respired $\delta^{13}C_R$ in the vial was measured using an IRMS (Isoprime; Elementar, Germany) interfaced to an autosampler (Microgas, GV, Manchester, UK), with an overall precision of SD < 0.2‰. CO_2 respiration (*R*) given in ppm cm^{-2} was calculated from the sample CO_2 peak area and referred to leaf area.

Extraction of respiratory substrates

Sampled leaf material was lyophilized and homogenized with a ball mill. Extraction and purification of metabolic fractions was performed according to previous studies (Richter *et al.*, 2009; Streit *et al.*, 2013). In brief: 43-100 mg of the leaf material (depending on the harvest biomass) were transferred into a reaction vial and re-suspended in 1.5 ml

deionized H_2O. The total water soluble organic content was extracted for 30 min at 85°C and the solution was centrifuged for 2 min at 10000 g. The supernatant was separated into three metabolic fractions (labelled as sugars, organic acids, amino acids) by ion exchange chromatography (Dowex 50WX8 in H^+-form and Dowex 1X8 in $NaCOO^-$-form; both 100-200 mesh, Sigma-Aldrich, Buchs, Switzerland). All eluted fractions were subsequently frozen in a -20°C freezer, lyophilized, and the pellet re-suspended in deionized H_2O. Additionally, the organic acid fractions were purified with 0.45 µm PTFE syringe filters (Infochroma AG, Zug, Switzerland). All described methods were tested with standards of analytical grade to be free of carbon isotope fractionation.

Isotopic analysis of respiratory substrates

Aliquots of sugar ($\delta^{13}C_{sugar}$) and amino acid fractions ($\delta^{13}C_{amino}$) were pipetted into tin capsules and oven-dried at 60°C and, as well as $\delta^{13}C$ of bulk leaves ($\delta^{13}C_{leaf}$), measured with EA-IRMS (Delta^{Plus}XP with Flash EA, Thermo Fisher Scientific, Bremen, Germany) as described in Werner *et al.* (1999), with a precision SD ≤ 0.12‰ for quality control long-term standards. Referencing followed the protocol of Werner & Brand (2001).

$\delta^{13}C$ and concentrations of organic acids were measured with a high performance liquid chromatography system (HPLC), which was connected via a Finnigan LC-Isolink to a Delta V Advantage IRMS (Thermo Fisher Scientific, Bremen, Germany) as described in Krummen *et al.* (2004). Organic acids were separated on a 4.6 x 300 mm Allure organic acid HPLC column (Restek, Bellefonte, USA, Hettmann *et al.*, 2005) at 8°C, using a 100 mM KH_2PO_4 buffer (pH = 3) as mobile phase with a flow of 500 µl min^{-1}. The 100% conversion of the analyte to CO_2 in the 99°C oxidation reactor was performed with an oxidation solution ($Na_2S_2O_8$) at low pH (H_3PO_4). Laboratory standards of malate and

oxalate in a range of 10-180 ng C μl^{-1} were analyzed every 10 samples. $\delta^{13}C$ values of these standards were used to correct for the compound specific offset to $\delta^{13}C$ values measured with the EA coupling, as described in Rinne *et al.* (2012). Peak areas of these standards were used to determine the sample organic acid concentration. The overall measurement precision of $\delta^{13}C$ analysis of all standards was SD < 0.4‰.

Statistical Analysis

p-values derived from linear mixed effects models are given, including treatments (light, temperature, and species) and time as fixed effects, while the individual plant number was used as a random effect to respect for non-independence of repeated measurements. If applicable, data was logarithmically transformed to ensure normal distribution and homoscedasticity. All statistical analyses were performed in R 3.0.2 (R Core Team, 2013).

Results

Interrupted light treatment under equal light duration

To investigate the effect of interrupted light on $\delta^{13}C_R$ and LEDR*, H. halimifolium* plants were shaded for 10 min every two hours and compared to an unshaded control group exposed to the same total duration of illumination (Fig. 1). During daytime, we observed a general progressive daytime increase in $\delta^{13}C_R$ and dark respiration (*R*), with highest values at the end of the light period, except for *R* values under interrupted light. Shortly upon darkening at the end of the light period, a transient increase in $\delta^{13}C_R$ and *R* under both treatments was followed by an exponential decline, approaching lowest values at the end of the dark period, except for *R* values under interrupted light. $\delta^{13}C_R$ was up to

2.8‰ more negative under interrupted light compared to continuous light conditions during the diurnal cycle ($p \leq 0.05$). Also *R* values were 30% lower under interrupted light, but only during daytime and the beginning of the dark period ($p \leq 0.001$). In summary, despite the same total light duration the interrupted light reduced the ^{13}C enrichment of leaf dark-respired CO_2 during the light period, as well as the amounts of leaf dark-respired CO_2.

Species-specific variations in $\delta^{13}C_R$ and LEDR under different temperatures

To characterize the relationship of increased $\delta^{13}C_R$ values and *LEDR* after illumination we measured both over the diurnal cycle in three species of different functional groups under two temperature conditions (Fig. 2). Interestingly, plants with marked *LEDR* (high *R* variations) showed the lowest amplitude in $\delta^{13}C_R$ and *vice versa*, however, the interspecific relationship between $\delta^{13}C_R$ and *R* was low ($r = 0.35$, $p \leq 0.001$, Pearson correlation)**.** Among all species, *H. halimifolium* plants reached highest diurnal variations under both temperature treatments in $\delta^{13}C_R$ (up to 15.9‰), but showed the lowest variations in *R*. *O. triangularis* and *S. officinalis* plants showed lower diurnal variations in $\delta^{13}C_R$ (6‰ and 11.1‰, respectively), but exhibited up to 3-fold higher variations in *R* than *H. halimifolium* plants. The characteristic patterns of $\delta^{13}C_R$ and *LEDR* were thereby consistent under both temperature treatments in all species, with highest values immediately upon darkening followed by an exponential decline, with the exception of *O. triangularis* where the $\delta^{13}C_R$ peak was delayed

Diurnal changes in $\delta^{13}C_R$ and in $\delta^{13}C$ and concentrations of respiratory substrates

Furthermore, we measured potential respiratory substrates (sugar, amino acids, and organic acids) along the diurnal cycle in different species. Similar to the first experiment, daytime $\delta^{13}C_R$ values were less negative than nighttime $\delta^{13}C_R$ values, with highest diurnal variations in *H. halimifolium* (up to 8‰) and lowest in *O. triangularis* (up to 6.6‰; $p \leq 0.001$; Fig. 3). $\delta^{13}C_{malate}$ varied significantly during the diurnal cycle by up to 2.9‰ ($p \leq 0.002$), especially in *O. triangularis* with lowest $\delta^{13}C$ values of malate at 10:00 h and highest at 19:30 h. In other species, $\delta^{13}C_{malate}$ tended to increase during daytime, but showed higher variability between replicates. Moreover, clear species-specific differences were observed for all measured parameters ($p \leq 0.001$), especially for $\delta^{13}C_{malate}$ with differences of up to 11.1‰. C_4 like isotopic signatures of malate ($\delta^{13}C_{malate}$ of -10.9‰ to -13.3‰) were found in the C_3-plant *O. triangularis*, whereas $\delta^{13}C_{malate}$ was lower in *H. halimifolium* and *A. unedo,* ranging from -18.4‰ to -22‰ (Fig. 3). $\delta^{13}C_{malate}$ was less negative than $\delta^{13}C_R$ during nighttime (8:00 and 22:00; up to 4‰) and more negative during daytime (10:00 - 19:30; up to 3.7‰) in *H. halimifolium* and *A. unedo*, while it was always less negative in *O. triangularis* (up to 13.5‰). All other measured compounds ($\delta^{13}C_{leaf}$, $\delta^{13}C_{sugar,}$ and $\delta^{13}C_{amino}$) were always more negative compared to $\delta^{13}C_R$ and $\delta^{13}C_{malate}$ and showed no temporal variations. Moreover, malate concentrations were highest in *A. unedo* (5 µg mg^{-1}), lowest in *H. halimifolium* (0.6 µg mg^{-1}; Fig. 4), and showed no significant temporal changes. Additionally, very high oxalate concentrations up to 74 µg mg^{-1} with $\delta^{13}C$ of about -22‰ were found in *O. triangularis* (Figs. 3, 4).

Discussion

Light-dependent carbon pool drives changes in $\delta^{13}C_R$ and LEDR

Leaf dark-respired CO_2 was observed to be ^{13}C enriched during the *LEDR* period (Werner *et al.*, 2009; Wegener *et al.*, 2010), suggesting a similar metabolic origin for isotopic compostion and amount of respired CO_2. It has been suggested that *LEDR* is probably fed by a ^{13}C enriched metabolite pool with high turn-over rates, e.g. a malate pool (Barbour *et al.*, 2007; Gessler *et al.*, 2009; Barbour *et al.*, 2011). In fact, a decrease in $\delta^{13}C_R$ and *R* under interrupted light compared to continuous light conditions was observed during daytime and during light-dark transition (Fig. 1). Thus, the consecutive light interruptions may have caused a consecutive break-down of the potential ^{13}C enriched pool, indicating that both, $\delta^{13}C_R$ and *LEDR,* might be driven by the buildup of a light-dependent internal carbon pool. These findings are also in line with previous studies, showing that a ^{13}C enrichment in leaf dark-respired CO_2 coincided with higher *LEDR* peaks, at least within one species (Barbour *et al.*, 2007).

Low interspecific relationship between $\delta^{13}C_R$ and LEDR

However, in contrast to our expectations, the interspecific relationship between $\delta^{13}C_R$ and *R* was low and therefore highly species-specific (Fig. 2). Highest variations in $\delta^{13}C_R$ and lowest in *LEDR* were observed after darkening in *H. halimifolium,* while the inverse was true for *O. triangularis*. *S. officinalis* showed an intermediate pattern. The temperature treatment caused also non-synchronous changes in $\delta^{13}C_R$ and *R* values across all species, indicating no consistent relationship between both parameters, thereby pointing towards species-specific differences in metabolic origins and associated isotope fractionation. These species-specific variations may be explained by differences

in the respiratory pathways in plant functional groups (Priault *et al.*, 2009; Werner *et al.*, 2009; Wegener *et al.*, 2010), which have been proposed to differ in the pivotal branching point of the pyruvate dehydrogenase reaction (PDH). PDH releases ^{13}C enriched CO_2 from pyruvate, whereas the remaining ^{13}C depleted acetyl-CoA residue contributes either to (1) dark respiration, which would diminish the ^{13}C enrichment in respiratory CO_2 and cause higher *LEDR* as observed in *S. officinalis* and *O. triangularis*, or (2) to biosynthesis of secondary metabolites or lipids (Jardine *et al.*, 2012), which would increase the ^{13}C enrichment in respiratory CO_2 and cause lower *LEDR* as observed in *H. halimifolium* (Jardine *et al.*, 2014). Furthermore, the characteristic patterns of $\delta^{13}C_R$ and *LEDR* itself were very similar under both temperature conditions. This is particularly important for the comparability of daytime $\delta^{13}C_R$ measurements often taken shortly after illumination (Hymus *et al.*, 2005; Prater *et al.*, 2006; Werner *et al.*, 2007; Werner *et al.*, 2009; Wegener *et al.*, 2010), especially under field conditions with dynamic temperature variations throughout the day (Sun *et al.*, 2009; Unger *et al.*, 2010; Dubbert *et al.*, 2012).

Malate is not the sole respiratory carbon source

Furthermore, it has been hypothesized that changes in $\delta^{13}C_R$ and *LEDR* are strongly dependent on an organic acid pool. In particular, the respiratory use of daytime accumulated malate has been extensively discussed as a key substrate for *LEDR* (Hill & Bryce, 1992; Atkin *et al.*, 1998; Barbour *et al.*, 2007; Gessler *et al.*, 2009; Werner & Gessler, 2011; Werner *et al.*, 2011). Indeed, malate was ^{13}C enriched compared to other substrates in all species (Fig. 3). However, neither diurnal changes in $\delta^{13}C_{malate}$ (maximum of 2.9‰) nor those in $\delta^{13}C_{sugar}$ and $\delta^{13}C_{amino}$ can explain the observed

changes in $\delta^{13}C_R$ up to 8‰ in *H. halimifolium* and *A. unedo*. Moreover, none of our species exhibited significant changes in malate concentrations during the diurnal cycle (Fig. 4), in contrast to findings for *Ricinus communis* where a decrease in the malate pool shortly upon darkening was sufficient to explain the observed ^{13}C enrichment in leaf dark-respired CO_2 (Gessler *et al.*, 2009). Thus, our results suggest that malate is not the ubiquist respiratory substrate explaining the high variation in leaf dark-respired CO_2 and associated isotope fractionations.

To a certain extend this opposing result might be explained by the heterogeneous intramolecular isotopic pattern of malate with a ^{13}C enriched C-4 position (Melzer & O'Leary, 1987; Melzer & O'Leary, 1991; Savidge & Blair, 2004), which could lead to fragmentation fractionation under uncompleted respiration of malate. It can be assumed that C-1 and C-4 positions of malate have similar isotopic compositions due to isotope randomization by the fumarase reaction (Gout *et al.*, 1993; Voet & Voet, 2011), and that C-2 and C-3 positions of malate stems from $\delta^{13}C$ of sugars broken down during glycolysis. Under these assumptions and with averaged data of $\delta^{13}C_{malate}$ (-20‰ or -21.5‰; Fig. 3) and $\delta^{13}C_{sugar}$ (-31.5‰ or -27.5‰; Fig. 3) we can calculate a $\delta^{13}C$ value for both carboxyl groups of malate ($\delta^{13}C_{carboxyl}$) in *H. halimifolium* or *A. unedo*, respectively:

$$\delta^{13}C_{carboxyl} = \delta^{13}C_{malate} * 2 - \delta^{13}C_{sugar}$$

This would result in -8.5‰ or -15.5‰ for $\delta^{13}C_{carboxyl}$, so that respired CO_2 derived from $\delta^{13}C_{carboxyl}$ and released by NAD malic enzyme or KC reactions could then be 10.5‰ and 7‰ more enriched in ^{13}C compared to averaged data of $\delta^{13}C_R$ (-19‰ or -22.5‰; Fig. 3) in *H. halimifolium* or *A. unedo*, respectively. However, such high fragmentation

fractionation would only be possible under unlikely metabolic conditions under which the C-2 and C-3 positions of malate are only weakly respired compared to $\delta^{13}C_{carboxyl}$ (Werner *et al.*, 2009). Moreover, respiration of malate has been shown to contribute only to a small extend to LEDR (Gessler *et al.*, 2009; Lehmann *et al.*, 2015 - in review), indicating that further processes, or other substrates must be involved in the high $\delta^{13}C_R$ variations. In summary, we propose that the high variations in $\delta^{13}C_R$ can only partially be explained with $\delta^{13}C_{malate}$, and only when assuming a potential intramolecular isotopic heterogeneity of malate with very ^{13}C enriched positions for the carbonyl groups and under metabolic conditions with strong isotope fractionation.

Oxalate accumulation may cause highly ^{13}C enriched malate

In addition, we found extraordinarily high $\delta^{13}C_{malate}$ values (up to -10.9‰) in *O. triangularis* in comparison to the other species (Fig. 3), which is particularly remarkable for a plant with C_3 metabolism. Since *O. triangularis* plants had lowest variations in $\delta^{13}C_R$ as observed in previous studies (Priault *et al.*, 2009; Wegener *et al.*, 2010), this suggests a shift in metabolic pathways, causing a ^{13}C enrichment in respiratory substrates instead of a ^{13}C enrichment in respiratory CO_2. A possible explanation for this notable result may be related to the very high oxalate accumulation found in *O. triangularis*, which was not found in other species (Fig. 3). Glyoxlate is discussed to be a precursor for oxalate biosynthesis via interactions of KC reactions with reactions of the glyoxlate cycle (Fig. 5; modified after Yu *et al.*, 2010). Glyoxylate stems from the acetyl-CoA part of isocitrate (derived from the KC) and is released by the isocitrate lyase reaction (ICL). The withdrawal of isocitrate from the KC must be compensated by an anapleurotic reaction (Yu *et al.*, 2010), e.g., via PEPC causing a ^{13}C enrichment in oxalacetate and malate. In

addition, a possible back-transport of ^{13}C enriched succinate (the other product of the ICL reaction) towards the KC resembles one cycling of the KC without any decarboxylation reactions and therefore without releasing ^{13}C enriched leaf dark-respired CO_2 (Heldt & Piechulla, 2011). Such a mechanism could explain the ^{13}C enrichment in malate in one or in both carboxyl groups without increasing $\delta^{13}C_R$ values. Moreover, the strong discrepancy between $\delta^{13}C_R$ and $\delta^{13}C_{malate}$ in *O. triangularis* indicates that malate may be compartmented in at least two pools, one for dark respiration and one for other metabolic processes such as storage, stomata regulation and/or the biosynthesis of oxalate. The pronounced pools might also differ in $\delta^{13}C_{malate}$ and malate concentrations. Overall, mechanisms leading to dark respiration in *O. triangularis* seem to differ from other species, causing less ^{13}C enriched respired CO_2 in spite of highly ^{13}C enriched malate and clearly demonstrate so far unknown species-specific isotope fractionation in metabolic pathways, suggesting that we only scratched the surface of the complexity of carbon isotope fractionation in plants yet.

