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Effects of a genetically modified potato line with altered starch metabolism on carbon fluxes within the plant-soil system and on microbial community structure and function in the rhizosphere

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My contribution to the publications

I.– III. I took part in planning the experiments, performed the samplings and conducted the subsequent laboratory analyses. In addition, all statistical evaluation of the results was done by myself. The manuscripts are based mainly on my input.

Wollte man warten, bis man etwas so gut könnte,

dass niemand etwas daran auszusetzen fände,

brächte man nie etwas zuwege...

(Friedrich Nietzsche)

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Summary

Despite the many benefits offered by genetically modified (GM) plants, their commercialization is still highly controversially discussed in many countries, raising questions about potential adverse effects on human and environmental health. Among the major concerns are the possibility of creating invasive plant species, the unintended consequences of transgene flow to indigenous plants and microorganisms, development of super pests, and the effects of transgenic plants on non-target organisms. Although the importance of soil microbes for ecosystem functioning and plant performance is well acknowledged, the impact of GM plant cultivation on belowground biomass has long been neglected in risk assessment. As plants contribute substantially to soil carbon content via root exudation, they are hypothesized to govern the development of beneficial microbial communities in their rhizosphere, which in turn promote plant growth and health by various mechanisms like nutrient mobilization, production of plant growth hormones, induction of systemic resistance in the host plant and/or by acting as biocontrol agents. Therefore, it has to be evaluated if genetic engineering affects carbon fluxes from plant into soil and thus microbial community structure.

Hence, to assess potential impacts of the growth of amylopectin-accumulating GM potato line #1332 (*Solanum tuberosum* L., Bayerische Landesanstalt für Landwirtschaft (LfL)) on associated soil microorganisms, two greenhouse experiments and a 1-year-1-site field study included in a great scale release of the respective GM potato line were conducted in a randomized experimental design. Besides the parental variety 'Walli', a second non-transgenic potato cultivar was planted, in order to relate possible GM-dependent effects to natural variation among different plant genotypes. Rhizosphere samples were taken at young leaf developmental and at flowering stage of potatoes. For investigation of carbon fluxes within the plant-rhizosphere system and microbial community structure, ^{13}C stable isotope probing (SIP) in combination with phospholipid fatty acid (PLFA) analysis was chosen. To get a more detailed insight into rhizosphere microbial populations, abundance pattern of important potato pathogens (*Clavibacter michiganensis*, *Phytophthora infestans*), plant beneficial microbes (*Pseudomonas* spp., *Trichoderma* spp.), and functional groups involved in soil mineralization processes were examined using quantitative real-time PCR (qPCR). Additionally, total bacterial and fungal abundance pattern (based on 16S rRNA, ITS rRNA) were determined.

The genetic modification present in potato line #1332 had no influence on carbon partitioning within the plant, on plant derived carbon within the pool of water extractable organic carbon (WEOC) in the

rhizosphere, or on rhizosphere community structure and activity. Furthermore, no differences in abundance pattern of phylogenetic groups and functional genes under investigation between the GM line and its parental variety ‘Walli’ were observed. Nevertheless, the non-transgenic potato cultivars varied significantly regarding to carbon partitioning from plant into soil, rhizosphere population structure and microbial gene abundance pattern. Moreover, also plant developmental stage affected carbon fluxes via plant into rhizosphere and, subsequently, microbial community structure and gene abundance.

When comparing data obtained from greenhouse and field, the impact of cultivar and plant vegetation stage on microbial populations was much more pronounced in the latter. Furthermore, the rhizosphere of field-grown potatoes showed a significantly higher abundance of microorganisms than that of plants grown under greenhouse conditions.

Altogether, genetic modification of potato line #1332 seemed not to influence carbon partitioning through plant into soil or microbial community structure and abundance in the rhizosphere. However, it has to be considered that a generalization in respect of risk assessment and monitoring is not possible, as data were obtained in greenhouse studies under optimal growth conditions and a 1-year-1-site field trial. Therefore, for evaluation of long-term effects further field studies lasting several years and including different sites have to be conducted.

Zusammenfassung

Obwohl gentechnisch veränderte (GV) Pflanzen durchaus Vorteile mit sich bringen, wird ihr Anbau in vielen Ländern immer noch kontrovers diskutiert, v.a. hinsichtlich möglicher negativer Auswirkungen auf die menschliche Gesundheit und die Umwelt. Dabei spielen hauptsächlich folgende Bedenken eine große Rolle: die Entstehung invasiver Pflanzenarten, der mögliche Gentransfer von GV Pflanzen zu einheimischen Pflanzen beziehungsweise zu im Boden lebenden Mikroorganismen, die Entstehung sogenannter Super-Schädlinge sowie mögliche Effekte auf Nicht-Ziel-Organismen. Besonders der Einfluss von GV Pflanzen auf Bodenmikroorganismen wurde im Rahmen einer Risikoabschätzung lange Zeit vernachlässigt, obwohl diese essentiell für ein funktionierendes Ökosystem sind und maßgeblich zum Pflanzertrag beitragen. Dadurch, dass die von den Pflanzen über die Wurzel abgegebenen Exsudate einen wesentlichen Teil des Boden-Kohlenstoffs (C) ausmachen, sind Pflanzen vermutlich in der Lage, die Entwicklung ihrer mikrobiellen Rhizosphären-Gemeinschaft zu steuern und somit hinsichtlich solcher Mikroorganismen zu selektieren, die die Nährstoffaufnahme der Pflanze verbessern, pflanzliche Wachstumshormone produzieren, systemische Resistenz in der Pflanze induzieren und/oder biokontrollaktiv sind - kurz gesagt, die für die Pflanze von Vorteil sind. Im Hinblick darauf muss untersucht werden, ob eine genetische Modifikation unbeabsichtigterweise den Kohlenstoff-Fluss durch die Pflanze in den Boden und damit auch die Struktur der mikrobiellen Gemeinschaft beeinflusst.

Im Rahmen dieser Doktorarbeit sollten mögliche Anbau-Effekte der Amylose-freien GV Kartoffellinie #1332 (*Solanum tuberosum* L., Bayerische Landesanstalt für Landwirtschaft (LfL)) auf die mit den Wurzeln assoziierten Mikroorganismen untersucht werden. Dafür wurden zwei Gewächshausexperimente sowie eine 1-jährige Feldstudie durchgeführt, wobei letztere mit einer großflächigen Freisetzung der entsprechenden GV-Kartoffellinie auf dem Versuchsgelände der LfL einherging. Um etwaige GV-Effekte mit der natürlichen Variabilität zwischen verschiedenen Sorten vergleichen zu können, wurde neben der parentalen Sorte 'Walli' zusätzlich eine zweite kommerzielle Kartoffelsorte in einem randomisiertem Versuchsdesign angepflanzt. Die Beprobung der Rhizosphäre erfolgte während des frühen Blattentwicklungsstadiums sowie während der Blüte der Kartoffelpflanzen. Um den Kohlenstoff-Fluss durch die Pflanze in den Boden verfolgen zu können, wurden die Kartoffeln zu den entsprechenden Entwicklungsstadien einer ^{13}C-CO_2-Atmosphäre ausgesetzt. Damit konnten die „schweren" Photosyntheseprodukte mithilfe komponentenspezifischer

Isotopenanalyseverfahren innerhalb der Pflanze, im Boden sowie in der mikrobiellen Biomasse detektiert werden. Die Identifizierung der mikrobiellen Gruppen, die Wurzelexsudate aktiv aufnehmen, erfolgte anhand ihrer Phospholipid-Fettsäure-Muster (PLFA). Um einen genaueren Einblick in die mit den Wurzeln assoziierten mikrobiellen Gemeinschaften zu erhalten, wurde außerdem die Abundanz wichtiger Kartoffelschädlinge (*Clavibacter michiganensis*, *Phytophthora infestans*), für die Pflanze nützlicher Mikroorganismen (*Pseudomonas* spp., *Trichoderma* spp.) sowie funktioneller, in Boden-Mineralisierungsprozesse involvierter Gruppen mittels quantitativer PCR (qPCR) erfasst. Zusätzlich wurde die gesamte bakterielle und pilzliche Abundanz (basierend auf 16S rRNA bzw. ITS rRNA) bestimmt.

Die in Kartoffellinie #1332 vorliegende genetische Modifikation hatte weder Auswirkungen auf den Kohlenstoff-Fluss durch die Pflanze in den Boden noch auf die Zusammensetzung der mikrobiellen Rhizosphären-Gemeinschaft oder deren Aktivität. Ebenso konnte kein Unterschied zwischen der GV Kartoffel und ihrer parentalen Linie hinsichtlich der Abundanz der untersuchten phylogenetischen Gruppen oder der funktionellen Gene beobachtet werden. Die beiden nicht-transgenen Sorten hingegen unterschieden sich signifikant hinsichtlich ihrer Kohlenstoff-Verteilungsmuster sowie der Zusammensetzung ihrer Mikrobengemeinschaft und deren Gen-Abundanzmustern. Außerdem konnte gezeigt werden, dass auch das Pflanzenentwicklungsstadium einen signifikanten Einfluss auf die untersuchten Parameter hatte.

Die Unterschiede zwischen den natürlichen Sorten sowie der Effekt des Pflanzenentwicklungsstadiums waren deutlicher ausgeprägt bei Pflanzen aus dem Feldversuch. Ebenso war die mikrobielle Abundanz in der Rhizosphäre deutlich erhöht gegenüber Kartoffeln, die unter kontrollierten Bedingungen in Gewächshaus angepflanzt wurden.

Zusammengefasst, scheint die genetische Modifikation der Kartoffellinie #1332 weder den Kohlenstoff-Fluss durch die Pflanze in den Boden noch die Struktur der mikrobiellen Rhizosphären-Gemeinschaft oder deren Gen-Abundanzmuster zu beeinflussen. Allerdings ist eine Verallgemeinerung hinsichtlich einer Risikoabschätzung nicht möglich, da die zugrundeliegenden Daten auf Gewächshausexperimenten und einer 1-jährigen Feldstudie an nur einem Standort beruhen. Um Langzeiteffekte auszuschließen, müssten zusätzlich noch mehrjährige Freilandstudien durchgeführt werden, optimalerweise an verschiedenen Standorten.

1. Introduction to the Ph.D. thesis

Microorganisms play a key role for soil ecosystem functioning by driving nutrient cycling, degrading pollutants and contributing to plant performance via various mechanisms like nutrient mobilization, production of plant growth hormones, induction of systemic resistance in the associated plant and/or acting as biological control agents (Raaijmakers *et al*., 2009). In turn, they benefit from nutrients provided by root exudates and consequently, their abundance and community structure is highly affected by the plant developmental stage, the plant species and also the plant genotype (Grayston *et al*., 1998; Gyamfi *et al*., 2002; Kowalchuk *et al*., 2002; Marschner *et al*., 2006; Smalla *et al*., 2001).
Although gene technology allows a more targeted modification of the plant genome than classical breeding approaches (Catchpole *et al.*, 2005), an impact of the whole plant metabolism is conceivable and might result in altered carbon and nitrogen allocation to the roots and thus modified root exudation. Consequently, also microbial communities closely associated to the plant roots might be affected by genetic engineering. Concerning the acknowledged importance of soil microorganisms for plant growth and health, the influence of increased cultivation of genetically modified (GM) crops on these microbes has to be evaluated for each modification event separately.

The present Ph.D. thesis was performed in the frame of a project funded by the Bayerisches Staatsministerium für Umwelt, Gesundheit und Verbraucherschutz (StMUGV), in order to assess potential effects of the GM amylopectin-accumulating potato line #1332 on microbial communities in the rhizosphere, which is defined as the soil zone influenced by plant roots (Soerensen, 1997). The respective GM line was generated by the Bayerische Landesanstalt für Landwirtschaft (LfL). Besides the parental cultivar, 'Walli', a second non-transgenic potato variety was included, allowing the relation of potential GM-caused impacts to natural cultivar variability. Furthermore, rhizosphere communities were examined at different plant vegetation stages, because root exudation pattern might also change during plant development (Jones *et al.*, 2004).
Regarding to the various ecological functions of microorganisms in soil ecosystems, the aim of this work was to evaluate the influence of genetic modification (i) on functional microbial populations contributing to mineralization (**Publication** I), (ii) on important potato pathogens (*Clavibacter michiganensis, Phytophthora infestans*) respectively plant beneficial microorganisms (*Pseudomonas* spp., *Trichoderma* spp.) (**Publication** II), and (iii) on photosynthate partitioning through the plant into

soil and into rhizosphere (**Publication III**). For the investigation of microbial populations, a polyphasic approach including phenotypic and genotypic biomarkers of microbes was chosen. Furthermore, stable isotopes were used to follow carbon fluxes through the plant-rhizosphere system.

2. Genetically modified plants

For thousands of years, humans altered the genomes of agricultural used plants by domestication and subsequent breeding, in order to improve growth characteristics, taste and nutritional values as well as disease / pest / herbicide resistance. However, the trait selection in classical plant breeding depends on generation time and development pattern and is thus relatively slow and laborious. Besides, the whole plant genome is impacted, which often leads to adverse effects on other plant characteristics when improving the desired trait (Kreuzer & Massey, 2001). Contrary, genetic modification of plants allows the target-oriented engineering by the specific transfer of single, well characterized genes into the plant genome. Therefore, unintended trait effects can be minimized. Furthermore, the genes of interest can arise from any organism, resulting in plant properties not achievable by conventional breeding, which is restricted to the use of closely related species (Thomson, 2006).

According to Food and Agriculture Organization of the United Nations (FAO, 2002), a genetically modified (GM) organism is defined as “an organism that has been modified by the application of recombinant DNA technology”. Since the first large scale commercial release of GM crops in 1996, the area cultivated with GM plants increased approximately 80-fold to 134 million hectares, representing 9% of the arable land in the world (James, 2009). This unprecedented high growth rate makes GM crops the fastest adopted plant technology in recent agricultural history. In 2009, 14 million farmers cultivated genetically engineered crops, whereby 90% were small and resource-poor farmers in developing countries (James, 2009). Furthermore, in the same year the number of countries growing GM crops increased to 25, including 16 developing and 9 industrial countries. Additionally, 32 countries permitted GM plants for import (James, 2009), resulting in a total of 57 countries granting regulatory approvals for planting GM crops and/or importing products derived from them. According to James (2009), GM soybean was, with an amount of 77% of the global soybean production of 90 million hectares, the most frequently cultivated biotech crop in 2009, followed by GM maize (26% of

158 million hectares grown worldwide), GM cotton (49% of 33 million hectares), and GM canola (21% of 31 million hectares).

Although, currently most of the planted GM crops were engineered for herbicide tolerance and/or insect resistance, the development of GM plants with traits beneficial for consumers increased in the last years. These "second generation" plants focused on food and feed with enhanced nutritional values, premium quality, low allergenicity or better processing characteristics, including rice with high β-carotene content, zeaxanthin-enriched potatoes, oil crops with enhanced polyunsaturated fatty acid content, rice containing low albumin and potatoes with modified starch composition (Yonekura-Sakakibara & Saito, 2006; Weinert *et al.*, 2009). Furthermore, the development of a "third generation" of GM crops able to produce biodegradable plastics or pharmaceutical compounds (hormones, vaccines, antibodies) is in progress (Poirier *et al.*, 1995; Yonekura-Sakakibara & Saito, 2006).

Overall, the cultivation of GM crops offers a number of advantages leading to a more sustainable agriculture, as it allows the reduction of applied harmful pesticides, herbicides and insecticides. Since commercial release of GM plants in 1996, the amount of applied pesticides declined by 352 million kilograms, which means a saving of 8.4%. Moreover, genetically engineered crops reduced greenhouse gas emissions by more than 14 billion kilogram of CO_2, resulting from both the less usage of fossil-based fuels associated with fewer insecticide and herbicide sprays and the savings from conservation tillage as less or no ploughing facilitated by herbicide tolerant plants (Brookes & Barfoot, 2010a, b). Besides those ecological advantages, GM plants also have economical benefits, as farmers profit from lower production costs, more practical crop management, an increase in yields and quality and higher social benefits (James, 2009).

Nevertheless, despite all these advantages, the release of GM crops is still highly controversial in many countries, raising questions about potential negative effects on human health and environment. Among the major concerns are the possibility of creating invasive plant species, the unintended consequences of transgene flow to indigenous plants and microorganisms, development of super pests, and the effects of transgenic plants on non-target organisms (Wolfenbarger & Phifer, 2000). Hence, GM crops have to undergo detailed food, feed and environmental safety evaluations controlled by different agencies like the European Food Safety Authority (EFSA) for the European market and the Federal Office of Consumer Protection and Food Safety (BVL) for the German market, before receiving approval.

3. Legislation regarding genetically modified plants in the European Union

According to the general food law Regulation (EC) No 178/2002, three different rules govern the approval of genetically engineered plants in the European Union (EU): Regulation (EC) No 1829/2003, addressing authorization and labelling provisions of all GM food and feed consisting of, containing or being produced from GM plants (EC, 2003a), Regulation (EC) No 1830/2003, concerning the traceability of GM plants, GM food and GM feed (EC, 2003b), and Directive 2001/18/EC, regulating the deliberate release of GM plants into the environment (EC, 2001). Furthermore, the EFSA Guidance Document provides independent scientific advice for applications within the framework of those legislations (EFSA, 2006).

Corresponding to EU law, all GM plants and derived products which should enter the food or feed market have to be evaluated by EFSA. Therefore, an authorization application containing scientific safety assessment, labelling, detection method and post-market monitoring adapted for the respective GM plant and product must be submitted to the European Commission by the approval seeking company. The European Commission passes the proposal along to EFSA, where a scientific expert committee implements a detailed risk assessment. Regarding to EFSA's recommendation, the European Commission judges on market approval and presents its decision to the Standing Committee on the Food Chain and Animal Health. This Committee, consisting of representatives from all EU Member States, finalizes the decision on market permission. Granted approval is valid for 10 years within the EU.