Conclusions

Here we investigated the phenomenon of ^{13}C-enriched dark-respired CO_2 after illumination of leaves. Our results indicate the build-up of a ^{13}C enriched respiratory substrate pool over the light period. However, contrary to our expectations we found no clear relationship between $\delta^{13}C_R$ and *LEDR* pattern across different species. Furthermore, neither diurnal variation in $\delta^{13}C$ values nor in concentration of malate could fully explain the examined phenomenon. Therefore, we have to reject the hypothesis that the ^{13}C enrichment in respiratory CO_2 after illumination is directly coupled to LEDR and that it originates from ^{13}C enriched malate as sole respiratory

carbon source, suggesting that additional substrates and processes must be involved. However, this might differ in plant species with stronger leaf malate concentrations than the ones of this study. Moreover, we found unusual species-specific differences in the isotopic composition of malate (up to 11.1‰). Reasons for that most likely depend on species-specific respiratory processes, the intramolecular isotope composition of malate, and on $\delta^{13}C$ and concentration variations in different leaf malate pools. Thus, position-specific ^{13}C analyses of different substrates are required to shed further light into metabolic processes during respiration.

Acknowledgements

We gratefully thank Nina Buchmann (ETH Zurich) and Rolf TW Siegwolf (Paul-Scherrer-Institute) for valuable discussions and comments. We acknowledge the technical support given by Lola Schmid, Yves Letz, and Sweety at PSI Villigen (Paul-Scherrer-Institute); by Annika Ackermann and EA Burns at ETH Zurich; and by Dina Heinze, Babsi Teichner and Elke Fürlkröger at University Bielefeld. The study was financed by the Swiss National Science Foundation (SNF, CIFRes, No. 205321_132768) and by the German Research Foundation (DFG, ECORES, No. WE2681/5-1). MML acknowledges a short-term scientific mission supported by the COST Action ES0806 SIBAE.

References

Atkin OK, Millar AH, Gardestroem P, Day DA 2000. Photosynthesis, carbohydrate metabolism and respiration in leaves of higher plants. In: Leegood RC, Sharkey TD, Von Caemmerer S eds. *Photosynthesis and Metabolism.* Dordrecht, the Netherlands: Kluwer Academic, pp. 153-175.

Atkin OK, Evans JR, Siebke K. 1998. Relationship between the inhibition of leaf respiration by light and enhancement of leaf dark respiration following light treatment. *Australian Journal of Plant Physiology* **25**: 437-443.

Barbour MM, Hunt JE, Kodama N, Laubach J, McSeveny TM, Rogers GN, Tcherkez G, Wingate L. 2011. Rapid changes in $\delta^{13}C$ of ecosystem-respired CO_2 after sunset are consistent with transient ^{13}C enrichment of leaf respired CO_2. *New Phytologist* **190**: 990-1002.

Barbour MM, McDowell NG, Tcherkez G, Bickford CP, Hanson DT. 2007. A new measurement technique reveals rapid post-illumination changes in the carbon isotope composition of leaf-respired CO_2. *Plant, Cell and Environment* **30**: 469-482.

Bowsher C, Steer M, Tobin A 2008. Plant Biochemistry. New York, NY, USA: Garland Science, pp. 143-193.

Brueggemann N, Gessler A, Kayler Z, Keel SG, Badeck F, Barthel M, Boeckx P, Buchmann N, Brugnoli E, Esperschutz J, et al. 2011. Carbon allocation and carbon isotope fluxes in the plant-soil-atmosphere continuum: a review. *Biogeosciences* **8**: 3457-3489.

Dubbert M, Rascher KG, Werner C. 2012. Species-specific differences in temporal and spatial variation in $\delta^{13}C$ of plant carbon pools and dark-respired CO_2 under changing environmental conditions. *Photosynthesis Research* **113**: 297-309.

Duranceau M, Ghashghaie J, Badeck F, Deleens E, Cornic G. 1999. $\delta^{13}C$ of CO_2 respired in the dark in relation to $\delta^{13}C$ of leaf carbohydrates in *Phaseolus vulgaris* L. under progressive drought. *Plant, Cell and Environment* **22**: 515-523.

Farquhar GD, Ehleringer JR, Hubick KT. 1989. Carbon isotope discrimination and photosynthesis. *Annual Review of Plant Physiology and Plant Molecular Biology* **40**: 503-537.

Gessler A, Tcherkez G, Karyanto O, Keitel C, Ferrio JP, Ghashghaie J, Kreuzwieser J, Farquhar GD. 2009. On the metabolic origin of the carbon isotope composition of CO_2 evolved from darkened light-acclimated leaves in *Ricinus communis*. *New Phytologist* **181**: 374-386.

Ghashghaie J, Badeck FW. 2014. Opposite carbon isotope discrimination during dark respiration in leaves versus roots - a review. *New Phytologist* **201**: 751-769.

Ghashghaie J, Duranceau M, Badeck FW, Cornic G, Adeline MT, Deleens E. 2001. $\delta^{13}C$ of CO_2 respired in the dark in relation to $\delta^{13}C$ of leaf metabolites: comparison between *Nicotiana sylvestris* and *Helianthus annuus* under drought. *Plant, Cell and Environment* **24**: 505-515.

Gout E, Bligny R, Pascal N, Douce R. 1993. ^{13}C nuclear magnetic resonance studies of malate and citrate synthesis and compartmentation in higher plant cells. *The Journal of Biological Chemistry* **268**: 3986-3992.

Hanning I, Heldt HW. 1993. On the function of mitochondrial metabolism during photosynthesis in spinach (*Spinacia-Oleracea* L.) leaves - Partitioning between respiration and export of redox equivalents and precursors for nitrate assimilation products. *Plant Physiology* **103**: 1147-1154.

Heldt HW, Piechulla B 2011. Plant Biochemistry - 4th edition. Waltham, MA, USA: Academic Press, Elsevier, pp. 388-393.

Hettmann E, Gleixner G, Juchelka D. 2005. IRM-LC/MS: $\delta^{13}C$ analysis of organic acids in plants. *Application note 30075, Thermo Fisher Scientific*.

Hill SA, Bryce JH 1992. Malate metabolism and light-enhanced dark respiration in barley mesophyll protoplasts. In: Lambers H, Van der Plas LHW eds. *Molecular,*

Biochemical and Physiological Aspects of Plant Respiration: SPB Academic Publishing, The Hague, The Netherlands, pp. 221-230.

Hymus GJ, Maseyk K, Valentini R, Yakir D. 2005. Large daily variation in ^{13}C-enrichment of leaf-respired CO_2 in two *Quercus* forest canopies. *New Phytologist* **167**: 377-384.

Igamberdiev AU, Romanowska E, Gardeström P. 2001. Photorespiratory flux and mitochondrial contribution to energy and redox balance of barley leaf protoplasts in the light and during light-dark transitions. *Journal of Plant Physiology* **158**: 1325-1332.

Jardine K, Wegener F, Abrell L, van Haren J, Werner C. 2014. Phytogenic biosynthesis and emission of methyl acetate. *Plant, Cell and Environment* **37**: 414-424.

Jardine K, Barron-Gafford GA, Norman JP, Abrell L, Monson RK, Meyers KT, Pavao-Zuckerman M, Dontsova K, Kleist E, Werner C, et al. 2012. Green leaf volatiles and oxygenated metabolite emission bursts from mesquite branches following light-dark transitions. *Photosynthesis Research* **113**: 321-333.

Krummen M, Hilkert AW, Juchelka D, Duhr A, Schluter HJ, Pesch R. 2004. A new concept for isotope ratio monitoring liquid chromatography/mass spectrometry. *Rapid Communications in Mass Spectrometry* **18**: 2260-2266.

Lehmann MM, Wegener F, Barthel M, Maurino VG, Siegwolf RTW, Buchmann N, Werner C, Werner RA. 2015. Disentangling biochemical processes determining $\delta^{13}C$ of leaf-respired CO_2 during light-dark transitions. *Plant, Cell and Environment*: - in review.

Melzer E, O'Leary MH. 1991. Aspartic-acid synthesis in C_3 plants. *Planta* **185**: 368-371.

Melzer E, O'Leary MH. 1987. Anapleurotic CO_2 fixation by phosphoenolpyruvate carboxylase in C_3 plants. *Plant Physiology* **84**: 58-60.

Prater JL, Mortazavi B, Chanton JP. 2006. Diurnal variation of the $\delta^{13}C$ of pine needle respired CO_2 evolved in darkness. *Plant, Cell and Environment* **29**: 202-211.

Priault P, Wegener F, Werner C. 2009. Pronounced differences in diurnal variation of carbon isotope composition of leaf respired CO_2 among functional groups. *New Phytologist* **181**: 400-412.

R Core Team. 2013. R: A language and environment for statistical computing. *R foundation for statistical computing, Vienna, Austria*.

Rascher KG, Maguas C, Werner C. 2010. On the use of phloem sap $\delta^{13}C$ as an indicator of canopy carbon discrimination. *Tree Physiology* **30**: 1499-1514.

Richter A, Wanek W, Werner RA, Ghashghaie J, Jaeggi M, Gessler A, Brugnoli E, Hettmann E, Gottlicher SG, Salmon Y, et al. 2009. Preparation of starch and soluble sugars of plant material for the analysis of carbon isotope composition: a comparison of methods. *Rapid Communications in Mass Spectrometry* **23**: 2476-2488.

Rinne KT, Saurer M, Streit K, Siegwolf RTW. 2012. Evaluation of a liquid chromatography method for compound-specific $\delta^{13}C$ analysis of plant carbohydrates in alkaline media. *Rapid Communications in Mass Spectrometry* **26**: 2173-2185.

Savidge WB, Blair NE. 2004. Patterns of intramolecular carbon isotopic heterogeneity within amino acids of autotrophs and heterotrophs. *Oecologia* **139**: 178-189.

Streit K, Rinne KT, Hagedorn F, Dawes MA, Saurer M, Hoch G, Werner RA, Buchmann N, Siegwolf RTW. 2013. Tracing fresh assimilates through *Larix decidua* exposed

to elevated CO_2 and soil warming at the alpine treeline using compound-specific stable isotope analysis. *New Phytologist* **197**: 838-849.

Sun W, Resco V, Williams DG. 2009. Diurnal and seasonal variation in the carbon isotope composition of leaf dark-respired CO_2 in velvet mesquite (*Prosopis velutina*). *Plant, Cell and Environment* **32**: 1390-1400.

Sweetlove LJ, Beard KFM, Nunes-Nesi A, Fernie AR, Ratcliffe RG. 2010. Not just a circle: flux modes in the plant TCA cycle. *Trends in Plant Science* **15**: 462-470.

Tcherkez G, Cornic G, Bligny R, Gout E, Ghashghaie J. 2005. In vivo respiratory metabolism of illuminated leaves. *Plant Physiology* **138**: 1596-1606.

Tcherkez G, Farquhar G, Badeck F, Ghashghaie J. 2004. Theoretical considerations about carbon isotope distribution in glucose of C_3 plants. *Functional Plant Biology* **31**: 857-877.

Tcherkez G, Nogues S, Bleton J, Cornic G, Badeck F, Ghashghaie J. 2003. Metabolic origin of carbon isotope composition of leaf dark-respired CO_2 in French bean. *Plant Physiology* **131**: 237-244.

Unger S, Maguas C, Pereira JS, Aires LM, David TS, Werner C. 2010. Disentangling drought-induced variation in ecosystem and soil respiration using stable carbon isotopes. *Oecologia* **163**: 1043-1057.

Voet D, Voet JG 2011. Biochemistry, 4th edition. New York, NY, USA: Wiley, pp. 789-822.

Wegener F, Beyschlag W, Werner C. 2010. The magnitude of diurnal variation in carbon isotopic composition of leaf dark respired CO_2 correlates with the difference between $\delta^{13}C$ of leaf and root material. *Functional Plant Biology* **37**: 849-858.

Werner C, Schnyder H, Cuntz M, Keitel C, Zeeman MJ, Dawson TE, Badeck FW, Brugnoli E, Ghashghaie J, Grams TEE, et al. 2012. Progress and challenges in using stable isotopes to trace plant carbon and water relations across scales. *Biogeosciences* **9**: 3083-3111.

Werner C, Gessler A. 2011. Diel variations in the carbon isotope composition of respired CO_2 and associated carbon sources: a review of dynamics and mechanisms. *Biogeosciences* **8**: 2437-2459.

Werner C, Wegener F, Unger S, Nogues S, Priault P. 2009. Short-term dynamics of isotopic composition of leaf-respired CO_2 upon darkening: measurements and implications. *Rapid Communications in Mass Spectrometry* **23**: 2428-2438.

Werner C, Hasenbein N, Maia R, Beyschlag W, Maguas C. 2007. Evaluating high time-resolved changes in carbon isotope ratio of respired CO_2 by a rapid in-tube incubation technique. *Rapid Communications in Mass Spectrometry* **21**: 1352-1360.

Werner RA, Buchmann N, Siegwolf RTW, Kornexl BE, Gessler A. 2011. Metabolic fluxes, carbon isotope fractionation and respiration - lessons to be learned from plant biochemistry. *New Phytologist* **191**: 10-15.

Werner RA, Brand WA. 2001. Referencing strategies and techniques in stable isotope ratio analysis. *Rapid Communications in Mass Spectrometry* **15**: 501-519.

Werner RA, Bruch BA, Brand WA. 1999. ConFlo III - an interface for high precision $\delta^{13}C$ and $\delta^{15}N$ analysis with an extended dynamic range. *Rapid Communications in Mass Spectrometry* **13**: 1237-1241.

Yu L, Jiang JZ, Zhang C, Jiang LR, Ye NH, Lu YS, Yang GZ, Liu E, Peng CL, He ZH, et al. 2010. Glyoxylate rather than ascorbate is an efficient precursor for oxalate biosynthesis in rice. *Journal of Experimental Botany* **61**: 1625-1634.