In contrast, applications needing only authorization for the deliberate release of GM plants into the environment have to be submitted to federal authorities, including scientific safety assessment, a standardized method for GM plant detection and a detailed monitoring concept. After initial evaluation by national agencies, the documents will be forwarded to the national authorities of the EU Member States and to the European Commission, which may decide on approval without involving EFSA. If also an authorization for the use of the respective GM plant on food or feed market is wanted, the company may submit both applications directly to EFSA. There, the scientific expert committee performs a detailed risk assessment and examines the applied monitoring concept due to its suitability. Afterwards, the proceeding will continue as provided in Regulation (EC) No 1829/2003.

4. Monitoring process for genetically modified plants

Monitoring serves to evaluate adverse effects on human and animal health or the environment that may emerge from a GM crop or its use. Therefore, according to EU legislation, each authorization application requires a detailed monitoring plan oriented on the environmental risk assessment for the respective GM plant, submitted by the company and evaluated by the responsible European administrations and/or EFSA. The post-market environmental monitoring starts with the cultivation approval by Directive 2001/18/EC and should provide further information about the interrelationship between the GM plant and the environment under farming practice conditions. Therefore, it has to fulfill two main objectives: (i) To examine the assumptions of the environmental compatibility test (e.g. concerning the effects on biodiversity of flora and fauna) submitted in the previous authorization application under agricultural cultivation (*case specific monitoring*), and (ii) to detect unexpected (adverse) long-time effects (*general surveillance*). While the need for *case specific monitoring* depends on the nature of the genetic modification, *general surveillance* is obligatory for all approved GM plants and derived products.

Although post-market environmental monitoring of released genetically engineered crops is mandatory in the EU, no uniform monitoring concept exists so far. Therefore, several strategies and methods focusing on accuracy, practicability and cost-effectiveness were developed in the last years within a number of safety research programs. Summarized, environmental monitoring should consider the following potential impacts of GM crops: Release and persistence of transgenic DNA or product into the environment, persistence of the GM plant, risk of invasiveness, toxicity, allergenicity, susceptibility to pathogens, effects on non-target organisms, unintended effects on target organisms (e.g. resistance development), unintended adverse effects (e.g. increased antibiotic resistance as result of horizontal gene transfer, new virus strains due to introduction of viral sequences in plant genomes and possible recombination), change in biogeochemical cycles, and change in management (Sparrow, 2010). Furthermore, due to practice relevance of the monitoring, GM plant cultivation ought to be integrated in a typical arable crop rotation on soils already used for the respective natural plant species.

5. Genetically modified potatoes with altered starch quality

5.1. Industrial applications of starch

Although the significance of potato as staple food is steadily declining, growing importance is attached to one of its constituents, namely starch. While industrial starch extraction has tripled since the eighties, potatoes became an important starch supplier throughout the EU (http://www.gmo-safety.eu/; Jobling, 2004). Towards its competitors wheat and maize, potato plants offer several advantages as they provide high yields, contain larger starch grains and produce a higher swelling and more viscous starch (Ellis *et al.*, 1998). This may explain the extensively increasing interest in potato starch particularly in Germany, where in 2008 already 62% of the raw material for starch production was supplied from potato (http://www.gmo-safety.eu/). According to its ability to bind and thicken, starch is an extremely versatile raw material used mainly in the food industry for long time. However, as starch is a renewable resource, many additional product applications in the non-food sector were developed in recent years, including biodegradable plastic. Since starch is also a component of paper, construction materials, adhesives and many more non-food products, today more than 40% of starch consumption amounts to the non-food sector, whereby the major part is used by the paper industry (http://www.gmo-safety.eu/). The main industrial starch applications in the non-food sector are listed in Table 1.

Table 1. Key industrial applications for starch in the non-food sector

Paper and cardboard	Wrapping paper, newspaper, corrugated cardboard
Building materials	Plasterboard, mineral fiberboard
Textile manufacturing	Sizing, finishing and stiffening agents
Adhesives	Wallpaper paste, glues for chipboard
Biotechnology	Starch as a food source for microorganisms
Plastics	Packaging and films, moulds
Cleaning products	Laundry soaps, washing powder, detergent base materials
Cosmetics	Toothpaste, cream, face powder, dry shampoo
Pharmaceuticals	Tablets, antibiotics, vitamin C

5.2. The two starch components: Amylose and amylopectin

Starch is the major storage compound of higher plants and is synthesized in amyloplasts (for longtime storage) or in chloroplasts (for the transient storage of photosynthetic products). It consists mainly of the two polysaccharides amylose and amylopectin in a ratio of 1:4 to 1:5, dependent on plant species. For example, reserve starch in potato tubers contains 18-23% amylose (Shannon & Garwood, 1984). Both starch components differ in their chemical structure, which is shown in Figure 1: Whereas amylose consists of α–(1→4) linked glucose molecules and is thus linear, about 5% of the glucose units of amylopectin are additional α–(1→6) linked, resulting in a branched molecule structure (Jobling, 2004).

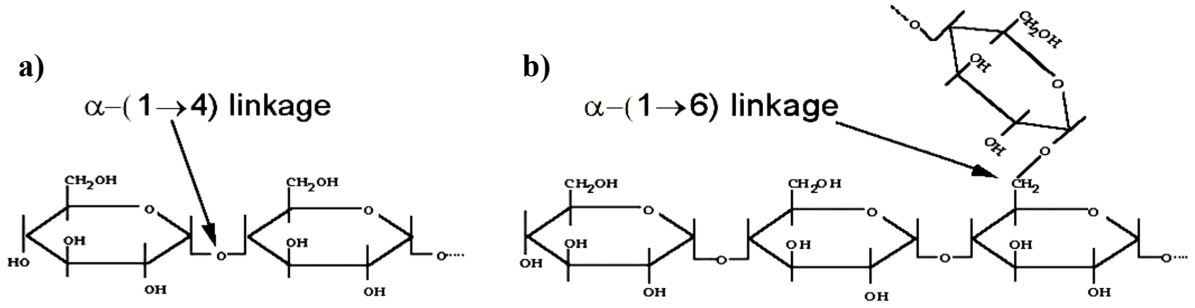

Fig. 1 The chemical structure of **(a)** amylose and **(b)** amylopectin (adapted from http://www.cheng.cam.ac.uk/research/groups/polymer/RMP/nitin/Starchstructure.html)

Due to their chemical structure, both starch components have typical characteristics which determine their industrial utilization. Amylose shows a high gelling strength which can be only applied in the food industry, especially for producing sweets. Besides, its film-forming ability keeps the coating on fried products crispy and reduces fat uptake during cooking. Moreover, high amylose starches can be processed into ”resistant starch” with nutritional benefits (Bird *et al.*, 2000; Jobling, 2004). In contrast, the high swelling power of amylopectin leads to clear, viscous pastes that will not gel. Due to this gelatinization ability, amylopectin can be applied much more versatile: For stabilization and thickening of many food products, but also in the paper and textile industry, for production of wall plasters and wall paper paste or generally as raw material for adhesives (http://www.gmo-safety.eu/; Jobling, 2004).

5.3. The amylopectin-accumulating potato

Starch is, as a renewable raw material, very attractive for many industrial areas. But, since the two starch components, amylose and amylopectin, show adverse physico-chemical properties, they have to be separated for industrial utilization (Kraak, 1993). To avoid the high production and energy costs incurred thereby (Young, 1984; Visser *et al.*, 1991), the development of a potato tuber (*Solanum tuberosum* L.) producing pure amylopectin starch is an important aim in plant breeding, mainly for paper industry. As the granule-bound starch synthase (GBSS) was identified as one of the key enzymes responsible for amylose synthesis (Robyt, 1984), a GM potato line containing a *gbss* gene fragment in antisense direction was constructed. Consequently, transcription leads to the formation of *gbss* dsRNA and thus inhibition of *gbss* expression and amylose production (Visser *et al.*, 1991; Kuipers *et al.*, 1994). Hence, the tuber starch of the respective GM line, named #1332, consists of more than 99% amylopectin, while the starch granules of the non-transgenic parental line 'Walli' contain only 75-80% amylopectin.

An analogical engineered transgenic potato variety, 'Amflora', (BASF Plant Science GmbH) was already accepted for commercial field release and utilization in feed products in the EU in March 2010. Moreover, the Dutch company AVEBE submitted an authorization application regarding cultivation and usage in feed products for another amylopectin-accumulating GM potato line called 'Modena' to the European Commission in 2009 (http://www.gmo-compass.org/). Similarly to the GM potato line investigated in this Ph.D. thesis, 'Modena' was transformed without antibiotic or herbicide selection marker genes by a newly developed method based on PCR screening (http://gmoinfo.jrc.ec.europa.eu/gmp_report.aspx?CurNot=B/DE/03/155; de Vetten *et al.*, 2003) to overcome public concerns about increasing resistance development in the environment through horizontal gene transfer (Wolfenbarger & Phifer, 2000).

Besides gene technology, also traditional mutation breeding techniques resulted in potato varieties with almost pure amylopectin content: According to Jacobsen *et al*. (1989), an amylose-free mutant could be identified after Röntgen-irridiation of potato clones. After backcrossing with high-yield varieties, the resulting amylopectin-accumulating potato line called 'Eliane' was introduced on market in 2005 (AVEBE). This long development time is a central problem in classical plant breeding, as on the one hand non-directional point mutations affecting the whole plant genome were induced, resulting in the necessity of several backcrossing events to counteract unintended adverse effects, and on the

other hand, trait selection depends on generation time and development pattern. The latter could be overcome by the so-called TILLING (targeting induced local lesions in genomes) method where chemical or X-ray mutagenesis of plants is combined with high-throughput screening for point mutations on DNA (Henikoff *et al.*, 2004). Using TILLING, the German company Bioplant was able to develop another amylose-free potato line within six years (http://www.biotechnologie.de/).

Although these classically bred cultivars were developed by man-made mutagenesis, they do not fall within the EU legislation regarding GM plants. Hence, for their commercialization no scientifically proved safety assessment or monitoring concept is regulated by law in Europe. Nevertheless, due to the importance of plant-microbe interactions for soil fertility and plant performance, potential effects on root-associated microbial communities should not be neglected.

6. The rhizosphere

6.1. General definition

The soil influenced by plant roots was first termed “rhizosphere” by Lorenz Hiltner (1904), describing a zone of high microbial activity due to large quantities of carbon and other nutrients provided by root exudates. Today, the root itself is divided into three compartments: The *endorhizosphere*, covering root cell layers, endodermis, cortex layer and epidermis, the root surface called *rhizoplane*, and the *ectorhizosphere*, comprising several millimetres of root surrounding soil (Soerensen, 1997). Furthermore, associations with mycorrhizal fungi formed in this area were named “mycorrhizosphere” (Lynch, 1990). The compartmentalisation of the rhizosphere is shown in Figure 2.

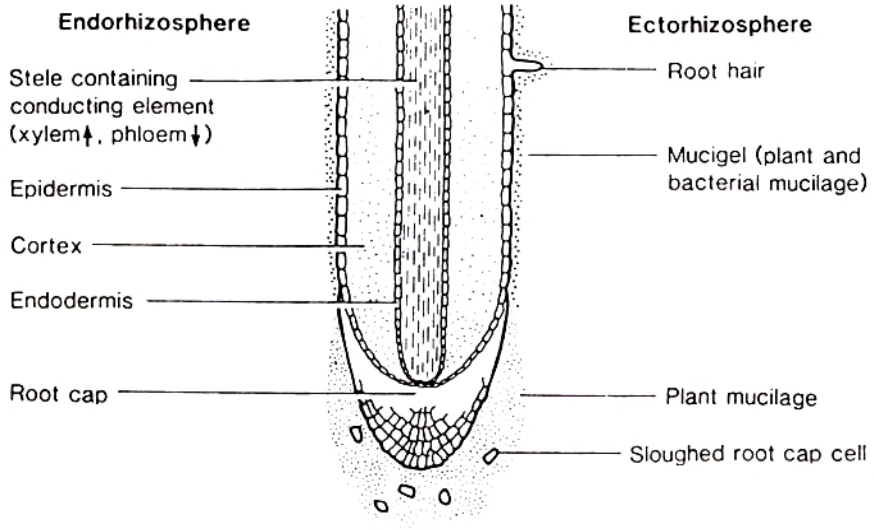

Fig. 2 Compartmentalization of the rhizosphere (adapted from Brimecombe *et al.*, 2001)

The rhizosphere forms around each growing root due to chemical, physical and biological changes in the direct proximity of each root (Uren, 2001). Although it lacks physically true boundaries (Campbell & Greaves, 1990), the area of soil affected by roots is 1-2 mm wide (Darrah & Roose, 2001). The total carbon entering the soil in form of root exudates is called “rhizodeposition” (Whipps & Lynch, 1985). On the one hand, this includes water-insoluble, high-molecular-weight substances like root debris (e.g. cell walls, sloughed cells and tissues) and mucilage, which functions as lubricant to push the root through the soil, especially in young roots (Neumann & Römheld, 2001). However, those substances have to be mobilized by extracellular enzymes before they can be used by microbes. On the other hand, water soluble, low-molecular-weight substances including sugars, amino acids, organic acids, hormones and vitamins are released by roots, mainly by secretion (Brimecombe *et al.*, 2001). These molecules can be readily assimilated by a wide range of microorganisms, and thus are responsible for the higher microbial growth and activity in the rhizosphere compared to the surrounding bulk soil, known as the ”rhizosphere effect” (Whipps, 1990; Uren, 2001). Due to this nutrient input from the plant, microbial populations in the rhizosphere differ clearly from that of bulk soil, regarding to both abundance and community composition (Marilley *et al.*, 1998; Smalla *et al.*, 2001; Costa *et al.*, 2006).
As photosynthetically assimilated CO_2 released via rhizodeposition is the primary carbon source in soils during plant vegetation period, microorganisms are also strongly dependent on biotic and abiotic factors changing the composition of root exudates, such as plant age, plant species, plant cultivar, nutritional status of the plant, soil structure, water content and temperature (Grayston *et al.*, 1998;

Neumann & Römheld, 2001; Smalla *et al.*, 2001; Gyamfi *et al.*, 2002; Kowalchuk *et al.*, 2002; Marschner *et al.*, 2006). Furthermore, it has been hypothesized that plants strategically govern their rhizosphere populations via quality and quantity of root exudates and select for beneficial microorganisms (Lynch & Whipps, 1990; Singh *et al.*, 2007), which in turn promote plant growth and health (Raaijmakers *et al.*, 2009). Moreover, as within the root system of a single plant root exudation occurs in a patchy way, microbial communities vary also between different parts of the root (Brimecombe *et al.*, 2001): Whereas microbes only able to utilize easily degradable sugars and organic acids are associated to the root tip, bacteria and fungi adapted to oligotrophic conditions are often found on older root zones, where carbon is deposited primarily as sloughed cells and consists of more recalcitrant materials including lignified cellulose and hemicellulose (Yang & Crowley, 2000). Especially Gram-positive bacteria are often found on elder roots, because they are able to tolerate decreasing exudation rates through spore formation (Brimecombe *et al.*, 2001).

Considering the strong and complex interactions between plant and microorganisms within the rhizosphere, it represents a highly dynamic habitat depending on many different factors, where especially the microbes need to adapt quickly to changing water-, oxygen- and nutrient-conditions.

6.2. Plant-microbe interactions

Depending on the respective microorganisms, the plant and the prevalent environmental conditions, plant-microbe interactions can be either harmful, beneficial or neutral to the plant (Lynch, 1990).

6.2.1. Harmful interactions

Harmful interactions describe both interactions with phytopathogens causing plant diseases or with deleterious rhizomicrobes inhibiting plant growth without inducing visible disease symptoms, but reducing yields (Nehl *et al.*, 1997). Decreased growth may thereby be caused by phytotoxins, phytohormones, competition with plant beneficial bacteria or inhibition of mycorrhiza or root-nodule formation (Brimecombe *et al.*, 2001). In this Ph.D. thesis, the two most devastating pathogens associated with potato, *Clavibacter michiganensis* and *Phytophthora infestans*, were examined.

Clavibacter michiganensis, an aerobic, non-sporulating, Gram-positive bacterium belonging to the order Actinomycetales, causes bacterial wilt in various agriculturally important plants. Therefore, it may be considered as the most important bacterial pathogen worldwide, including subspecies specific for alfalfa (*C. michiganensis* ssp. *insidiosus*), maize (*C. michiganensis* ssp. *nebraskensis*), wheat (*C. michiganensis* ssp. *tesselarius*), tomato (*C. michiganensis* ssp.*michiganensis*) and potato (*C. michiganensis* ssp. *sepedonicus*). The last two are subject to international quarantine regulations restricting import and export at strict zero tolerance in Canada, the USA and Europe (de Boer & Slack, 1984; European Union, 1995). Infection of the host plant usually occurs through wounds, followed by invasion and degradation of the vascular tissue causing plant wilt (Wallis, 1977). An infection at late plant developmental stage often remains symptomless, leading to unwittingly disease spreading by the use of contaminated seeds or, in the case of potato, infected tubers. Furthermore, some *Clavibacter* spp. can survive latently in the absence of their hosts on farm machinery, sacks and bins for several months (Hayward, 1974), additionally hampering disease control. As chemical disease control by antibiotics or copper compounds is ineffective and besides causes severe environmental problems, the only possible way of disease prevention so far is the utilization of certified seeds or transplants tested to be free of *C. michiganensis* (Mansfeld-Giese, 1997).

Although *C. michiganensis* ssp. *michiganensis* and *C. michiganensis* ssp. *sepedonicus*, the causal agents of cancer and wilt in tomato respective ring rot in potato, lead to huge economic losses worldwide, only little is known about the mechanisms involved in virulence and infection. Like most soil bacteria, they produce exopolysaccharides (EPS) determining multiple biological functions: EPS protect against dehydration by generating a water-saturated matrix around the bacterium, concentrate nutrients, bind toxic components, prevent recognition by the plant defence system, mediate adhesion to abiotic and biological surfaces and may promote infection and colonization of the host plant (Jahr *et al.*, 1999). Moreover, the viscous, high-molecular-weight EPS combined with the high titre of bacteria in the xylem vessels may cause a severe water stress directly contributing to plant wilt (van Alfen *et al.*, 1987). Besides, *Clavibacter* spp. produce different extracellular enzymes capable of attacking and degrading plant cell walls like cellulases, pectinases and glucanases. For *C. michiganensis* ssp. *sepedonicus*, only two virulence factors are determined so far: an endo-b-1,4-glucanase containing a cellulose-binding domain and an expansin-like domain, and a not fully characterized hypersensitive response including protein necessary for full development of disease (Nissinen *et al.*, 1997; Laine *et*

al., 2000; Jahr *et al.*, 1999). Furthermore, amylase has been found to affect virulence, probably important in conjunction with the rotting of potato tubers (Jahr *et al.*, 1999).