Figures

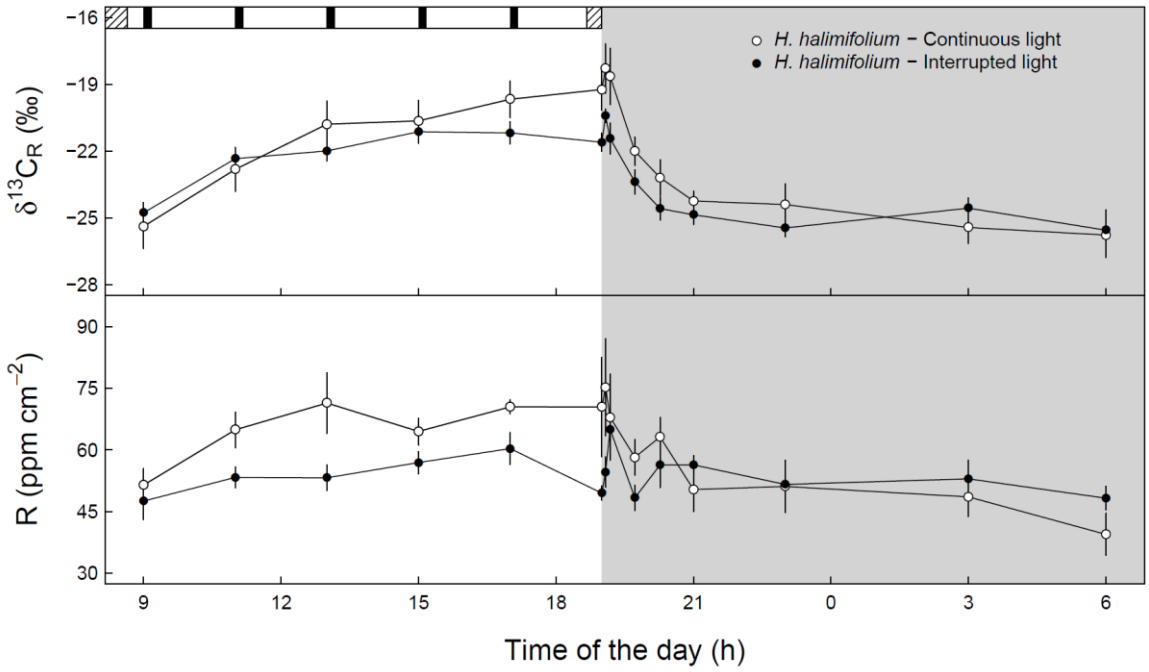

Figure 1: Diurnal cycle of the carbon isotopic composition of leaf dark-respired CO_2 ($\delta^{13}C_R$, ‰) and dark respiration (R, ppm cm^{-2}) under continuous (open circles) and interrupted light (closed circles) in *H. halimifolium*. The interrupted light treatment is indicated by the upper bar: black boxes denote periodical interruptions of the light period for 10 min, while hashed boxes indicate additional light periods (in total 50 min) to ensure the same total light duration under both conditions. The grey area denotes nighttime. Means ± SE are given (n = 3-6).

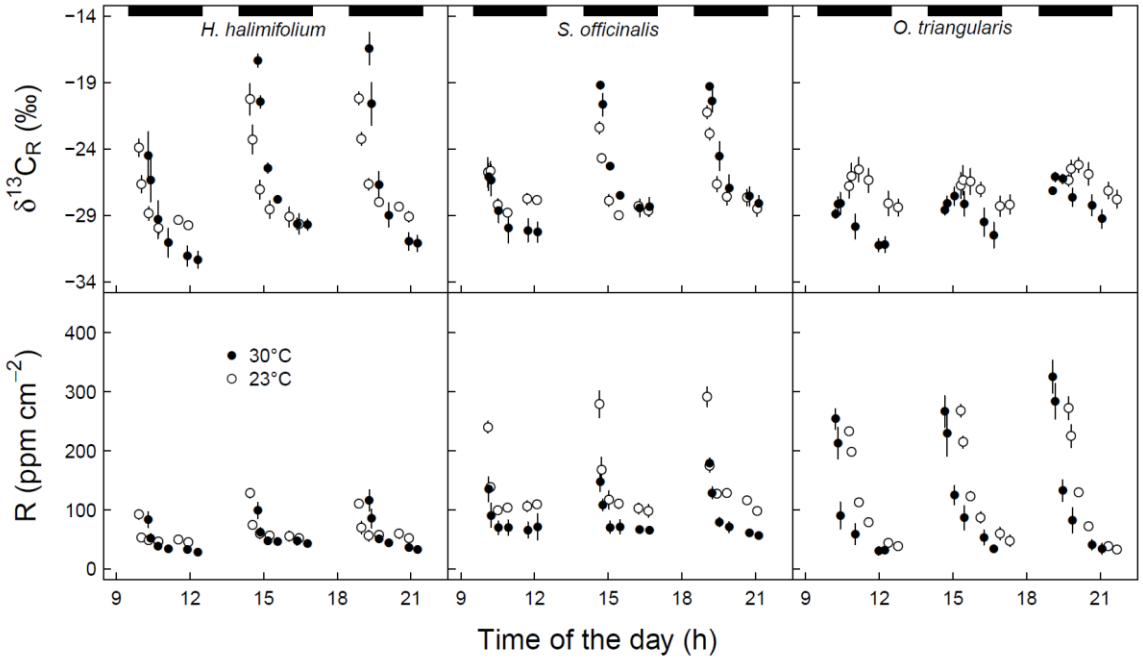

Figure 2: Carbon isotopic composition of leaf dark-respired CO_2 ($\delta^{13}C_R$, ‰) and dark respiration (R, ppm cm^{-2}) under high (closed circles) and low temperature conditions (open circles) for three different species. Plants were darkened at three points in time for two hours (black bars) during the light period to induce *LEDR*. Means ± SE are given (n = 3-4).

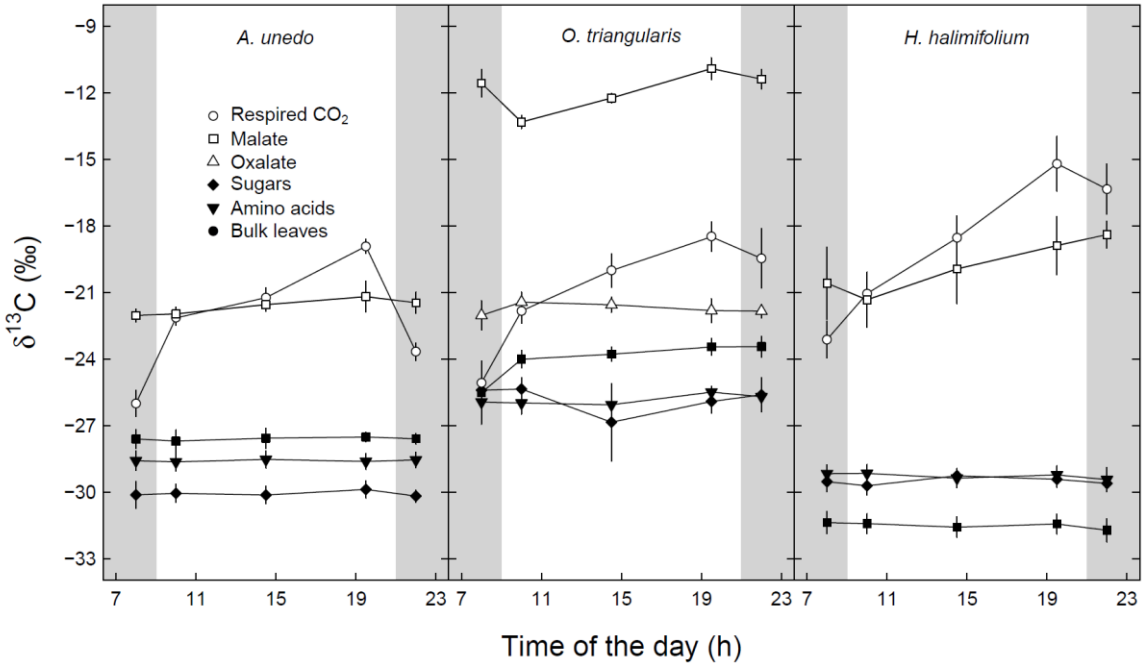

Figure 3: Diurnal cycle of the carbon isotopic composition ($\delta^{13}C$, ‰), of leaf dark-respired CO_2, bulk leaves, water soluble organic fractions of sugars and amino acids, and malate in three different species, as well as $\delta^{13}C$ of oxalate in *O. triangularis*. Grey areas denote nighttime. Means ± SE are given (n = 3-5).

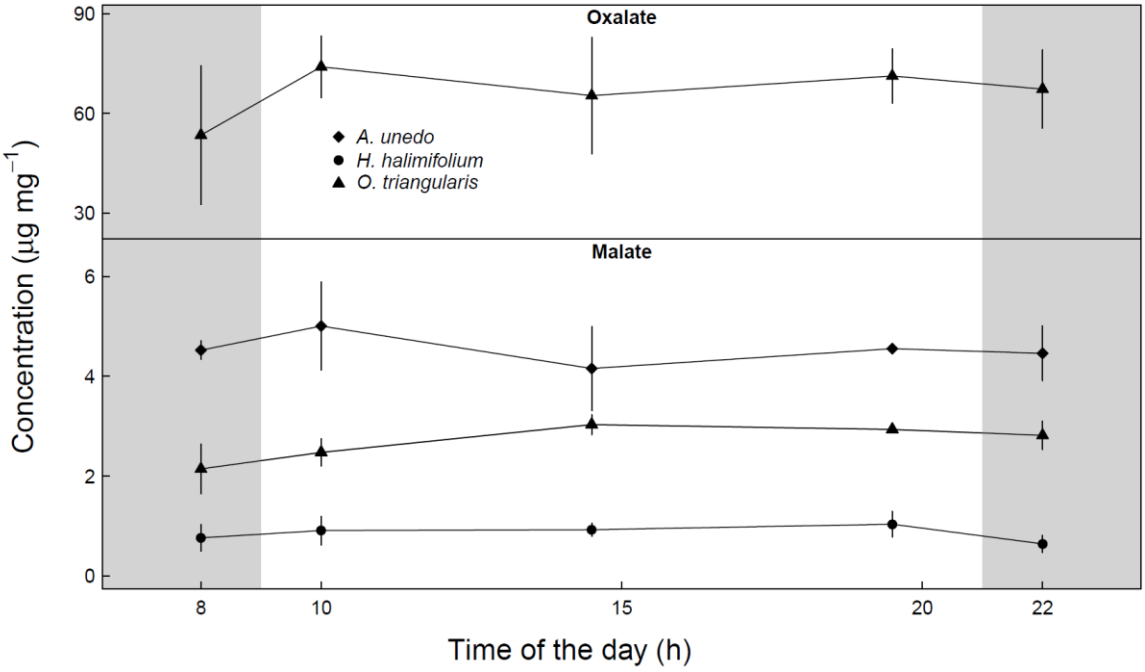

Figure 4: Diurnal cycle of malate concentration (µg mg^{-1}) in three different species, as well as the oxalate concentration in *O. triangularis*. Grey areas denote nighttime. Means ± SE are given (n = 3-4).

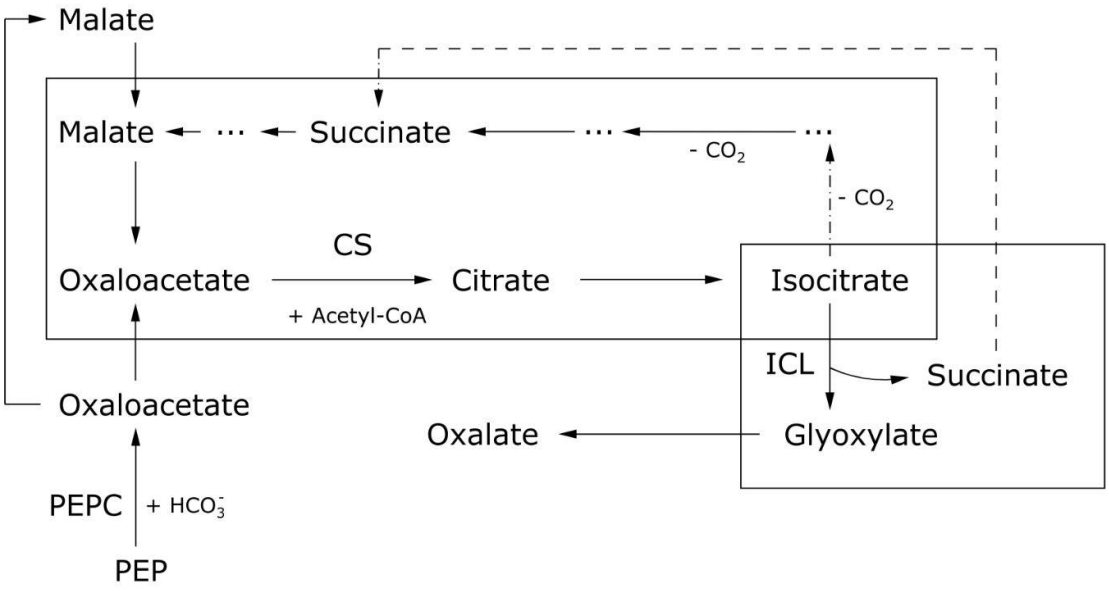

Figure 5: The production of ^{13}C enriched malate in the oxalate accumulating plant *Oxalis triangularis* in interaction with reactions of the Krebs cycle (KC) and the glyoxylate cycle. Glyoxylate is discussed as precursor of oxalate (Yu *et al.*, 2010). Isocitrate can be withdrawn from the KC by the glyoxylate cycle, where the isocitrate lyase reaction (ICL) produces glyoxylate. The C_2 skeleton of glyoxylate originates from the acetyl-CoA part of isocitrate, which derives from citrate produced by the citrate synthase reaction (CS). The anapleurotic flux from the phosphoenolpyruvate carboxylase reaction (PEPC) supplies KC with ^{13}C enriched compounds such as oxaloacetate or malate. A possible back-transport of ^{13}C enriched succinate towards the KC (dashed line) causes one cycling of the KC without any decarboxylation reactions, leading to a ^{13}C enrichment in malate but not in leaf dark-respired CO_2.

Chapter 4

4. Disentangling biochemical processes determining $\delta^{13}C$ of leaf-respired CO_2 during light-dark transitions

Marco M. Lehmann[1], Frederik Wegener[2], Matti Barthel[1], Verónica G. Maurino[3], Rolf T. W. Siegwolf[4], Nina Buchmann[1], Christiane Werner[2], Roland A. Werner[1]

[1]Institute of Agricultural Sciences, ETH Zurich, Universitätsstr. 2, CH-8092 Zurich, Switzerland;

[2]Agro-Ecosystem Research, BAYCEER, University of Bayreuth, Universitätsstr. 30, D-95447 Bayreuth, Germany;

[3]Institute of Developmental and Molecular Biology of Plants, CEPLAS, Heinrich-Heine-University Düsseldorf, Universitätsstr. 1, D- 40225 Düsseldorf, Germany;

[4]Laboratory of Atmospheric Chemistry, Paul Scherrer Institute (PSI), CH-5232 Villigen, Switzerland

Manuscript was submitted to *Plant, Cell & Environment*.

Abstract

Enhanced CO_2 releases in leaves during light-dark transitions, defined as "light enhanced dark respiration", show often strong increases in their carbon isotopic composition ($\delta^{13}C_{LEDR}$). However, the underlying biochemical processes resulting in increased $\delta^{13}C_{LEDR}$ are poorly understood, but might be associated with decarboxylation of a potentially ^{13}C enriched C-4 position of malate by the NAD-malic enzyme reaction (NAD-ME). We fed leaves with position-specific ^{13}C-labelled malate or pyruvate and measured label-derived leaf $^{13}CO_2$ respiration (R_{Label}) during light-dark transitions of *Arabidopsis thaliana* and of species with high (*Halimium halimifolium*) and low increased $\delta^{13}C_{LEDR}$ (*Oxalis triangularis*), using laser spectroscopy. *Arabidopsis* mutants (lacking functional NAD-ME) fed with ^{13}C-4 malate, did not differ significantly in R_{Label} from wild type plants during light-dark transitions, indicating that NAD-ME had no significant effect on $\delta^{13}C_{LEDR}$. Highest R_{Label} was found in *Halimium halimifolium*, but not in *Oxalis triangularis*, when fed with ^{13}C-1 pyruvate, suggesting high mitochondrial pyruvate dehydrogenase (mtPDH) activity in *Halimium halimifolium*. Both species showed increased R_{Label} after being fed with ^{13}C-1 and ^{13}C-4 malate substrates, but not after ^{13}C-2 pyruvate feeding. Thus, our results suggest that strong natural increases in $\delta^{13}C_{LEDR}$ are mainly determined by mtPDH, using ^{13}C-1 pyruvate as substrate, while NAD-ME plays only a minor role.