Phytophthora infestans belongs to the Oomycetes, which are separated from the true fungi by classification into the kingdom Protoctista or Chromista and are closely related to brown algae and diatoms (Fry & Goodwin, 1997). Oomycetes are characterized by cellulose-glucan cell walls without chitin, zoospores with heterokont flagella borne in sporangia, diploid nuclei in vegetative cells and sexual reproduction through antheridia and oogonia (Fry & Goodwin, 1997). The genus *Phytophthora* represents a large group of devastating pathogens causing important diseases in a wide variety of plant species, including potato, tomato, soybean and valuable forest trees (Erwin & Ribeiro, 1996). A typical infection cycle starts with the asexual germination of dormant spores resting in soil, in infected roots or infected tubers, leading to the development of sporangia. Germination can occur by two pathways: At soil temperatures >14°C, direct germination appears, resulting in the development of hyphae which emerge through the sporangial wall and colonize plants through openings like stomata, lenticel or wounds. Indirect germination, which represents the predominant spreading form, occurs at cooler temperatures and high soil moisture, leading to the release of motile, biflagellate zoospores from the sporangia. These are chemotactically attracted by substances released from roots. Upon surface contact, the zoospores encyst rapidly, germinate and develop appressoria allowing active penetration of the plant tissue. Afterwards, the mycelium migrates through the stem towards the leaves, where hyphae grow throughout the epidermis and new sporangia were produced (Judelson & Blanco, 2005). Those leaf-attached sporangia can be detached from the sporangiophore by wind and water, disseminating *Phytophthora* infection over several kilometres (Aylor, 2003). According to its heterothallic nature, *Phytophthora* is additionally able to reproduce sexually by interaction of the two mating types A1 and A2, resulting in the development of oogonia and antheridia. Following, each oogonium produces a single oospore, which can germinate and develop sporangia (Fry & Goodwin, 1997).

Contrary to the most other *Phytophthora* spp., *P. infestans* has a rather narrow host range involving mainly Solanaceae. It is the causal agent of late blight disease in potato and tomato and was responsible for the great potato famines in Ireland and Scotland in the 19th century. If left uncontrolled, late blight epidemics can result in up to 100% crop losses when weather conditions are favourable

(Platt *et al.*, 1999). Common disease symptoms are brownish spots on the leave surface, while the distinctive white mycelium at the lower leave surface can be observed only at high humidity.

6.2.2. Beneficial interactions

Beneficial interactions are facilitated by various microorganisms like plant growth promoting rhizobacteria (PGPR) or arbuscular mycorrhizal fungi (AMF), which directly or indirectly contribute to plant growth and health. Direct effects include nutrient mobilization, production of plant growth hormones or inducing systemic resistance in the plant. Additionally, various microorganisms improve plant growth and health indirectly by reducing the growth of plant pathogens. These microbes were commonly called biological control agents (BCA). Altogether, around two thirds of the cultivable rhizosphere microorganisms are able to express plant growth-promoting traits (Cattelan *et al.*, 1999; Furnkranz *et al.*, 2009), whereas up to 35% showed antagonistic capacity by inhibiting pathogen growth in vitro (Berg *et al.*, 2002, 2006). Due to their strong positive impact on plants, *Pseudomonas* spp. and *Trichoderma* spp. are among the most widely studied plant beneficial microbes.

***Pseudomonas* spp.** belong to the Gram-negative Gammaproteobacteria. According to both their motility and ability to utilize a variety of low-molecular-weight substances as well as some more complex compounds as carbon and energy source, pseudomonads appear ubiquitous in soil. Furthermore, they are excellent root colonizers and found to be the predominant bacteria in the rhizosphere of many plant species, including wheat, canola, ryegrass, bentgrass and clover (Grayston *et al.*, 1998; Siciliano & Germida, 1999). Their high colonization rate is due to large competition abilities: additionally to their (i) broad substrate spectrum, pseudomonads (ii) are known to produce lytic exoenzymes like proteases and chitinases, which can degrade cell walls of concurrent microorganisms (Adesina *et al.*, 2007). Moreover, they compete successfully for nutrients by (iii) secreting high-affinity chelating agents, e.g. siderophores linking iron. Although iron is the fourth most common element on earth, it prevails mainly in its oxidized and insoluble form as ferric hydroxide, impeding uptake. Therefore, iron-deficiency can be circumvented by releasing siderophores, as is done especially by fluorescent pseudomonads (Raaijmakers *et al.*, 2009). The siderophore-iron-complexes have to be reabsorped by specific membrane receptors, preventing uptake by competitors (Raaijmakers *et al.*, 1995). This nutrient competition is in turn beneficial for the plant, as it deprives

pathogenic fungi of iron, as these in general produce lower-affinity siderophores than bacteria (O'Sullivan & O'Gara, 1992). Furthermore, many fluorescent pseudomonads (iv) secrete biosurfactants and secondary antimicrobial metabolites (antibiosis) like 2,4-diacethylphloroglucinol (DAPG), pyrrolnitrin and phenazines to outcompete other microorganisms. As these compounds act actively against a wide range of phytopathogenic microbes by affecting the cell membrane integrity or the electron transport chain (Raaijmakers *et al.*, 2009), this antagonism also provides benefits to the associated plant.

Besides those indirect contributions to plant welfare, *Pseudomonas* spp. may also directly benefit to plant growth and health: On the one hand, their presence can activate plant defence mechanisms against a broad range of phytopathogens. This phenomenon termed “induced systemic resistance” (ISR) is triggered by bacterial determinants like siderophores, lipopolysaccharides and salicylic acid, which, when recognized by plant receptors, induce jasmonate- and ethylene-regulated pathways in the respective plant resulting in enhanced resistance against diseases caused by viruses, bacteria, fungi, parasitic plants and nematodes (Vallad & Goodman, 2004). On the other hand, pseudomonads are known to increase plant growth by releasing phytohormons such as indole-3-acetic acid (IAA; Xie *et al.*, 1996) and by nutrient mobilization. Moreover, they may lower growth-inhibiting levels of ethylene in plant tissues by production of 1-aminocyclopropane-1-carboxylic acid (ACC)-deaminase, an enzyme which degrades the ethylene precursor ACC (Glick *et al.*, 1998; Patten & Glick, 2002).

Fungi of the genus ***Trichoderma*** are characterized by rapid growth, mostly bright green conidia and a repetitively branched conidiophore structure (Gams & Bissett, 1998). They account for the major portion of the fungal biomass in agricultural soils (Danielson & Davey, 1973) and are also successful colonizers of the plant rhizosphere. Their ubiquitous abundance is due to their efficient utilization of various substrates as well as their competition strength. Similarly to pseudomonads, *Trichoderma* spp. release lytic enzymes like chitinases and glucanases as part of their defence actions (Viterbo *et al.*, 2002). This so-called “mycoparasitism” by *Trichoderma* includes the constitutive production of fungal cell wall degrading enzymes coupled with very sensitive receptors for the respective cell wall fragments released from target fungi. Detection stimulates directional growth towards the target microorganisms. After physical contact, *Trichoderma* coils around and may form appresoria on the surface of the host. Enhanced secretion of mycolytic enzymes leads to degradation of the target hyphae and finally to host penetration (Viterbo *et al.*, 2002; Shoresh *et al.*, 2010). Additionally,

mycotoxins and more than 100 metabolites with antibiotic activity including polyketides, pyrones, terpenes and polypeptides were secreted by *Trichoderma* spp. in order to compete in their ecological niche (Sivasithamparam & Ghisalberti, 1998; Schuster & Schmoll, 2010). By synergistic application of mycoparasitism and antibiosis, *Trichoderma* is able to control phytopathogens belonging to ascomycetes, basidiomycetes and oomycetes (Monte, 2001; Benitez *et al.*, 2004), and therefore contributes highly to plant health. Besides these indirect benefits to plant fitness, *Trichoderma* spp. can also increase plant growth and health directly by enhancing nutrient uptake in plants, producing auxin-like phytohormons and inducing systemic resistance in the host plant (Contreras-Cornejo *et al.*, 2009; Shoresh *et al.*, 2010).

Furthermore, according to their high plant beneficial potential, several strains of *Pseudomonas* and *Trichoderma* are nowadays commercially available bioinoculants used in agriculture to substitute fungicide application (Berg, 2009).

6.2.3. Neutral interactions

Neutral interactions are formed with saprophytic microorganisms maintaining nutrient cycles by the decomposition of organic material in soil. Although the plant is not directly influenced by these microbes, their presence is essential for the functioning of soil ecosystems. Furthermore, they also play an important role in microbe-microbe interactions in the rhizosphere. Consequently, their absence would strongly impact soil quality and thus plant health.