Keywords: isotope fractionation, laser spectroscopy, light enhanced dark respiration (LEDR), malate, plant respiration, pyruvate, respired CO_2, stable carbon isotopes

Abbreviations: $\delta^{13}C_{LEDR}$, carbon isotopic composition of leaf-respired CO_2 during LEDR; Δ_{Label}, label-induced ^{13}C enrichment; cyIDH and mtIDH, cytosolic and mitochondrial isocitrate dehydrogenase; LEDR, light enhanced dark respiration; NAD-ME, NAD-malic enzyme; OGDH, 2-oxoglutarate dehydrogenase; cpPDH and mtPDH, chloroplast and mitochondrial pyruvate dehydrogenase; PEPC, phosphoenolpyruvate carboxylase; R_{Label}, label-derived leaf $^{13}CO_2$ respiration; TCA cycle, tricarboxylic acid cycle

Introduction

During dark respiration plants metabolize photosynthetic assimilates via glycolysis and the tricarboxylic acid (TCA) cycle, providing intermediates, reducing equivalents, and chemical energy for numerous plant metabolic reactions. Ongoing anabolic and catabolic reactions in many mitochondrial and chloroplast respiratory pathways are fuelled by small molecules, such as the organic acids malate and pyruvate (Plaxton & Podesta, 2006). During this process, these substrates are progressively broken down by CO_2 producing reactions.

However, the amount of CO_2 released during dark respiration is not always constant. Shortly after darkening, leaves show an enhanced CO_2 release during a transition period defined as "light enhanced dark respiration" (LEDR; Atkin *et al.*, 1998), linking photosynthetic and respiratory processes. LEDR is often described to last about 20 to 30 min (Atkin *et al.*, 1998; Griffin & Turnbull, 2012), with a maximum CO_2 release within the first 5 min. It was shown that the magnitude of the CO_2 release increases with the light intensity of the previous photoperiod (Atkin *et al.*, 2000). Several studies have hypothesized that organic acids such as malate accumulate during the light period due to an open and only partially active TCA cycle (Tcherkez *et al.*, 2005; Sweetlove *et al.*, 2010), and that their rapid breakdown supports the enhanced CO_2 release during LEDR (Hill & Bryce, 1992; Xue *et al.*, 1996; Atkin *et al.*, 1998). On the other hand, with studies using stable carbon isotopes it was shown that the carbon isotopic composition of leaf-respired CO_2 during LEDR ($\delta^{13}C_{LEDR}$) was up to 15.9‰ enriched in ^{13}C compared to water soluble organic compounds (such as carbohydrates, amino and organic acids) and that this initial peak in $\delta^{13}C_{LEDR}$ was followed by a progressive decrease (Hymus *et al.*, 2005;

Prater *et al.*, 2006; Werner *et al.*, 2009; Wegener *et al.*, 2010; Werner & Gessler, 2011). Such course of $\delta^{13}C_{LEDR}$ was also observed in a grassland ecosystem shortly after sunset, using laser-based, on-line measurements (Barbour *et al.*, 2011), demonstrating that changes observed under controlled conditions are also relevant at ecosystem scales. Moreover, several studies showed that $\delta^{13}C_{LEDR}$ differs among plant functional groups (Priault *et al.*, 2009; Wegener *et al.*, 2010): evergreen, slow-growing or aromatic species showed highest increases in $\delta^{13}C_{LEDR}$ during the day (up to 14.8‰), while fast-growing, non-aromatic herbaceous species had lower increases in $\delta^{13}C_{LEDR}$ (up to 3.4‰; Wegener *et al.*, 2010). Nevertheless, the reasons for the increases and species-specific differences in $\delta^{13}C_{LEDR}$ are not fully understood so far (Werner & Gessler, 2011; Ghashghaie & Badeck, 2014).

The current hypothesis for increases in $\delta^{13}C_{LEDR}$ is based on the "respiratory malate mechanism". Several studies have suggested that CO_2 derived from malate by the mitochondrial NAD-malic enzyme reaction (NAD-ME) might determine $\delta^{13}C_{LEDR}$ (Barbour *et al.*, 2007; Gessler *et al.*, 2009; Werner *et al.*, 2011). In particular, the C-4 position of malate is expected to be enriched in ^{13}C due to an anapleurotic flux refilling TCA cycle intermediates via the phosphoenolpyruvate carboxylase reaction (PEPC), which fixes CO_2 with a net isotope fractionation against ^{13}C of -5.7‰ (Melzer & O'Leary, 1987; Farquhar *et al.*, 1989; Melzer & O'Leary, 1991; Savidge & Blair, 2004). Differences in respiration of the potentially ^{13}C enriched C-4 position by NAD-ME might explain species-specific differences in $\delta^{13}C_{LEDR}$. However, experimental evidence supporting this hypothesis is still missing.

Besides the ^{13}C enrichment in malate, $\delta^{13}C_{LEDR}$ can also be influenced by the heterogeneous intramolecular isotope distribution within other putative respiratory substrates, which is defined as "fragmentation fractionation" (Tcherkez *et al.*, 2004). For instance, the C-3 and C-4 positions of glucose are enriched in ^{13}C compared to other positions of the same molecule (Rossmann *et al.*, 1991; Gilbert *et al.*, 2012), probably due to an equilibrium isotope effect of the aldolase reaction (Gleixner & Schmidt, 1997). On the one hand, ^{13}C enriched positions of glucose molecules broken down during glycolysis are relocated to the C-1 position of pyruvate molecules and can be released as CO_2 by the mitochondrial pyruvate dehydrogenase reaction (mtPDH) in the dark. Additionally, chloroplast PDH (cpPDH) can consume photosynthetically produced pyruvate in the light (Tovar-Mendez *et al.*, 2003). On the other hand, ^{13}C depleted positions of glucose molecules relocated in the acetyl-CoA residue (C-2 and C-3 position of pyruvate) can be used for respiration or for biosynthesis of various substrates such as amino acids and lipids. Several feeding studies have been carried out with leaves of different species, using position-specific ^{13}C-labelled glucose or pyruvate, to investigate the fractionation occurring during molecule fragmentation and to identify the contribution of decarboxylating enzymatic reactions to leaf-respired CO_2 (Tcherkez *et al.*, 2005; Priault *et al.*, 2009; Werner *et al.*, 2009; Wegener *et al.*, 2010; Jardine *et al.*, 2014). However, no experiments with position-specific ^{13}C-labelled malate solutions have been performed to date, and thus, the respiratory and metabolic fate of C-1 and C-4 positions of malate during LEDR and their associated respiratory mechanisms imprinting on $\delta^{13}C_{LEDR}$ remains to be proven. Further insights may be gained by conducting position-specific ^{13}C-labelled experiments in *Arabidopsis thaliana* mutants due to their large

potential to unravel the metabolic fluxes within the respiratory network of plants (Sweetlove *et al.*, 2010; Araújo *et al.*, 2012).

Here we hypothesize that $\delta^{13}C_{LEDR}$ is mainly determined by decarboxylation of a potentially ^{13}C enriched C-4 position of malate via NAD-ME during light-dark transitions, with the expectation to observe species-specific differences in the intensity of this reaction. Our main objectives are (1) to identify species-specific differences in the respiration of position-specific ^{13}C-labelled malate and pyruvate during light-dark transitions, including LEDR; (2) to understand the associated respiratory mechanisms determining increases in $\delta^{13}C$ of leaf-respired CO_2 in the light and in the dark during LEDR; and (3) to evaluate the non-respiratory fate of position-specific ^{13}C-labelled malate and pyruvate. Thus, we fed leaves of an *Arabidopsis thaliana* mutant lacking any functional NAD-ME, as well as wild type plants, with ^{13}C-4 malate via the transpiration stream. Moreover, leaves of two species with known high (*Halimium halimifolium*) and low (*Oxalis triangularis*) increases in $\delta^{13}C_{LEDR}$ were fed with different position-specific ^{13}C-labelled malate (^{13}C-1, ^{13}C-4) and pyruvate (^{13}C-1, ^{13}C-2). We measured the label-derived leaf $^{13}CO_2$ respiration during light-dark transitions on-line with isotope laser spectroscopy and determined the ^{13}C label allocation into leaf metabolic fractions.

Material & Methods

Plant material

We chose species from two functional groups, as described in Priault *et al.* (2009): the evergreen Mediterranean shrub *Halimium halimifolium* L. and the fast-growing herb *Oxalis triangularis* A. St.-Hil. While *H. halimifolium* plants were potted in 5 L pots filled

with sand (plant height 40-60 cm), *O. triangularis* plants were potted in 1 L pots with potting soil (plant height 10-15 cm). In addition, we used five to six week old *Arabidopsis thaliana* wild type Col-0 and *nadme1x2* plants potted in 0.2 L pots filled with potting soil. The double mutant *nadme1x2*, defined here as NAD-ME mutant, lacks functionality of both genes expressing NAD-malic enzyme and therefore no functional NAD-ME can be observed (Tronconi *et al.*, 2008). Additionally, no growth or developmental phenotype was observed as described previously (Tronconi *et al.*, 2008). All plants were grown under controlled conditions in walk-in climate chambers at the University of Bayreuth, with a constant temperature of 23°C and relative humidity of about 60% during a 12 h light period (09:00–21:00 h) with a photosynthetic photon flux density (PPFD) of about 770 µmol m^{-2} s^{-1}.

Synthesis of position-specific ^{13}C-labelled malate

^{13}C-1 and ^{13}C-4 labelled malate substrates were synthesized with coupled enzymatic reactions, which were modified after Rosenberg & O`Leary (1985): 4.4 mmol NADH disodium salt (Roth, Arlesheim, Switzerland), 2.8 mmol 2-oxoglutarate (Sigma-Aldrich, Buchs, Switzerland), 2.9 mmol ^{13}C-1 or ^{13}C-4 aspartate (99%, both Sercon, Crewe, UK), and 100 U glutamate oxalate transaminase (Roche, Rotkreuz, Switzerland) were dissolved in 50 ml of 0.2 M KH_2PO_4 (pH 7.5) buffer solution. Subsequently, pH 7.5 was readjusted with 11 ml of 1 M KOH and the reaction solution continuously stirred for 10 min at 25°C. The enzymatic reaction was then started by adding 100 U of a malate dehydrogenase solution (Roche, Rotkreuz, Switzerland). Aliquots of the reaction solution were analyzed at 340 nm with a 96-well microplate reader (ELx800, BioTek, Luzern, Switzerland) to follow the NADH degradation, which ended after 2 h. Thereafter, all

enzyme residues of the reaction solution were removed with pre-washed centrifugation filters with a molecular weight cut-off of 5000 da (Vivaspin 15R, 5000 MWCO HY, Sartorius, Göttingen, Germany). Subsequently, aliquots of the reaction solutions were separated by ion exchange chromatography, using Dowex 50WX8 in H^+ form and Dowex 1X8 in $NaCOO^-$ form (both 100-200 mesh, Sigma-Aldrich, Buchs, Switzerland). Malate containing fractions of the reaction solution were eluted from Dowex 1X8 columns with 40 ml 1 M HCl, frozen (-20°C), and lyophilized. Finally, pellets were re-suspended in deionized H_2O and aliquots merged to one labelling solution. Synthesis of ^{13}C-1 and ^{13}C-4 labelled malate was verified with an enzymatic kit (L-malic acid, R-biopharm, Darmstadt, Germany), showing the presence of malate in both malate labelling solutions, with a recovery of about 80%.

Experimental setup for laser-based on-line measurements

Leaf $^{13}CO_2$ respiration was determined on-line with a cavity ringdown laser spectrometer (CRDS, G2101-I, Picarro, Santa Clara, CA, USA). The CRDS system holds a wavelength monitor, which quantifies the spectral signature of CO_2 isotopologues with a time-based measurement technique in an optical cavity at 1603 nm. Temperature and pressure within the cavity were constantly at 40°C and 140 Torr, respectively. Data were monitored continuously with a temporal resolution of 0.75 Hz and 45 single values were averaged per minute for further analysis.

For $^{13}CO_2$ measurements of sample gas ($^{13}CO_{2_{SG}}$), leaves or twigs were placed in transparent cylindrical glass chambers, with a volume of 500 ml. The open bottom site of the plant chamber was fully sealed with airtight plastic foil (FEP, 4PTFE, Stuhr, Germany)

to prevent isotope fractionation. For $^{13}CO_2$ measurements of reference gas ($^{13}CO_{2_{RG}}$), an empty plant chamber of the same size, sealed with the same foil, was used. Inlets of all plant chambers were flushed continuously with fresh air at a volumetric flow rate of 500 ml min^{-1} for *Arabidopsis* experiments and 950 ml min^{-1} for *H. halimifolium* and *O. triangularis* experiments. Outlets were connected via Teflon tubing to the CRDS, thus determining $^{13}CO_{2_{SG}}$ or $^{13}CO_{2_{RG}}$. Before and after each $^{13}CO_{2_{SG}}$ measurement of about 40 to 60 min, $^{13}CO_{2_{RG}}$ was analyzed for about 10 to 20 min. $^{13}CO_{2_{RG}}$ concentrations (µmol * mol^{-1}) were interpolated with a generic regression equation (y = mx + b) for the period of $^{13}CO_{2_{SG}}$ measurements. Switching between $^{13}CO_{2_{SG}}$ and $^{13}CO_{2_{RG}}$ was done manually by re-plugging the CRDS Teflon tubing. $^{13}CO_2$ readings were discarded within the first minutes after switching between gases. To detect any drift in $^{13}CO_2$ measurements during the experiments, compressed air from tanks (4.4 ppm $^{13}CO_2$, Riessner-Gase, Lichtenfels, Germany) was analyzed two times per day for about 10-20 min, showing a total variation of SD ≤ 0.008 ppm in $^{13}CO_2$.

Tests with dilution series of ^{13}C-labelled malate (^{13}C-1, ^{13}C-4) in *H. halimifolium* showed that 2 mM malate solutions produced sufficient respired $^{13}CO_2$ without changing the total CO_2 respiration ($^{12}CO_2$ + $^{13}CO_2$), thus this concentration was applied for all further experiments. For pyruvate, 6 mM solutions of ^{13}C-1 pyruvate (Cambridge Isotope Laboratories, Tewksbury, MA, USA) and ^{13}C-2 pyruvate (Sigma-Aldrich, Buchs, Switzerland) yielded the best signals and were used in all experiments. Differences in molar strength by a factor of 3 for the labelling solutions were accounted for when computing the label-derived leaf $^{13}CO_2$ respiration and the label-induced ^{13}C enrichment

(see equations 1, 2). In general, no significant changes in total CO_2 respiration occurred after feeding plants of both species with any position-specific ^{13}C-labelled substrate, indicating that concentrations of malate and pyruvate solutions were in physiologically reasonable ranges. Air temperature within the plant chambers increased up to 29°C, while PPFD was about 720 µmol m^{-2} s^{-1} during our measurements.

Equation 1 was used to calculate label-derived leaf $^{13}CO_2$ respiration (R_{Label}), which was corrected for non-labelled leaf $^{13}CO_2$ respiration:

$$R_{Label} = \left(\frac{f\,(^{13}CO_{2_{SG}} - {}^{13}CO_{2_{RG}})}{a}\right) - R_{NL} \qquad \textbf{(Eqn 1)}$$

where *f* is the mass flow rate (µmol s^{-1}), *a* the leaf area (mm^{-2}), $^{13}CO_{2_{SG}}$ the sample gas, $^{13}CO_{2_{RG}}$ the reference gas measurement, and R_{NL} the non-labelled leaf $^{13}CO_2$ respiration.

For *Arabidopsis* experiments, three detached rosette leaves were immersed in a reaction vial with either ^{13}C-4 malate solution or deionized water to correct for non-labelled leaf $^{13}CO_2$ respiration. These reaction vials were placed in a plant chamber, which was subsequently sealed with airtight plastic foil. Thereafter, R_{Label} was measured for about 20 min in the light (to ensure uptake of the labelling solution via the transpiration stream) and 20 min in the dark by covering the plant chamber with a light impermeable cloth. Experiments with ^{13}C-4 labelled malate were repeated three to four times, whereas experiments with deionized water were measured once for each species.

For further experiments, one twig with leaves of *H. halimifolium* or three leaves of *O. triangularis* still attached to the potted plant were enclosed in the plant chambers.

The system was sealed with airtight plastic foil around the chamber and the protruding stem or leaf petiole. Respiration rates of twigs and leaves were measured in the first 10 to 20 min to correct for non-labelled leaf $^{13}CO_2$ respiration. Subsequently, twigs or leaves within the plant chamber were detached from the plant by cutting and immediately transferred into tap water and cut again under water to prevent air embolism in the xylem. Stems were then directly transferred into a reaction vial containing 1 ml position-specific ^{13}C-labelled malate or pyruvate solutions. All ^{13}C labelling solutions were refilled during the experiments to continuously provide ^{13}C-labelled substrates to the plant. Subsequently, R_{Label} was measured for about 20 min in the light and 20 min in the dark, as described above. Three to five replicates were measured with each position-specific ^{13}C-labelled substrate for each species. Leaf area of all leaves was determined after each experiment.

Isotopic analysis of leaf metabolic fractions

Similar to the above-described experiments regarding respiration, an additional experiment was carried out to determine the non-respiratory fate of position-specific ^{13}C-labelled malate and pyruvate. One twig with leaves of *H. halimifolium* or three leaves of *O. triangularis* were placed into reaction vials containing one of the four different position-specific ^{13}C-labelled substrates or deionized water to correct for non-labelling conditions. To trace the ^{13}C allocation in leaf metabolic fractions during light dark-transitions, leaves of both species were harvested after 20 min in the light, as well as after 40 min (20 min in the light and 20 min in the dark), using additional twigs/leaves of the same plants. Subsequently, leaves from both species were immediately frozen with liquid N_2, lyophilized, and milled to a fine powder with a ball mill. 100 mg of the plant

powder was solved in a 1.5 ml MCW (methanol, chloroform, water, 12:3:5, v:v:v) and boiled for 30 min at 70°C in a water bath to extract the water soluble fraction, as described in Richter *et al.* (method I2, 2009). Samples were centrifuged for 2 min at 10000 g, and 800 ml of the supernatant were transferred into a new reaction vial. After adding 250 µl chloroform, samples were shaken intensively and centrifuged for 2 min at 10000 g. For the isotopic analysis of lipids, aliquots of the lower chloroform phase were carefully taken to avoid contamination with the upper phase and transferred into tin capsules. In a next step, 1.2 ml of the upper phase was transferred into a new reaction vial, mixed roughly with 500 µl chloroform, and centrifuged 2 min at 10000 g. Finally, 1 ml of the upper phase, which contained the total hydrophilic fraction of the extract, was transferred into a new reaction vial and stored at -20°C.

The hydrophilic fraction was further separated into sugar, organic acid, and amino acid fractions with ion-exchange chromatography, using Dowex material as described above. Eluates of all three fractions were frozen at -20°C, lyophilized, and the pellets re-suspended in deionized water. Subsequently, aliquots of the fractions were transferred into tin capsules. All capsules (including lipids) were oven-dried at 60°C and $\delta^{13}C$ values analyzed with an EA-IRMS (Thermo Fisher Scientific, Bremen, Germany). Measurements and referencing for the IRMS were done after Werner et al. (1999) and Werner & Brand (2001). The IRMS long-term precision of quality control standards was SD ≤ 0.12‰. Three replicates were measured with each position-specific ^{13}C-labelled substrate and deionized water for each species.

Equation 2 was used to calculate the label-induced ^{13}C enrichment (Δ_{Label}) in leaf metabolic fractions, which was corrected for $\delta^{13}C$ values under non-labelling conditions:

$$\Delta_{Label} = \delta^{13}C_L - \delta^{13}C_{NL} \qquad \textbf{(Eqn 2)}$$

where $\delta^{13}C_L$ are the $\delta^{13}C$ values in leaf metabolic fractions (lipids, sugars, amino and organic acids) of plants treated with position-specific ^{13}C-labelled substrates, and $\delta^{13}C_{NL}$ is the mean $\delta^{13}C$ value of the corresponding leaf metabolic fraction under non-labelling conditions.

Statistics

One-way analysis of variance (ANOVA) and Tukey-HSD post-hoc tests were performed to test for differences in sums of R_{Label} and in Δ_{Label} among position-specific ^{13}C-labelled substrates for *H. halimifolium* and *O. triangularis*. Welch's *t*-test was used to determined species-specific differences in sums of R_{Label} in *Arabidopsis* lines. Unless otherwise specified, means and standard errors are given. All statistical analyses were performed in R (version 3.1.1; R Core Team, 2013)

Results

Leaf $^{13}CO_2$ respiration derived from ^{13}C-4 malate in Arabidopsis

In order to elucidate if the NAD-malic enzyme reaction (NAD-ME) determines increases in $\delta^{13}C_{LEDR}$ by releasing the C-4 position of malate as CO_2, we analyzed the label-derived leaf $^{13}CO_2$ respiration (R_{Label}) derived from ^{13}C-4 malate in *A. thaliana* wild-type and NAD-ME mutant plants. R_{Label} in the *Arabidopsis* plants was very low in the light, while a clear peak was observed within the first 3 min after darkening during LEDR, which was followed by a continuous decrease (Fig. 1). However, no significant differences between the *Arabidopsis* plants were found for sums of R_{Label} in the light ($t = 1.22$, $p = 0.289$, *t*-

test) nor in the dark (t = 2.4, p = 0.125, *t*-test), although R_{Label} tended to lower values in NAD-ME mutants during darkness. Similarly, no differences were observed between *Arabidopsis* lines for non-labelled leaf $^{13}CO_2$ respiration (data not shown), verified with in-tube incubation measurements of $\delta^{13}C_{LEDR}$ at natural isotope abundances (Fig. S1). Overall, we found no significant differences during light-dark transitions neither in *Arabidopsis* lines treated with ^{13}C-4 malate nor at natural isotope abundances.

Leaf $^{13}CO_2$ respiration of different position-specific ^{13}C-labelled substrates and species

Previous studies showed clear species-specific differences in $\delta^{13}C_{LEDR}$ at natural isotope abundances, with high increases in *H. halimifolium* and low increases in *O.* triangularis (Priault *et al.*, 2009; Wegener *et al.*, 2010). Thus, we treated those plants with different position-specific ^{13}C-labelled malate and pyruvate with the expectation to observe species-specific differences in R_{Label} during light-dark transitions and to identify substrates and mechanisms imprinting on $\delta^{13}C_{LEDR}$.

R_{Label} from all ^{13}C-labelled substrates was generally lower in the light than in the dark in both species (Figs. 2, 3; Tab. 1). R_{Label} from ^{13}C-1 and ^{13}C-4 malate in *H. halimifolium* was low in the light, but steeply increased shortly after darkening during LEDR, with a peak of about 0.02 µmol $^{13}CO_2$ m^{-2} s^{-1} (Fig. 2), showing unexpectedly no significant difference between the malate treatments in sums of R_{Label} in the light and in the dark (Tab. 1). Similar results were found in *O. triangularis* plants under both malate treatments (Fig. 3; Tab. 1). Thus, respiration based on CO_2 release by an isocitrate dehydrogenase reaction (IDH) and/or by mtPDH in combination with a preceding NAD-ME (^{13}C-1 malate) did not differ significantly from respiration based on the activity of the 2-oxoglutarate

dehydrogenase reaction (OGDH) and/or NAD-ME (^{13}C-4 malate) during light-dark transitions in both species.

In contrast to ^{13}C-labelled malate substrates, R_{Label} from ^{13}C-1 pyruvate reflects directly the CO_2 release by a PDH reaction. R_{Label} from ^{13}C-1 pyruvate was higher compared to all other treatments in *H. halimifolium* during light-dark transitions (Fig. 2), with high R_{Label} values of about 0.025 µmol $^{13}CO_2$ m^{-2} s^{-1} in the light, and a peak of 0.06 µmol $^{13}CO_2$ m^{-2} s^{-1} in the dark during LEDR, which was followed by a clear continuous decrease. Similarly, sums of R_{Label} from ^{13}C-1 pyruvate were highest compared to all other position-specific ^{13}C-labelled substrates in the light and in the dark in *H. halimifolium* (Tab. 1). A very different pattern for R_{Label} from ^{13}C-1 pyruvate was found in *O. triangularis*, with no clear peak during LEDR (Fig. 3) and no differences in sums of R_{Label} among position-specific ^{13}C-labelled substrates in the light and in the dark (Tab. 1). Plants of both species fed with ^{13}C-2 pyruvate, reflecting the activity of CO_2 releasing reactions within the TCA cycle, showed hardly any increase in R_{Label} (Figs. 2, 3) and the lowest sums of R_{Label} in the dark (Tab. 1). In summary, clear species-specific differences during light-dark transitions were only observed in R_{Label} derived from ^{13}C-1 pyruvate.

^{13}C allocation into leaf metabolic fractions

Carbon isotope ratios in leaf metabolic fractions (lipids, sugars, amino and organic acids) of *H. halimifolium* and *O. triangularis* plants did not show significant light-dark differences between the two points in time (20 min light or 20 min light and 20 min dark) and were thus pooled for further analysis, i.e., to identify the non-respiratory fate of position-specific ^{13}C-labelled substrates. However, one exception occurred: *O.*

triangularis plants fed with ^{13}C-2 pyruvate showed a strong ^{13}C enrichment in amino (p-value < 0.01; ANOVA) and organic acids (p-value < 0.05; ANOVA) in the dark compared to values in the light (data not shown).

Generally, the ^{13}C label was mainly allocated to amino and organic acids rather than to lipids or sugars irrespective of substrate and species (Fig. 4). The label-induced enrichment (Δ_{Label}) of amino acids was high compared to all other metabolic fractions in both species, with highest values in *H. halimifolium* fed with ^{13}C-labelled pyruvate solutions. No significant differences for Δ_{Label} of amino acids among position-specific ^{13}C-labelled substrates were observed in *O. triangularis*. Δ_{Label} of organic acids in both species was more enriched in ^{13}C when plants were fed with any ^{13}C-labelled malate than with ^{13}C-labelled pyruvate, except for *H. halimifolium* treated with ^{13}C-2 pyruvate. Moreover, plants of both species treated with ^{13}C-labelled pyruvate substrates had less ^{13}C incorporated into lipids and sugars than plants treated with ^{13}C-labelled malate substrates, except for Δ_{Label} of sugars in *O. triangularis*, where no distinct ^{13}C incorporation was found. In summary, ^{13}C label was mainly allocated from the malate treatments to organic acids, while ^{13}C label from the pyruvate treatments was incorporated into amino acids.

Discussion

Leaf $^{13}CO_2$ respiration in the dark is predominantly determined by mtPDH

Transferring light acclimated leaves into darkness causes a transformation of various biochemical processes, inhibiting photosynthesis, increasing respiration. This reorganization of plant metabolism can take about half an hour (Florez-Sarasa *et al.*,

2012; Griffin & Turnbull, 2012), and transitionally leading to "light enhance dark respiration" (LEDR) and an associated increase in the natural carbon isotopic composition of the leaf-respired CO_2 ($\delta^{13}C_{LEDR}$). Based on this observation, previous studies suggested that the decarboxylation of a potentially ^{13}C enriched C-4 position of malate to pyruvate and CO_2 by the NAD-malic enzyme reaction (NAD-ME) mainly determines increases in $\delta^{13}C_{LEDR}$ (Barbour *et al.*, 2007; Gessler *et al.*, 2009; Werner *et al.*, 2011), but our *Arabidopsis* experiments contrast with these findings. Against the expectation, that the *Arabidopsis* NAD-ME mutants should show lower ^{13}C-4 malate respiration in comparison to wild type plants, we found no significant differences between the *Arabidopsis* lines, neither after the ^{13}C-4 malate treatment (Fig. 1), nor at natural isotope abundances in $\delta^{13}C_{LEDR}$ (Fig. S1). Thus, this indicates a minor influence of NAD-ME on increases in $\delta^{13}C_{LEDR}$. In addition, R_{Label} in both *Arabidopsi*s lines followed the typical course of $\delta^{13}C_{LEDR}$, with an initial peak followed by steady decrease (Werner *et al.*, 2009). This shows that parts of the ^{13}C-4 labelled malate in the NAD-ME mutant are released by other reactions than by NAD-ME during LEDR, suggesting that the NAD-malic enzyme reaction does not exclusively determine increases in $\delta^{13}C_{LEDR}$.

Furthermore, our results suggest that the mitochondrial pyruvate dehydrogenase reaction (mtPDH) mainly determines increases in $\delta^{13}C_{LEDR}$ by breaking down pyruvate to acetyl-CoA and CO_2 rather than NAD-ME. This was shown by clear species-specific differences in R_{Label} from ^{13}C-1 pyruvate in *H. halimifolium* and *O. triangularis* during light-dark transitions, which were not observed for other substrates (Figs. 2, 3, Tab. 1). The amount of CO_2 released from ^{13}C-1 pyruvate was clearly higher during LEDR compared to any other position-specific ^{13}C-labelled substrate in *H. halimifolium,* the species with

high natural increases in $\delta^{13}C_{LEDR}$ (up to 14.8‰; Wegener *et al.*, 2010). In contrast, no differences between respiration of different substrates and only small increase in R_{Label} derived from ^{13}C-1 pyruvate were observed in *O. triangularis,* the species which did exhibit low natural increases in $\delta^{13}C_{LEDR}$ (up to 3.4 ‰; Wegener *et al.*, 2010). This clearly demonstrates a biochemical link between respiration of the C-1 position of pyruvate and the known high increase in $\delta^{13}C_{LEDR}$ in *H. halimifolium*. It also suggests high mtPDH activity during LEDR in these plants and shows that species can periodically differ in the use of respiratory substrates.

Nevertheless, both ^{13}C-1 and ^{13}C-4 malate were respired during LEDR in *H. halimifolium* and *O. triangularis* (Figs. 2, 3; Tab. 1), demonstrating that both carboxyl groups of malate contribute to respiration (Figs. 2, 3). Interestingly, the pattern of R_{Label} from ^{13}C-1 and ^{13}C-4 malate was very similar in both species, which was unexpected since the C-4 position of malate was expected to be of higher importance for dark respiration via NAD-ME (Barbour *et al.*, 2007; Gessler *et al.*, 2009; Werner *et al.*, 2011). An explanation for this observation is most likely strongly connected to the fumarase reaction, causing a partial isotope randomization of the carboxyl groups of malate (Gout *et al.*, 1993). Therefore, ^{13}C-1 malate could also be produced from ^{13}C-4 malate and *vice versa*. Subsequently, ^{13}C-1 malate derived from ^{13}C-4 malate might be decarboxylated by NAD-ME, producing ^{13}C-1 labelled pyruvate, which fuels mtPDH (Fig. 5). This pathway could also be a mechanistic explanation for R_{Label} derived from ^{13}C-4 malate in NAD-ME mutants during LEDR (Fig. 1). Moreover, if randomization of malate by the fumarase reaction is assumed, ^{13}C label from both carboxyl groups of malate could be indirectly respired in the TCA cycle by the mitochondrial isocitrate dehydrogenase reaction

(mtIDH), as well as by OGDH (Fig. 5), which would otherwise only release one carboxyl group of malate (C-1 by mtIDH and C-4 by OGDH).

Additionally, ^{13}C-2 pyruvate was only weakly respired in both species (Figs. 2, 3; Tab. 1), showing that the C-2 position of pyruvate (reflecting the acetyl-CoA residue) is predominantly used for biosynthesis of compounds during light-dark transitions such as the production of amino acids rather than for respiration within the TCA cycle (Fig. 5). The results are consistent with those of Priault *et al.* (2009), showing larger differences in $\delta^{13}C$ of leaf-respired CO_2 between ^{13}C-1 pyruvate and ^{13}C-2 pyruvate treated plants for *H. halimifolium* than for *O. triangularis*. In brief, our findings strongly suggest that mtPDH has a predominant role during LEDR, using pyruvate from glycolysis or from malate via preceding enzymatic reactions (NAD-ME and fumarase) as respiratory substrates.

Leaf $^{13}CO_2$ respiration in the light is mainly determined by cpPDH and ^{13}C-1 pyruvate

Generally, leaf respiration is known to be strongly inhibited in the light due to inactivation of respiratory key enzymes, blocking the unidirectional cycling of the TCA cycle (Hanning & Heldt, 1993; Tcherkez *et al.*, 2005; Sweetlove *et al.*, 2010; Araújo *et al.*, 2012). Nevertheless, we observed high R_{Label} from ^{13}C-1 pyruvate in *H. halimifolium* in the light, which was much higher compared to R_{Label} from all other ^{13}C-labelled substrates (Fig. 2; Tab. 1), and mirrored in *O. triangularis* plants, although at a much lower level (Fig. 3; Tab. 1). While this increase in R_{Label} from ^{13}C-1 pyruvate can be directly derived from mitochondrial or chloroplast PDH (cpPDH; Tovar-Mendez *et al.*, 2003), the fact that mtPDH is at least partially inhibited under light conditions (Budde & Randall, 1990) suggests that ^{13}C-1 pyruvate is probably primarily respired by cpPDH in

H. halimifolium. Note, hardly any ^{13}C-2 pyruvate was respired under light conditions in both species, supporting earlier results (Tcherkez *et al.*, 2005; Priault *et al.*, 2009; Werner *et al.*, 2009; Wegener *et al.*, 2010).