The degradation of organic compounds by microbial enzymes is of central importance for soil fertility, as carbon (C) and nitrogen (N) are mobilized from the dead biomass and returned to the global nutrient cycles (Ladd & Butler, 1972; Paul & Clark, 1996). Therefore, soil enzymes could be used as indicators for soil quality based on their importance in maintaining plant productivity and their sensitivity towards factors like amendment type, crop rotation, climate and tillage (Naseby & Lynch, 1997; Ndiaye *et al.*, 2001). In terrestrial ecosystems, the majority of soil N is present in organic macromolecules like proteins, nucleic acids and chitin (Schulten & Schnitzer, 1997; Lipson & Näsholm, 2001). Because these compounds cannot be assimilated by plants, plant growth is often N-limited (Paul & Clark, 1996). Therefore, the hydrolysis of proteins and chitin is particularly important in making N available for plants (Jones *et al.*, 2005a, b). The process of proteolysis in soil is conducted mostly by

peptidases of bacterial origin (Watanabe & Hayano, 1994). By selective inhibition of different groups of bacterial proteases, it has been shown that alkaline and neutral metalloproteases (Bach & Munch, 2000) are mainly responsible for protein degradation in arable soils. Furthermore, proteolytic communities in soils seemed to be dominated by *Pseudomonas* spp., especially *P. fluorescens* (Bach & Munch, 2000; Sakurai *et al.*, 2007), which are known to produce various exoenzymes, e.g. alkaline metallopeptideses. Moreover, also *Bacillus* spp. contribute mostly to protein degradation by producing neutral metalloproteases (Watanabe & Hayano, 1994; Bach & Munch, 2000; Bach *et al.*, 2002). Similarly, many bacteria produce chitinases to hydrolyze chitin as a nutrient source for growth (Xiao *et al.*, 2005) and thus also contribute largely to the nutrient recycling, as chitin is the second most abundant polymer on earth (Gooday, 1990a, b). Bacterial chitinases are divided into groups A, B and C based on differences in the amino acid sequences of their catalytic domain (Suzuki *et al.*, 1999). Due to their prevalence in nature, group A chitinases are most widely studied (Williamson *et al.*, 2000; Metcalfe *et al.*, 2002; Xiao *et al.*, 2005; Lian *et al.*, 2007). As the potential for the microbial degradation of diverse nitrogenous compounds in the soil depends on the indigenous microbial community containing genes encoding for the required enzymes, quantitative real-time PCR can be used to assess the mineralization capacity as marker for soil fertility.

7. Molecular tools to study microbial communities in soil ecosystems

Before the introduction of molecular techniques in microbial ecology, identification and quantification of microorganisms was mainly based on morphological and physiological traits determined microscopically and by cultivation on artificial media. Although those methods allowed a rough characterization, they depend on cultivability of microbes and thus could not give actual information about biological variety, composition of microbial populations and specific activity in soils. Not before the introduction of new molecular methods allowed identification of uncultivable bacteria in addition, a deeper insight into soil microbial communities was possible (Woese, 1987). Nowadays, various cultivation-independent techniques to investigate microbial communities in soil exist. Many were nucleic acid-based, targeting either DNA or RNA according to research objective. Whereas DNA-approaches provide primarily information about the presence of specific genes targeting phylogenetic or functional microbial groups, RNA-based methods allow the determination of gene expression

pattern (mRNA) or cellular growth and activity (rRNA). However, such transcriptome studies are only snapshots of a very narrow time frame, as most microbial mRNA shows halftimes ranging from a few minutes up to hours (Hambraeus *et al.*, 2003).

Despite the advantages of nucleic acid-based methods for the characterization of microbial soil communities, several inherent limitations occur: Firstly, the choice of the most suitable extraction procedure is crucial, as various commercial kits and protocols are available (DNA/RNA coextraction (Griffiths *et al.*, 2000b), DNA extraction (Yeates *et al.*, 1998), RNA extraction (Sessitsch *et al.*, 2002)). Among others, the minimization of co-extracted humic acids which may inhibit subsequent analyses is an important issue. Secondly, raw extracts of nucleic acids from soil usually contain insufficient target gene copies for direct analysis. Therefore, amplification of the gene of interest by subsequent PCR is required, leading to PCR-related biases like inadequate primer specifity, formation of secondary structures of the template, misincorporation of nucleic acids by the *Taq* polymerase, formation of chimeric molecules and heterogeneity of template sequences (Meyerhans *et al.*, 1990; Pallansch *et al.*, 1990; Eckert & Kunkel, 1991; von Wintzingerode *et al.*, 1997; Wang & Wang, 1997).

Additionally, investigation of microbial community could also be based on proteomics. But, as total protein extraction is difficult due to the heterogeneity of proteins, mostly only specific proteins are isolated. Therefore, only restricted information about microbial population can be obtained. Altogether, a combined approach based on the genome-transcriptome-proteome cascade would provide a great potential for understanding the complexity of soil microorganisms and their contribution to ecosystem functioning.

Besides these methods characterizing genotypic criteria, microbial populations can also be investigated via phenotypic traits, e.g. by phospholipid fatty acid (PLFA) analysis targeting essential membrane components of all cells alive (Zelles, 1999). Although this method offers a more powerful approach to recognize changes in total living microbial community structure than PCR-based techniques, it provides only limited insight into changes in specific populations (Ramsey *et al.*, 2006). Therefore, analysis method must be chosen according to research objective: if changes in total microbial community structure shall be assessed, the PLFA approach is most powerful, but if information on underlying changes in microbial populations is needed, a PCR-based analysis should be selected.

When investigating microbial communities in the rhizosphere, not only the population structure itself but also (i) the identification of microorganisms particularly promoted by plant root exudates and (ii) the

contribution of different microbial groups to carbon transformation processes in the plant-soil system is of interest. By growing plants in ^{13}C-CO_2 enriched atmosphere, carbon fluxes through plants into soil can be determined by stable isotope probing (SIP). By coupling with either nucleic acid- or PLFA-based approaches, characterization of microbial communities utilizing photosynthates exudated from plant roots into the rhizosphere is possible (Griffiths *et al.*, 2004; Cebron *et al.*, 2007; Esperschütz *et al.*, 2009; Wu *et al.*, 2009).

In order to gain insight into the complexity of microbial communities in the rhizosphere of different potato genotypes, a polyphasic approach including quantitative real-time PCR (qPCR) and PLFA analysis combined with stable isotope probing (PLFA-SIP) was chosen in this Ph.D. thesis.

7.1. Quantitative real-time PCR

The traditional “end point” PCR, where amplicons are only analyzed after completion of the final PCR cycle, allows qualitative description of microbial diversity indeed but often falsifies quantitative assessment. This is due to the inherent biases associated with the amplification of targets from mixed template community DNA (Reysenbach *et al.*, 1992; Suzuki & Giovannoni, 1996; Polz & Cavanaugh, 1998), leading to the problem that the proportion of the numerically dominant amplicons do not necessarily reflect the numerically dominant genes in complex environmental samples. To overcome these limitations, qPCR has recently been applied to environmental microbiology and now allows detection and quantification of microbial gene or transcript numbers from environmental samples without the “end point” PCR-related biases (Reysenbach *et al.*, 1992; Suzuki & Giovannoni, 1996; Lueders *et al.*, 2004).

In this method, a fluorescent dye accumulating in direct proportion to the yield of amplified PCR products enables template quantification during every cycle (namely in real-time) of the exponential amplification stage. Consequently, initial concentration of the target gene can be calculated by determining the cycle at which fluorescence (and thus amplicon accumulation) for the first time significantly exceeds background fluorescence (Heid *et al.*, 1996). At this point termed “threshold cycle” (C_t), amplified gene copy numbers are proportional to those of the initial template extracted from the sample. Hence, unknown samples can be quantified by comparing their C_t values to a standard curve constructed from known amounts of the gene of interest.

The following fluorescent chemistries can be used for quantification: (i) dyes binding to dsDNA, e.g. SYBR® Green I (ii) DNA sequence-specific probes, including *Taq*Man® probe, molecular beacon and dual hybridization probe, and (iii) DNA sequence-specific primer like Ampifluor®, Scorpion® and Light Upon eXtension® (LUX) primer (Zhang & Fang, 2006). In this Ph.D. thesis, both SYBR® Green I (**Publication I, II**) and *Taq*Man® probe (**Publication II**) were used as fluorescent reagents in qPCR.

SYBR® Green I intercalates all dsDNA, emitting a fluorescent signal following light excitation (Fig. 3a). Thus, with the accumulation of amplicon numbers during PCR also fluorescence increases. As SYBR® Green I does not discriminate between target amplicons and non-specific PCR products, the use of primer pairs highly specific for their target sequence is essential. Furthermore, primer should also not exhibit self-complementarity, in order to prevent primer-dimer formation. To overcome the unspecifity of SYBR® Green I, a post-PCR dissociation curve analysis generating melting peaks of the amplicons should be carried out, as it allows the distinct differentiation between target products, non-specific amplicons and primer-dimer (Rasmussen *et al.*, 1998). Moreover, as target genes of different organisms may diverge in their GC content, multiple melting peaks might occur. Then, amplicon specifity must be confirmed by agarose gel electrophoresis.

The *Taq*Man® method utilizes a linear oligonucleotide probe with a 5' end fluorophore, termed "reporter", and a 3' end quencher, that can hybridize to an additional conserved region within the target amplicon sequence (Livak *et al.*, 1995). Due to fluorescence resonance energy transfer, the fluorescence emitted by the "reporter" is dimmed by the nearby quencher. After DNA denaturation during PCR, primers and probe bind to their target sequences. During subsequent template elongation, the 5' exonuclease activity of the *Taq* polymerase cleaves the fluorophore from the probe. As now the "reporter" is no longer close to the quencher, a fluorescent signal is detected (Fig. 3b). Thus, template amplification is measured by the release and accumulation of the "reporter" during the extension stage of each PCR cycle. Contrary to the SYBR® Green I assay, the specifity of the *Taq*Man® probe ensures that the fluorescent signal generated during qPCR is derived only from amplification of the target sequence. The mechanisms of both fluorescent dyes are shown in Figure 3.

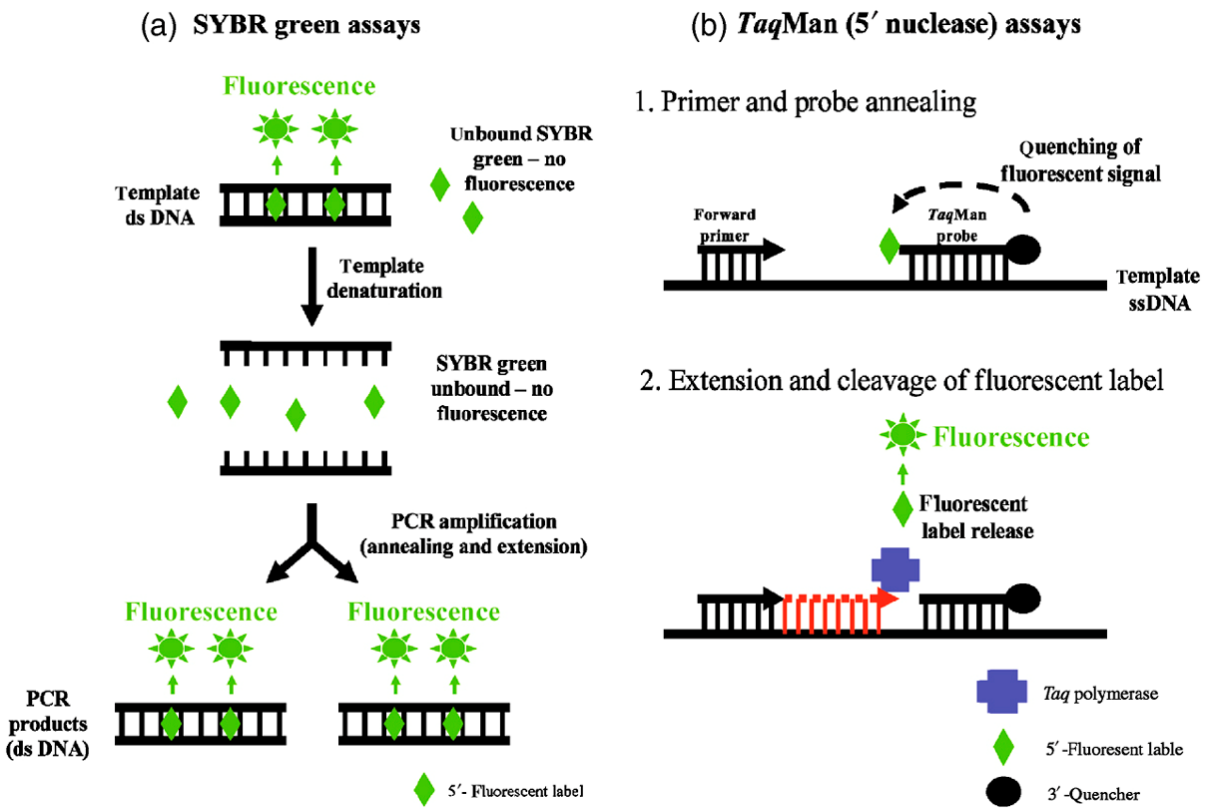

Fig. 3 Real-time PCR chemistries: **(a)** SYBR® Green I assay and **(b)** *Taq*Man® assay (adapted from Smith & Osborn, 2009)

Overall, for gene quantification with SYBR® Green I no complicated probe design is required, SYBR® Green I can be used in conjunction with any primer set, and the fluorescent reagent itself is less costly than a hybridization probe. In turn, the use of TaqMan probes allows the establishment of multiplex qPCR protocols combining differently labelled probes in the same PCR assay, and, due to the high probe specifity, no post-PCR analyses are necessary.

However, although qPCR has been shown to be a robust, highly reproducible and sensitive method to quantitatively track phylogenetic and functional gene changes under varying conditions, most limitations associated with all PCR-based approaches remain in force, including particularly the choice of extraction protocol and primer specifity. Furthermore, in order to obtain PCR efficiencies beyond 80%, generated amplicons should contain less than 500 bp (Pfaffl, 2001), which excludes most primer systems used for diversity analyses. Another important point for interpretation of abundance data is the possibility of multiple gene copies per cell, which has been described for 16S rRNA genes (Klappenbach *et al.*, 2001) and functional genes (Mrkonjic Fuka *et al.*, 2008). Thus, quantified gene

copy numbers cannot be converted directly to cell densities. Nevertheless, to understand microbial functioning in the environment at a molecular level, it is essential to look not only on the gene diversity but also on gene abundance and distribution within the environment, for which qPCR offers a powerful tool.

7.2. Phospholipid fatty acid analysis

Characterization of microbial community structure can be based on both genotypic and phenotypic criteria. By the use of nucleic acid-based approaches for analyses, such as DGGE, TGGE and T-RFLP, microbial community can be characterized down to subspecies level, thus offering a very detailed insight into population structure. However, these techniques require the amplification of target sequences, resulting in the typical “end point” PCR biases described above. Therefore, if such a high taxonomic resolution is not necessary, conventional phenotypic fingerprinting methods are preferable (Ramsey *et al.*, 2006).

Probably the most common phenotypic approach for characterization of microbial soil communities is PLFA analysis. Although this method cannot give the species composition of microbial communities because many phospholipids are not related to a special taxonomic group, it offers a powerful approach to determine changes in total living microbial community structure (Green & Scow, 2000; Ramsey *et al.*, 2006), as PLFA are essential membrane components of all cells (Zelles, 1999) but are rapidly degraded after cell death (White *et al.*, 1979). Basically, membrane fatty acids can be separated into straight-chain and branched-chain fatty acids, according to their biosynthetic relationship (Kaneda, 1991). But, as both the PLFA extract contains all fatty acids of the whole microbial community and different microbial genera may vary in their phospholipid composition due to diverse synthetic pathways, data interpretation for environmental samples is extremely difficult. To simplify, a summarization of the main PLFA groups found in soil is given in Table 2.

Table 2. Phospholipid fatty acid biomarkers commonly used for specific groups of microorganisms (according to Zelles, 1997, 1999; Leckie *et al.*, 2004)

PLFA type	Indicator value	Reference
SATFA	Bacteria, eucaryotes	Zelles, 1999
-br	Gram-positive bacteria	Lechevalier & Moss, 1977; Kroppenstedt, 1992
-iso / anteiso	Gram-positive bacteria	Lechevalier & Moss, 1977; Kroppenstedt, 1992
-cy	Gram-negative bacteria	Ratledge & Wilkinson, 1989
-br10	Actinomycetes	Lechevalier & Moss, 1977; Kroppenstedt, 1992
MUFA	Gram-negative bacteria	Ratledge & Wilkinson, 1989
PUFA	Eucaryotes	Zelles, 1999
18:2ω6,9	Fungi	Ratledge & Wilkinson, 1989
18:3	Fungi, plants	Ratledge & Wilkinson, 1989; Zelles, 1999
20:4ω6,9,12,15	Microeucaryotes	Lechevalier & Moss, 1977
PLOH	Gram-negative bacteria, fungi, plants	Zelles, 1997; Zelles, 1999
NEL-PLFA	Anaerobic bacteria	Zelles, 1999

Saturated fatty acids (SATFA) consist of straight-chain fatty acids (nor), branched-chain fatty acids (br) and cyclopropyl fatty acids (cy). Although straight-chain fatty acids are ubiquitous in cell membranes (Zelles, 1999), they represent mainly eucaryotes and higher plants when containing more than 20 carbon atoms. Branched-chain fatty acids (iso, anteiso or unknown methyl-branching) describe commonly Gram-positive bacteria without further subdivision, with exception of a methyl-branching at the tenth carbon atom (br10), which indicates specifically actinomycetes (Lechevalier & Moss, 1977; Kroppenstedt, 1992). Moreover, cyclopropyl fatty acids can be found in Gram-negative bacteria as well as in anaerobic Gram-positive bacteria (Ratledge & Wilkinson, 1989). Cyclopropyl and br10-fatty acids are derivates of straight-chain monounsaturated fatty acids, which has to be considered in interpretation.