Furthermore, we found low increases in R_{Label} from ^{13}C-1 and ^{13}C-4 malate in *H. halimifolium* and *O. triangularis* (Figs. 2, 3), most probably due to the cytosolic isocitrate dehydrogenase reaction (cyIDH, Fig. 5), at least for plants of the ^{13}C-1 malate treatments. Malate can be converted into oxaloacetate by the malate dehydrogenase reaction, which is formed in combination with glycolytic acetyl-CoA to citrate by the citrate synthase reaction. This citrate can then be released to the cytosol via the citrate shuttle, where it is converted into isocitrate and CO_2 is produced by cyIDH (Werner *et al.*, 2011). On the other hand, no mechanism is known for direct respiration of ^{13}C-4 malate in the light, since NAD-ME and TCA cycle reactions are known to be inhibited (Hill & Bryce, 1992; Igamberdiev *et al.*, 2001; Tcherkez *et al.*, 2005; Lee *et al.*, 2010; Araújo *et al.*, 2012). However, our results suggest that R_{Label} from ^{13}C-4 malate originates from the C-1 position of malate by cyIDH in the light, due to a preceding randomization of malate by the fumarase reaction (Fig. 5). In summary, our findings suggest that label-derived leaf $^{13}CO_2$ respiration in the light is mainly determined by the C-1 position of pyruvate and by cpPDH (Fig. 5).

Differences in the non-respiratory fate of malate and pyruvate

Our final objective was to determine the non-respiratory fate of position-specific ^{13}C-labelled substrates. We observed a label-induced ^{13}C enrichment (Δ_{Label}) in leaf metabolic fractions (lipids, sugars, amino and organic acids) primarily under light

conditions, while an additional 20 min period in the dark did not cause further ^{13}C enrichments. These observations showed that biosynthetic processes were clearly down-regulated during LEDR, and indicate that position-specific ^{13}C-labelled malate and pyruvate were instead used for respiratory processes (Figs. 2, 3). On the one hand, ^{13}C-labelled pyruvate substrates were mainly incorporated into amino acids, especially in *H. halimifolium* (Fig. 4), suggesting that pyruvate is used as an important precursor for the biosynthesis of different amino acids in both species (Fig. 5), most likely for amino acids derived from pyruvate and 2-oxoglutarate. On the other hand, ^{13}C-labelled malate substrates were incorporated into organic acids in both species, indicating that malate could be used for replenishing TCA cycle intermediates and for building up carbon storage in the form of citrate or malate (Fig. 5; Hanning & Heldt, 1993; Sweetlove *et al.*, 2010; Werner *et al.*, 2011). Nevertheless, ^{13}C label derived from malate substrates were as well allocated towards amino acids, especially in O. triangularis, most likely for biosynthesis of amino acids derived from oxaloacetate. Δ_{Label} values in lipids for both species indicated that only small amounts of the position-specific ^{13}C-labelled malate and pyruvate were used for lipogenesis (Figs. 4, 5), similar to results of a recent ^{14}C labelling study (Grimberg, 2014). Low Δ_{Label} values in sugars in *H. halimifolium* might be explained by higher mean photosynthetic rates (5.7 µmol CO_2 m^{-2} s^{-1}) in comparison to those in *O. triangularis* (4.6 µmol CO_2 m^{-2} s^{-1}). Overall, our results demonstrate that particularly in the light pyruvate is used for the biosynthesis of amino acids, while malate is used for the production of organic acids.

Conclusions

Here we studied the respiratory and the non-respiratory fate of two key biochemical compounds during the distinctive light-dark transition period in different plant species. Based on our experiments with position-specific ^{13}C-labelled malate or pyruvate, we rejected our initial hypothesis that mainly NAD-malic enzyme and a potentially ^{13}C-enriched C-4 position of malate determine the extraordinary high increases in the natural carbon isotopic composition of leaf-respired CO_2 during "light enhanced dark respiration". Instead, we postulate that the mechanism is more complex, probably due a predominant mitochondrial pyruvate dehydrogenase reaction, using pyruvate from glycolysis and from malate via several assisting enzymatic reactions (NAD-ME, fumarase). Furthermore, the use of pyruvate and malate as respiratory substrates in the dark and as biosynthetic precursors in the previous light period reflects rapid changes in biochemical processes during light-dark transitions and emphasizes the pivotal importance of the substrates. Finally, the observed species-specific differences in respiration derived from specific positions of malate and pyruvate show the enormous potential of position-specific labelling techniques to disentangle differences in metabolic pathways. Since amino acids may also act as respiratory substrates, experiments with different position-specific ^{13}C-labelled amino acids may provide further insights into the fast and complex biochemical mechanisms appearing during light-dark transitions.

Acknowledgements

We gratefully thank Barbara E. Kornexl (ETH Zurich) for experimental advice. Technical assistance was provided by Annika Ackermann, Noemi Umbricht, and EA Burns (all ETH

Zurich), as well as by Ilse Thaufelder (University of Bayreuth). This study was financially supported by the Swiss National Science Foundation (SNF project "CIFRes", contract number 205321_132768) and by the German Research Foundation (DFG project "ECORES", contract number WE2681/5-1). MML also acknowledges a grant by COST Action ES0806 SIBAE for a short-term scientific mission.

References

Araújo WL, Nunes-Nesi A, Nikoloski Z, Sweetlove LJ, Fernie AR. 2012. Metabolic control and regulation of the tricarboxylic acid cycle in photosynthetic and heterotrophic plant tissues. *Plant, Cell & Environment* **35**: 1-21.

Atkin OK, Millar AH, Gardestroem P, Day DA 2000. Photosynthesis, carbohydrate metabolism and respiration in leaves of higher plants. In: Leegood RC, Sharkey TD, Von Caemmerer S eds. *Photosynthesis and Metabolism.* Dordrecht, the Netherlands: Kluwer Academic, pp. 153-175.

Atkin OK, Evans JR, Siebke K. 1998. Relationship between the inhibition of leaf respiration by light and enhancement of leaf dark respiration following light treatment. *Australian Journal of Plant Physiology* **25**: 437-443.

Barbour MM, Hunt JE, Kodama N, Laubach J, McSeveny TM, Rogers GN, Tcherkez G, Wingate L. 2011. Rapid changes in $\delta^{13}C$ of ecosystem-respired CO_2 after sunset are consistent with transient ^{13}C enrichment of leaf respired CO_2. *New Phytologist* **190**: 990-1002.

Barbour MM, McDowell NG, Tcherkez G, Bickford CP, Hanson DT. 2007. A new measurement technique reveals rapid post-illumination changes in the carbon isotope composition of leaf-respired CO_2. *Plant, Cell and Environment* **30**: 469-482.

Budde RJA, Randall DD. 1990. Pea leaf mitochondrial pyruvate dehydrogenase complex is inactivated *in vivo* in a light-dependent manner. *Proceedings of the National Academy of Sciences, USA* **87**: 673-676.

Farquhar GD, Ehleringer JR, Hubick KT. 1989. Carbon isotope discrimination and photosynthesis. *Annual Review of Plant Physiology and Plant Molecular Biology* **40**: 503-537.

Florez-Sarasa I, Araújo WL, Wallstrom SV, Rasmusson AG, Fernie AR, Ribas-Carbo M. 2012. Light-responsive metabolite and transcript levels are maintained following a dark-adaptation period in leaves of *Arabidopsis thaliana*. *New Phytologist* **195**: 136-148.

Gessler A, Tcherkez G, Karyanto O, Keitel C, Ferrio JP, Ghashghaie J, Kreuzwieser J, Farquhar GD. 2009. On the metabolic origin of the carbon isotope composition of CO_2 evolved from darkened light-acclimated leaves in *Ricinus communis*. *New Phytologist* **181**: 374-386.

Ghashghaie J, Badeck FW. 2014. Opposite carbon isotope discrimination during dark respiration in leaves versus roots - a review. *New Phytologist* **201**: 751-769.

Gilbert A, Robins RJ, Remaud GS, Tcherkez GGB. 2012. Intramolecular ^{13}C pattern in hexoses from autotrophic and heterotrophic C_3 plant tissues. *Proceedings of the National Academy of Sciences of the United States of America* **109**: 18204-18209.

Gleixner G, Schmidt H-L. 1997. Carbon isotope effects on the fructose-1,6-bisphosphate aldolase reaction, origin for non-statistical ^{13}C distributions in carbohydrates. *Journal of Biological Chemistry* **272**: 5382-5387.

Gout E, Bligny R, Pascal N, Douce R. 1993. ^{13}C nuclear magnetic resonance studies of malate and citrate synthesis and compartmentation in higher plant cells. *The Journal of Biological Chemistry* **268**: 3986-3992.

Griffin KL, Turnbull MH. 2012. Out of the light and into the dark: post-illumination respiratory metabolism. *New Phytologist* **195**: 4-7.

Grimberg Å. 2014. Preferred carbon precursors for lipid labelling in the heterotrophic endosperm of developing oat (*Avena sativa* L.) grains. *Plant Physiology and Biochemistry* **83**: 346-355.

Hanning I, Heldt HW. 1993. On the function of mitochondrial metabolism during photosynthesis in spinach (*Spinacia-Oleracea* L.) leaves - Partitioning between respiration and export of redox equivalents and precursors for nitrate assimilation products. *Plant Physiology* **103**: 1147-1154.

Hill SA, Bryce JH 1992. Malate metabolism and light-enhanced dark respiration in barley mesophyll protoplasts. In: Lambers H, Van der Plas LHW eds. *Molecular, Biochemical and Physiological Aspects of Plant Respiration*: SPB Academic Publishing, The Hague, The Netherlands, pp. 221-230.

Hymus GJ, Maseyk K, Valentini R, Yakir D. 2005. Large daily variation in ^{13}C-enrichment of leaf-respired CO_2 in two *Quercus* forest canopies. *New Phytologist* **167**: 377-384.

Igamberdiev AU, Romanowska E, Gardeström P. 2001. Photorespiratory flux and mitochondrial contribution to energy and redox balance of barley leaf protoplasts in the light and during light-dark transitions. *Journal of Plant Physiology* **158**: 1325-1332.

Jardine K, Wegener F, Abrell L, van Haren J, Werner C. 2014. Phytogenic biosynthesis and emission of methyl acetate. *Plant, Cell and Environment* **37**: 414-424.

Lee CP, Eubel H, Millar AH. 2010. Diurnal changes in mitochondrial function reveal daily optimization of light and dark respiratory metabolism in *Arabidopsis*. *Molecular & Cellular Proteomics* **9**: 2125-2139.

Melzer E, O'Leary MH. 1991. Aspartic-acid synthesis in C_3 plants. *Planta* **185**: 368-371.

Melzer E, O'Leary MH. 1987. Anapleurotic CO_2 fixation by phosphoenolpyruvate carboxylase in C_3 plants. *Plant Physiology* **84**: 58-60.

Plaxton WC, Podesta FE. 2006. The functional organization and control of plant respiration. *Critical Reviews in Plant Sciences* **25**: 159-198.

Prater JL, Mortazavi B, Chanton JP. 2006. Diurnal variation of the $\delta^{13}C$ of pine needle respired CO_2 evolved in darkness. *Plant, Cell and Environment* **29**: 202-211.

Priault P, Wegener F, Werner C. 2009. Pronounced differences in diurnal variation of carbon isotope composition of leaf respired CO_2 among functional groups. *New Phytologist* **181**: 400-412.

R Core Team. 2013. R: A language and environment for statistical computing. *R foundation for statistical computing, Vienna, Austria*.

Richter A, Wanek W, Werner RA, Ghashghaie J, Jaeggi M, Gessler A, Brugnoli E, Hettmann E, Gottlicher SG, Salmon Y, et al. 2009. Preparation of starch and soluble sugars of plant material for the analysis of carbon isotope composition: a comparison of methods. *Rapid Communications in Mass Spectrometry* **23**: 2476-2488.

Rosenberg RM, O`Leary MH. 1985. Aspartate beta-decarboxylase from *Alcaligenes faecalis*: carbon-13 kinetic isotope effect and deuterium exchange experiments. *Biochemistry* **24**: 1598-1603.

Rossmann A, Butzenlechner M, Schmidt H-L. 1991. Evidence for a nonstatistical carbon isotope distribution in natural glucose. *Plant Physiology* **96**: 609-614.

Savidge WB, Blair NE. 2004. Patterns of intramolecular carbon isotopic heterogeneity within amino acids of autotrophs and heterotrophs. *Oecologia* **139**: 178-189.

Sweetlove LJ, Beard KFM, Nunes-Nesi A, Fernie AR, Ratcliffe RG. 2010. Not just a circle: flux modes in the plant TCA cycle. *Trends in Plant Science* **15**: 462-470.

Tcherkez G, Cornic G, Bligny R, Gout E, Ghashghaie J. 2005. In vivo respiratory metabolism of illuminated leaves. *Plant Physiology* **138**: 1596-1606.

Tcherkez G, Farquhar G, Badeck F, Ghashghaie J. 2004. Theoretical considerations about carbon isotope distribution in glucose of C_3 plants. *Functional Plant Biology* **31**: 857-877.

Tovar-Mendez A, Miernyk JA, Randall DD. 2003. Regulation of pyruvate dehydrogenase complex activity in plant cells. *European Journal of Biochemistry* **270**: 1043-1049.

Tronconi MA, Fahnenstich H, Weehler MCG, Andreo CS, Flugge UI, Drincovich MF, Maurino VG. 2008. *Arabidopsis* NAD-malic enzyme functions as a homodimer and heterodimer and has a major impact on nocturnal metabolism. *Plant Physiology* **146**: 1540-1552.

Wegener F, Beyschlag W, Werner C. 2010. The magnitude of diurnal variation in carbon isotopic composition of leaf dark respired CO_2 correlates with the difference between $\delta^{13}C$ of leaf and root material. *Functional Plant Biology* **37**: 849-858.

Werner C, Gessler A. 2011. Diel variations in the carbon isotope composition of respired CO_2 and associated carbon sources: a review of dynamics and mechanisms. *Biogeosciences* **8**: 2437-2459.

Werner C, Wegener F, Unger S, Nogues S, Priault P. 2009. Short-term dynamics of isotopic composition of leaf-respired CO_2 upon darkening: measurements and implications. *Rapid Communications in Mass Spectrometry* **23**: 2428-2438.

Werner RA, Buchmann N, Siegwolf RTW, Kornexl BE, Gessler A. 2011. Metabolic fluxes, carbon isotope fractionation and respiration - lessons to be learned from plant biochemistry. *New Phytologist* **191**: 10-15.

Werner RA, Brand WA. 2001. Referencing strategies and techniques in stable isotope ratio analysis. *Rapid Communications in Mass Spectrometry* **15**: 501-519.

Werner RA, Bruch BA, Brand WA. 1999. ConFlo III - an interface for high precision $\delta^{13}C$ and $\delta^{15}N$ analysis with an extended dynamic range. *Rapid Communications in Mass Spectrometry* **13**: 1237-1241.

Xue XP, Gauthier DA, Turpin DH, Weger HG. 1996. Interactions between photosynthesis and respiration in the green alga *Chlamydomonas reinhardtii* - Characterization of light-enhanced dark respiration. *Plant Physiology* **112**: 1005-1014.

Figures & Tables

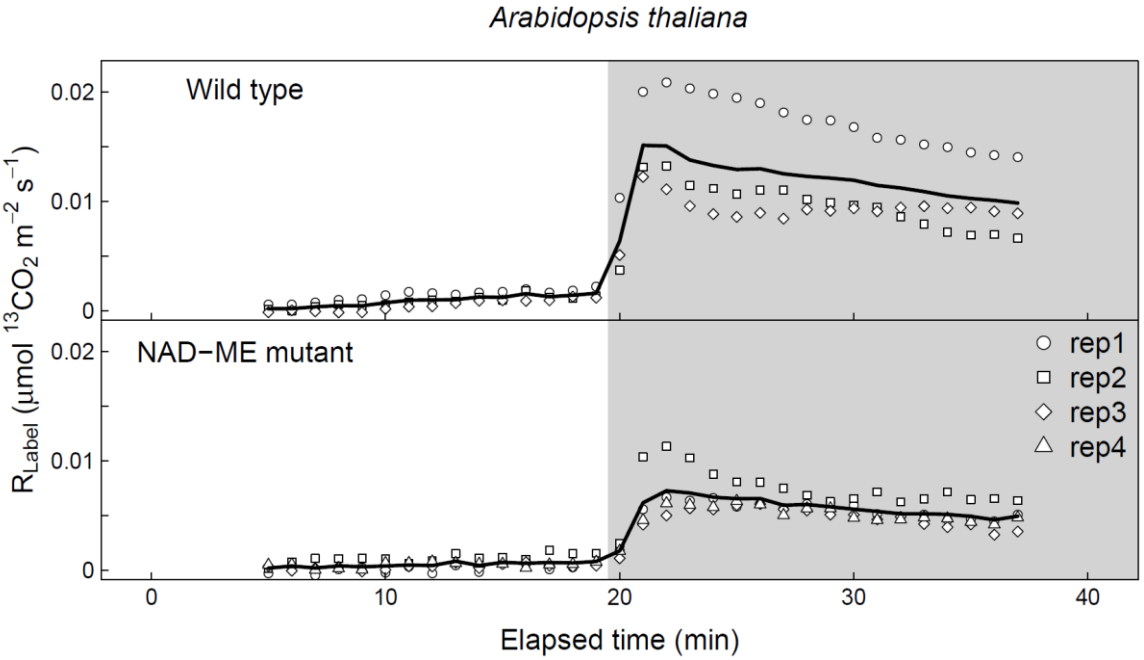

Figure 1: Label-derived leaf $^{13}CO_2$ respiration (R_{Label}, µmol $^{13}CO_2$ m^{-2} s^{-1}) during light-dark transitions in *Arabidopsis thaliana*. Rosette leaves of wild type and NAD-ME mutant (*nadme1x2*; double mutant of NAD depending mitochondrial malic enzyme) plants were fed with ^{13}C-4 malate for about 40 min (20 min in the light and 20 min in the dark). Grey areas denote dark periods. Black bold lines denote mean values, while symbols indicate single replicates (n = 3-4 individuals).

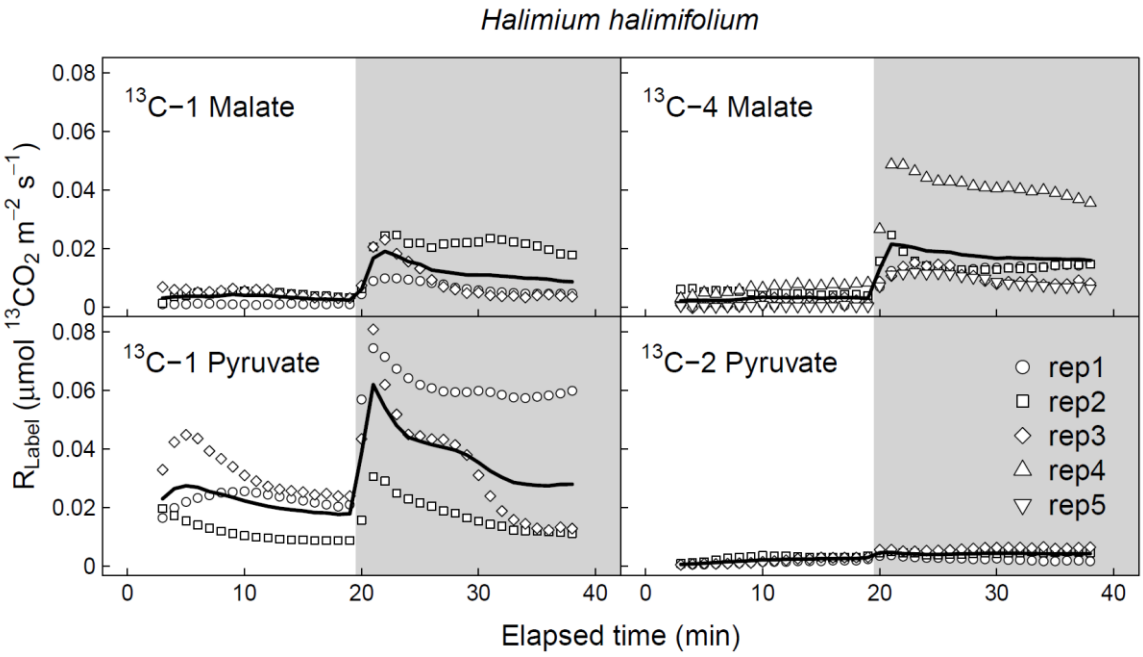

Figure 2: Label-derived leaf $^{13}CO_2$ respiration (R_{Label}, µmol $^{13}CO_2$ m^{-2} s^{-1}) during light-dark transitions in *Halimium halimifolium*. Twigs with leaves were fed with position-specific ^{13}C-labelled malate (^{13}C-1, ^{13}C-4) or pyruvate (^{13}C-1, ^{13}C-2) for about 40 min (20 min in the light and 20 min in the dark). Grey areas denote dark periods. Black bold lines denote mean values, while symbols indicate single replicates (n = 3-5 individuals).

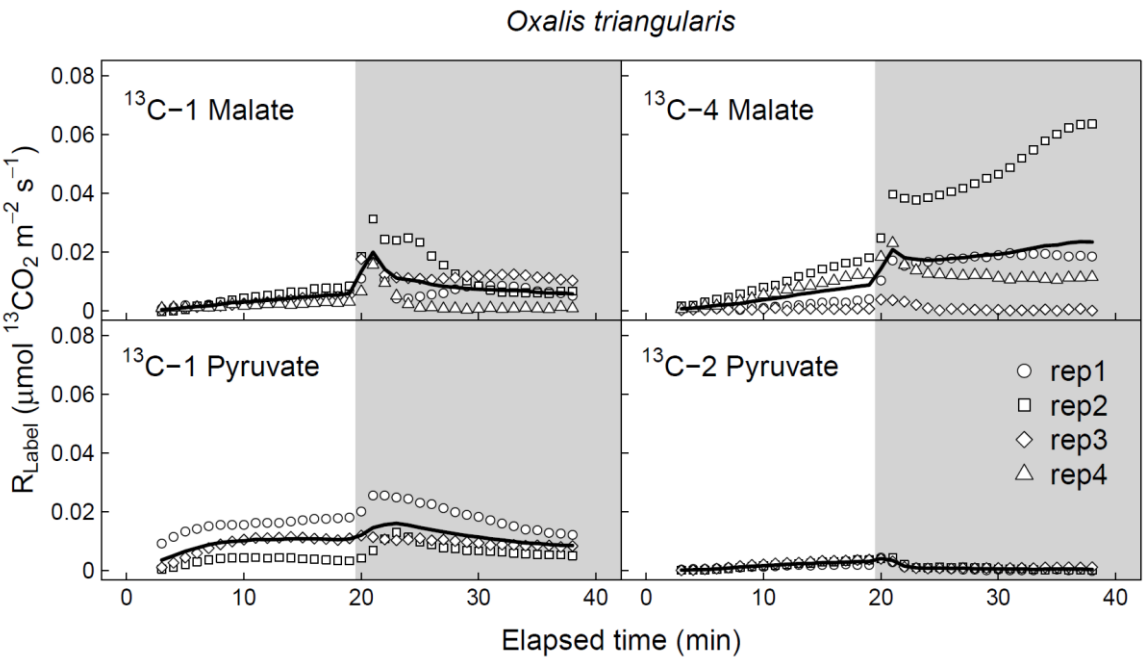

Figure 3: Label-derived leaf $^{13}CO_2$ respiration (R_{Label}, µmol $^{13}CO_2$ m^{-2} s^{-1}) during light-dark transitions in *Oxalis triangularis*. Leaves were fed with position-specific ^{13}C-labelled malate (^{13}C-1, ^{13}C-4) or pyruvate (^{13}C-1, ^{13}C-2) for about 40 min (20 min in the light and 20 min in the dark). Grey areas denote dark periods. Black bold lines denote mean values, while symbols indicate single replicates (n = 3-4 individuals).

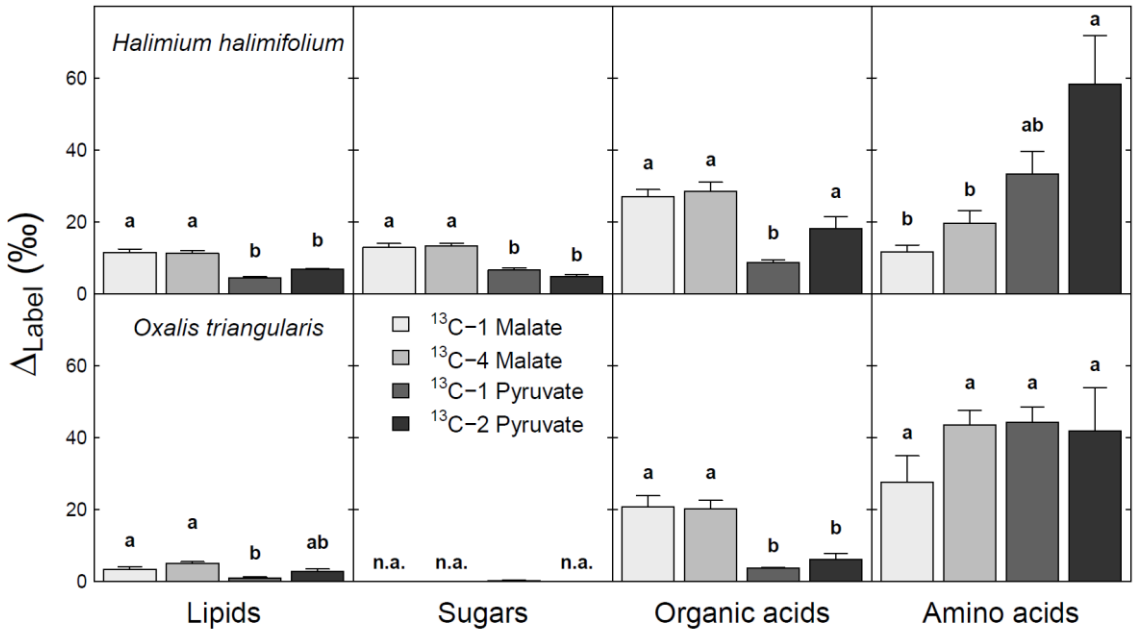

Figure 4: The non-respiratory fate of position-specific ^{13}C-labelled malate (^{13}C-1, ^{13}C-4) and pyruvate (^{13}C-1, ^{13}C-2) in *H. halimifolium* and *Oxalis triangularis*. The label-induced ^{13}C enrichment corrected for non-labelling conditions (Δ_{Label}, ‰) indicates the amount of total ^{13}C incorporated into leaf metabolic fractions. Data from the two harvests (20 min light or 20 min light and 20 min dark after labelling) were pooled for each ^{13}C-labelled substrate in both species, since no significant light-dark differences were observed for Δ_{Label} among leaf metabolic fractions and treatments in both species (except for *O. triangularis* plants fed with ^{13}C-2 pyruvate which showed higher $\delta^{13}C$ values in the dark than in the light in organic and amino acids). n.a. indicates no observable Δ_{Label}. Small letters indicate significant differences between ^{13}C-labelled substrates (ANOVA and Tukey-HSD). Means and SE are given (n = 5-6 individuals).

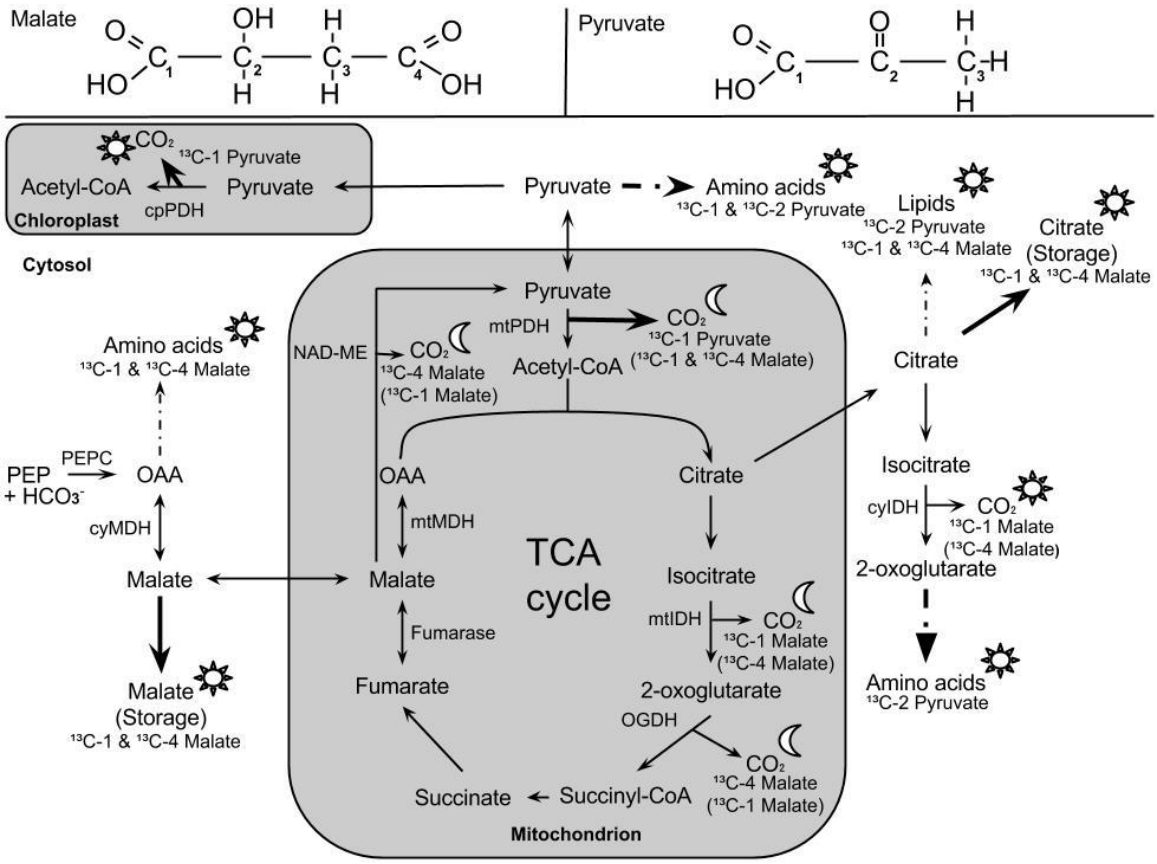

Figure 5: Overview of ^{13}C allocation from position-specific ^{13}C-labelled malate (^{13}C-1, ^{13}C-4) and pyruvate (^{13}C-1; ^{13}C-2) during light-dark transitions. Sun (☼) and half-moon (☾) symbols denote temporary ^{13}C allocation during light or dark periods (LEDR), respectively. Solid arrows indicate ^{13}C allocation of highest importance, while dashed arrows indicate reactions with intermediate steps. ^{13}C-labelled malate substrates in brackets denote alternative respiratory pathways due to the influence of NAD-ME (decarboxylation of C-4 position of malate) and fumarase (randomization of C-1 and C-4 position of malate). The following abbreviations are used: cyIDH and mtIDH, cytosolic and mitochondrial isocitrate dehydrogenase; cyMDH and mtMDH, cytosolic and mitochondrial malate dehydrogenase; NAD-ME, NAD-malic enzyme; OAA, oxaloacetate; OGDH, 2-oxoglutarate dehydrogenase; cpPDH and mtPDH, chloroplast and mitochondrial pyruvate dehydrogenase; PEPC, phosphoenolpyruvate carboxylase; TCA cycle, tricarboxylic acid cycle.

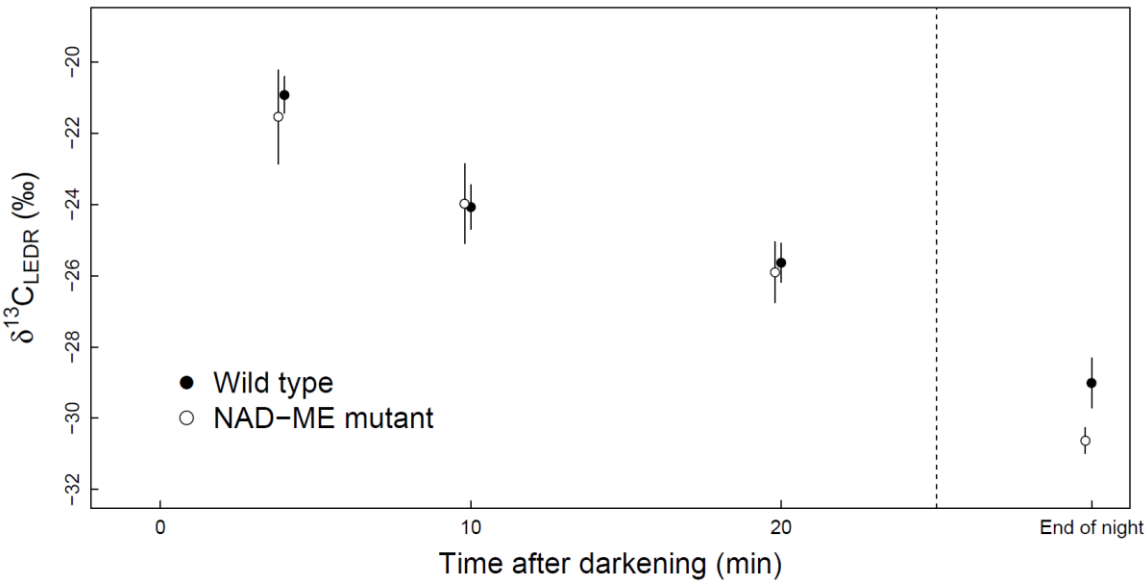

Figure S1: The natural carbon isotopic composition of leaf-respired CO_2 during light enhanced dark respiration ($\delta^{13}C_{LEDR}$) in *Arabidopsis thaliana* Col-0 (wild type) and *nadme1x2* (NAD-ME mutant) plants shortly upon darkening. Measurements were performed in the afternoon of a 12 h light period, with an in-tube incubation method (Werner et al., 2007). "End of night" denotes samples taken at the end of the following dark period.

Werner C., Hasenbein N., Maia R., Beyschlag W. & Maguas C. (2007) Evaluating high time-resolved changes in carbon isotope ratio of respired CO_2 by a rapid in-tube incubation technique. *Rapid Communications in Mass Spectrometry*, **21**, 1352-1360.

Table 1: Sums of label-derived leaf $^{13}CO_2$ respiration in the light ($R_{Label\text{-}light}$, µmol $^{13}CO_2$ m^{-2} s^{-1}) and in the dark ($R_{Label\text{-}dark}$, µmol $^{13}CO_2$ m^{-2} s^{-1}) in *H. halimifolium* and *O. triangularis* plants. Small letters indicate significant differences among ^{13}C-labelled substrates within either light or dark (ANOVA & Tukey-HSD). Means and SE are given (n = 3-5 individuals).

Species	Label	$R_{Label\text{-}light}$	$R_{Label\text{-}dark}$
H. halimifolium	^{13}C-1 Malate	0.06±0.02 b	0.22±0.08 ab
	^{13}C-4 Malate	0.05±0.02 b	0.33±0.11 ab
	^{13}C-1 Pyruvate	0.37±0.10 a	0.71±0.24 a
	^{13}C-2 Pyruvate	0.03±0.01 b	0.08±0.02 b
O. triangularis	^{13}C-1 Malate	0.05±0.01 a	0.17±0.05 a
	^{13}C-4 Malate	0.08±0.03 a	0.38±0.19 a
	^{13}C-1 Pyruvate	0.16±0.06 a	0.23±0.07 a
	^{13}C-2 Pyruvate	0.03±0.00 a	0.02±0.00 a

Chapter 5

5. General Discussion and Outlook

The isotopic composition of leaf dark-respired CO_2 ($\delta^{13}C_R$) *per se* is linked to the isotopic composition of respiratory substrates. $\delta^{13}C_R$ was often shown to be less negative compared to $\delta^{13}C$ of respiratory substrates and to vary up to 15‰ during a diel cycle (Duranceau *et al.*, 1999; Ghashghaie *et al.*, 2001; Werner *et al.*, 2009; Wegener *et al.*, 2010). Nevertheless, changes in $\delta^{13}C$ of putative respiratory substrates under different environmental conditions during a diel cycle are not well studied so far. This lack of knowledge limits the information gained by $\delta^{13}C_R$, which could be a useful indicator for plant physiological parameters, prevalent environmental conditions, and currently used respiratory substrates. In a first study (chapter 2), we showed that diel cycles of $\delta^{13}C_R$ and $\delta^{13}C$ of respiratory substrates changed similarly with environmental conditions in potato plants, with lower values under high temperatures compared to low temperatures and higher values under dry conditions compared to wet conditions. Interestingly, the combined impact of high temperature and dry conditions canceled out the individual effects of each factor, showing that $\delta^{13}C_R$ and $\delta^{13}C$ of respiratory substrates in plants under highest stress levels (high temperature and dry conditions) were most similar to plants with lowest stress levels (low temperature and wet conditions). This demonstrates that $\delta^{13}C_R$ of plants under non-stressful conditions can hardly be distinguished from $\delta^{13}C_R$ of plants under several stress conditions, which could

at least be true for plants comparable with potato plants. Moreover, we measured $\delta^{13}C$ of different putative respiratory substrates and found the closest relationship between $\delta^{13}C_R$ and $\delta^{13}C$ of malate across all environmental conditions during daytime and nighttime (chapter 2). Additionally, $\delta^{13}C$ of malate was typically less negative compared to other substrates, and also often less negative than $\delta^{13}C_R$ (chapter 2, 3), indicating a high potential to cause the ^{13}C enrichment often observed in leaf dark-respired CO_2 during a diel cycle. A multiple linear regression analysis revealed malate as the most explaining respiratory substrate of leaf dark-respired CO_2 in potato plants, strongly indicating a metabolic link.

Due to that reasons, we carried out malate measurements in species of different functional groups, comprising species with low and high variations in $\delta^{13}C_R$ (chapter 3). We found again that $\delta^{13}C$ of malate was less negative compared to other respiratory substrates such as sugars and amino acids, and also compared to leaf dark-respired CO_2. Interestingly, we found a strong ^{13}C enrichment in malate in *Oxalis triangularis*, with $\delta^{13}C$ values up to -10.9‰, indicating so far unknown isotope fractionations. Nevertheless, variations in $\delta^{13}C$ of malate during a diel cycle were lower in comparison to variations in $\delta^{13}C_R$ and highly species-specific. Also malate concentrations did not change significantly during the diel cycle. The results suggest that in these species $\delta^{13}C$ of malate cannot fully explain changes in $\delta^{13}C_R$ alone, indicating that additional substrates and processes must play a role.

The results are in contrast to studies suggesting malate as key respiratory substrates of relatively ^{13}C enriched leaf dark-respired CO_2 during a period of enhanced dark respiration rates shortly upon darkening, defined as light enhanced dark respiration

(LEDR; Barbour *et al.*, 2007; Gessler *et al.*, 2009; Werner *et al.*, 2009; Barbour *et al.*, 2011). The ^{13}C enrichment in respired CO_2 was supposed to derive from the C-4 position of malate via the mitochondrial malic enzyme reaction (ME). However, our labelling experiments with ^{13}C-4 malate and *Arabidopsis* mutants, without any functional ME, indicate that ME is not the central enzymatic reaction leading to changes in $\delta^{13}C_R$ during LEDR and that other enzymatic reactions must be involved (chapter 4). Moreover, respiration of ^{13}C-1 and ^{13}C-4 malate was lower compared to respiration of ^{13}C-1 pyruvate at least in a species with known high increases in $\delta^{13}C_R$. So we could show for the first time, that pyruvate rather than malate is the key substrate of $\delta^{13}C_R$ during LEDR. One explanation for this result could be the respiratory use of pyruvate via mitochondrial pyruvate dehydrogenase. The C-1 position of pyruvate can be released by pyruvate dehydrogenase reactions, causing ^{13}C enriched CO_2, while the reaction residue acetyl-CoA undergoes respiration or biosynthesis in the Krebs cycle. Our results clearly showed that respiration of ^{13}C-1 pyruvate was typically higher than ^{13}C-2 pyruvate during light and dark conditions, showing the importance of the C-1 position of pyruvate for dark respiration, while the C-2 position (reflecting acetyl-CoA residue) is used for anabolic reactions such as the production of amino acids or lipids (chapter 4). Additionally, we could show that malate and pyruvate are preferably used for the production of organic and amino acids (chapter 4). Subsequently, our results lead us to reject the common hypothesis that the mitochondrial malic enzyme reaction significantly influences $\delta^{13}C_R$ during LEDR. We rather propose that a more complex mechanism is causing the ^{13}C-enrichment in leaf dark-respired CO_2 during LEDR, most likely due to the mitochondrial pyruvate dehydrogenase reaction in connection with

several assisting enzymatic reactions, using pyruvate and malate as substrates. Such a mechanism most likely explains the known species-specific differences observed in $\delta^{13}C_R$ during LEDR for natural isotope abundances.

Overall, this work provided new knowledge about the substrates and environmental conditions imprinting on $\delta^{13}C_R$, indicating malate as main respiratory substrate in potato plants (chapter 2). However, the result could not be confirmed with other species such as shrubs and herbs, although we found strong species-specific differences in $\delta^{13}C_R$ and $\delta^{13}C$ of malate (chapter 3). That pyruvate rather than malate plays an important role for respiration, especially during LEDR, was finally demonstrated with leaf-labelling studies using ^{13}C position-specific labelled malate and pyruvate (chapter 4). Thus, processes leading to changes in $\delta^{13}C_R$ cannot be explained by one simple mechanism. However, based on our results more precise explanations can be given for observations in connection to plant respiration and isotope biochemistry at the leaf and plant scale.

Outlook

Several experimental approaches with different species were carried out in this study to investigate substrates, environmental conditions, and underlying mechanisms imprinting on $\delta^{13}C_R$. However, for a better understanding of the respiratory processes we still need more information about the temporal changes in activity of enzymatic reactions (such as PEPC, PDH, ME, MDH, Fumarase and more) and their substrate turnover rates in different plant species (Gessler *et al.*, 2009; Lee *et al.*, 2010). Moreover, experiments with other position-specific ^{13}C labelled substrates connected to dark respiration as used in this study would increase our understanding about plant respiration. For instance

leaf-labelling experiments with (1) ^{13}C labelled HCO_3^- could help to investigate processes associated with respiration, as well as with soil carbon uptake and allocation at the metabolic scale; (2) ^{13}C labelled glycine or succinate could help to get new insights into photorespiration and LEDR (Parys & Romanowska, 2000); (3) ^{13}C labelled amino acid such as glutamate and aspartate could give us a better understanding about their potential influence on plant respiration during light-dark transitions (Wallace *et al.*, 1984). Furthermore, experiments with plants under severe environmental conditions fed with ^{13}C labelled substrates could probably show which substrates are more respired under stress conditions and which substrates are not (Tcherkez *et al.*, 2003; Usadel *et al.*, 2008). Also experiments under reduced oxygen concentrations inhibiting photo-respiratory processes would be interesting, since LEDR were shown to be reduced under such conditions (Parys & Romanowska, 2000). This might help to prove if underlying mechanisms of the ^{13}C enrichment in CO_2 and the enhanced respiration rates during LEDR are related or not. Moreover, research with different mutants of *Arabidopsis thaliana* or other species could be useful to investigate or identify new isotope fractionation processes (Duranceau et al., 2001), e.g., knockout of distinct genes in connection to respiration might change the isotopic composition of respiratory substrates and leaf dark-respired CO_2 and would help to identify previously unknown isotope fractionation. Moreover, we propose NMR studies of intramolecular ^{13}C patterns within respiratory substrates such as glucose or malate extracted from different species at different points in time, to investigate species-specific and temporal differences (Gilbert *et al.*, 2009). Finally, determining $\delta^{13}C$ of individual substrates in different cell

compartments such as chloroplast, mitochondrion, and cytosol could clarify the existence of isotopic different carbon pools in plants.

References

Barbour MM, Hunt JE, Kodama N, Laubach J, McSeveny TM, Rogers GN, Tcherkez G, Wingate L. 2011. Rapid changes in $\delta^{13}C$ of ecosystem-respired CO_2 after sunset are consistent with transient ^{13}C enrichment of leaf respired CO_2. *New Phytologist* **190**: 990-1002.

Barbour MM, McDowell NG, Tcherkez G, Bickford CP, Hanson DT. 2007. A new measurement technique reveals rapid post-illumination changes in the carbon isotope composition of leaf-respired CO_2. *Plant, Cell and Environment* **30**: 469-482.

Duranceau M, Ghashghaie J, Brugnoli E. 2001. Carbon isotope discrimination during photosynthesis and dark respiration in intact leaves of Nicotiana sylvestris: comparisons between wild type and mitochondrial mutant plants. *Australian Journal of Plant Physiology* **28**: 65-71.

Duranceau M, Ghashghaie J, Badeck F, Deleens E, Cornic G. 1999. $\delta^{13}C$ of CO_2 respired in the dark in relation to $\delta^{13}C$ of leaf carbohydrates in *Phaseolus vulgaris* L. under progressive drought. *Plant, Cell and Environment* **22**: 515-523.

Gessler A, Tcherkez G, Karyanto O, Keitel C, Ferrio JP, Ghashghaie J, Kreuzwieser J, Farquhar GD. 2009. On the metabolic origin of the carbon isotope composition of CO_2 evolved from darkened light-acclimated leaves in *Ricinus communis*. *New Phytologist* **181**: 374-386.

Ghashghaie J, Duranceau M, Badeck FW, Cornic G, Adeline MT, Deleens E. 2001. $\delta^{13}C$ of CO_2 respired in the dark in relation to $\delta^{13}C$ of leaf metabolites: comparison between *Nicotiana sylvestris* and *Helianthus annuus* under drought. *Plant, Cell and Environment* **24**: 505-515.

Gilbert A, Silvestre V, Robins RJ, Remaud GS. 2009. Accurate Quantitative Isotopic ^{13}C NMR Spectroscopy for the Determination of the Intramolecular Distribution of ^{13}C in Glucose at Natural Abundance. *Analytical Chemistry* **81**: 8978-8985.

Lee CP, Eubel H, Millar AH. 2010. Diurnal changes in mitochondrial function reveal daily optimization of light and dark respiratory metabolism in *Arabidopsis*. *Molecular & Cellular Proteomics* **9**: 2125-2139.

Parys E, Romanowska E. 2000. Relationship between postillumination burst of CO_2 and enhancement of respiration in tall fescue leaves. *Acta Physiologiae Plantarum* **22**: 135-142.

Tcherkez G, Nogues S, Bleton J, Cornic G, Badeck F, Ghashghaie J. 2003. Metabolic origin of carbon isotope composition of leaf dark-respired CO_2 in French bean. *Plant Physiology* **131**: 237-244.

Usadel B, Blasing OE, Gibon Y, Retzlaff K, Hoehne M, Gunther M, Stitt M. 2008. Global transcript levels respond to small changes of the carbon status during

progressive exhaustion of carbohydrates in *Arabidopsis* rosettes. *Plant Physiology* **146**: 1834-1861.

Wallace W, Secor J, Schrader LE. 1984. Rapid accumulation of γ-aminobutyric acid and alanine in soybean leaves in response to an abrupt transfer to lower temperature, darkness, or mechanical manipulation. *Plant Physiology* **75**: 170-175.

Wegener F, Beyschlag W, Werner C. 2010. The magnitude of diurnal variation in carbon isotopic composition of leaf dark respired CO_2 correlates with the difference between $\delta^{13}C$ of leaf and root material. *Functional Plant Biology* **37**: 849-858.

Werner C, Wegener F, Unger S, Nogues S, Priault P. 2009. Short-term dynamics of isotopic composition of leaf-respired CO_2 upon darkening: measurements and implications. *Rapid Communications in Mass Spectrometry* **23**: 2428-2438.