Monounsaturated fatty acids (MUFA) are typical biomarkers for Gram-negative bacteria (Ratledge & Wilkinson, 1989). Although they are found additionally in Gram-positive bacteria, their relative amount to total PLFA content within this group is less than 20% (Zelles, 1999). According to previous studies, physiological stress in microbial community, e.g. through altered environmental conditions, results in an increased MUFA content (Ratledge & Wilkinson, 1989; Heipieper *et al.*, 1996).

Contrary to SATFA and MUFA, polyunsaturated fatty acids (PUFA) are distinctive for fungi, algae and protozoa and only rarely detected in bacteria (Ratledge & Wilkinson, 1989). The linoleic fatty acid PUFA 18:2ω6,9 was detected in particular high amounts in fungal cell walls, and a positive correlation with the fungal biomarker ergosterol has been postulated (Frostegård & Bååth, 1996). But for interpretation it has to be taken into account that linoleic and linolenic fatty acids are ubiquitous among eucaryotes in general.

Additionally, hydroxyl-substituted (PLOH) and non ester-linked (NEL-PLFA) fatty acids are present in environmental samples, but were not considered in this Ph.D. thesis. PLOH can indicate Gram-negative bacteria, fungi and plants, whereas NEL-PLFA are used as biomarker for anaerobic bacteria, as sphingolipids, plasmalogens and other aminolipids are typical for this fatty acid fraction (Zelles, 1999).

7.3. Stable isotope probing

A very common method to assess activities of microbial rhizosphere communities is to expose plants to a ^{13}C-CO_2 enriched atmosphere. By following the ^{13}C signature through plants into soil, it is possible to locate photosynthetically assimilated carbon within the plant itself, and to track the plant derived carbon into the soil pool of water-extractable organic carbon (WEOC) and the total microbial biomass. Subsequently, microbial communities utilizing carbon substrates exudated by plant roots can be identified by targeting DNA, RNA or PLFA. Approaches targeting labelled DNA or RNA for community characterization obtain lots of methodical problems, e.g. extraction, purification and the need of high label incorporation for separation from non-labelled template, resulting in long incubation time and/or high concentrations of the labelled substrate not reflecting natural environmental conditions. Therefore, most studies investigating plant-microbes interactions used PLFA-SIP (Treonis *et al.*, 2004; Prosser *et al.*, 2006; Williams *et al.*, 2006; Lu *et al.*, 2007; Esperschütz *et al.*, 2009; Wu *et al.*, 2009). This method allows a very sensitive detection of microorganisms metabolizing plant derived carbon, due to analysis by gas chromatography-combustion-isotope ratio mass spectrometry (GC/C/IRMS), which can detect very low amounts of stable isotope already. Therefore, potential cross-feeding problems by the need of long incubation time and/or high labelled substrate concentrations can be avoided.

When interpreting carbon fluxes within plant-soil systems, it must be considered that almost up to 50% of the assimilated carbon may be lost by plant respiration (Morgan & Austin, 1983). Additionally, also a minor part of the remaining carbon subsequently translocated into soil is released in form of CO_2, due to both endogenous respiration of soil microbes and exoenzyme activities (Kindler *et al.*, 2006). Therefore, only a certain amount of the photosynthetically fixed labelled carbon can be detected in plant, WEOC or soil microorganisms. Furthermore, it has to be taken into account that the ratio of fixed to respired carbon may differ between microbial groups, according to metabolic differences (Ostle *et al.*, 2007). Additionally, labelling incorporation is also determined by the amount of available labelled substrate. While fast growing *r*-strategists utilize the major part of root exudates (Fontaine *et al.*, 2003), only a low amount of labelled carbon remains for slow growing *k*-strategists. Consequently, considerably less ^{13}C could be detected in the latter. Besides, it is also important to consider isotopic fractionation when interpreting carbon fluxes. Some biochemical reactions within a plant-soil system are known to discriminate against one carbon isotope, resulting in the accumulation of the other one and thus in an enriched or depleted $\delta^{13}C$ value. As fractionation occurs first during photosynthesis, leaves show a lower $\delta^{13}C$ content than the surrounding atmosphere. Subsequent fractionation steps lead to a continuously decrease of $\delta^{13}C$ values from assimilation tissue to roots to WEOC to soil microbes (Damesin & Lelarge, 2003).

8. Outline of the Ph.D. thesis

Despite the annually increasing area under cultivation of GM plants (James, 2009), their release is still highly controversial in many countries. Apart from the many benefits offered by GM plants, the debate on their safety is ongoing, including the possibility of creating invasive plant species, the unintended consequences of transgene flow to indigenous plants and microorganisms, development of super pests, and the effects of transgenic plants on non-target above- and below-ground (Wolfenbarger & Phifer, 2000). Although traits of GM plants do not necessarily differ from those introduced by conventional breeding, regulatory mechanisms clarifying potential risks and benefits specifically for each introduced trait are needed to avoid generalisation according to unexplained observations (Barton & Dracup, 2000).

In the present Ph.D. thesis, the amylopectin-accumulating GM potato line #1332, generated by the Bayerische Landesanstalt für Landwirtschaft (LfL), was investigated for its potential impact on the structural and functional diversity of associated microbial communities in the rhizosphere. Therefore, greenhouse and field studies were conducted, whereby the latter were associated with a great-scale field release of the appropriate GM potatoes due to the conduction of GM plant monitoring in the frame of EU legislation. Despite the use of a tuber-specific promoter consisting of 5' regulatory GBSS sequences, it cannot be excluded that the genetic modification might affect the whole plant metabolism, resulting in altered carbon and nitrogen partitioning to the roots and thus modified root exudation. As root derived carbon is the primary C source in soil during plant vegetation period, an altered exudation pattern could impact the activity and composition of microbial rhizosphere communities. While most rhizosphere microorganisms provide benefits to their host plant, this in turn may reduce plant growth and health. Therefore, the aim of this Ph.D. thesis was to assess the potential effects of genetic modification (i) on microbial groups contributing in general to soil nutrient recycling by mineralization (**Publication I**), (ii) on plant beneficial microorganisms and potato pathogens in the rhizosphere (**Publication II**), and (iii) on photosynthate allocation within the plant and the rhizosphere microbial community (**Publication III**). For investigation of microbial populations, both qPCR (**Publication I, II**) and PLFA-SIP (**Publication III**) approaches were used. In order to relate the potential GM-caused impacts to natural variation among potato cultivars, which we postulate to exceed the potential effect of genetic modification, the GM line was compared to its parental cultivar 'Walli' and a second non-transgenic commercial potato variety. Furthermore, the influence of plant developmental stage was considered by sampling at different plant vegetation stages. Regarding to the ongoing debate about the necessity for field experiments in transgenic research, the experiments were conducted under both greenhouse and field conditions (**Publication I, II**).

As in soil ecosystems the majority of N is present in organic macromolecules like proteins, nucleic acids and chitin which cannot be assimilated by plants, their hydrolysis by soil microbes is particularly important for making N available to plants. Therefore, in **Publication I** the influence of the GM potato line on the bacterial mineralization potential was examined by quantifying copy numbers of key genes (gene encoding the bacterial alkaline metalloprotease (*apr*), gene encoding the bacterial neutral metalloprotease (*npr*), gene encoding the bacterial chitinase group A (*chi*A)) contributing to microbial degradation of diverse nitrogenous compounds in the rhizosphere, using qPCR. Although gene

abundances do not necessarily imply actual turnover rates, they may provide a microbial basis for understanding substrate fluxes (Sharma *et al.*, 2007).

Our results revealed no difference in gene abundance pattern between the GM potato and its parental cultivar but between the two natural varieties. Furthermore, the plant developmental stage seemed to be a more important driver for measured functional gene abundance than the plant genotype. Additionally, we found the *apr*-harbouring microorganisms to predominate proteolytic bacterial community. By comparing greenhouse and field data, no relevant differences were obtained, suggesting the greenhouse design as suitable model for the investigated parameters.

When evaluating the effect of genetic modification on rhizosphere microbes, also those microorganisms contributing to plant growth and health by nutrient mobilization, production of phytohormons, induction of systemic resistance in the host plant, and/or by acting as biocontrol agents should be addressed. In turn, the presence of plant pathogens must be considered, too. Hence, in **Publication Ⅱ**, the rhizosphere abundance of *Pseudomonas* spp. and *Trichoderma* spp. as model organisms for plant beneficial microbes was determined using qPCR. Moreover, gene copy numbers of the two most devastating potato pathogens, *Clavibacter michiganensis* and *Phytophthora infestans*, were quantified in the rhizosphere. Additionally, total bacterial and fungal abundance was measured by addressing 16S rRNA and ITS rRNA genes.

Our results revealed that the examined gene abundance pattern were not affected by genetic modification but by natural variation, as differences between the non-transgenic potato varieties were observed for all investigated genes, except ITS rRNA of *Trichoderma* spp.. Furthermore, also the influence of plant developmental stage could be shown. By comparing greenhouse and field data, field-grown plants showed a higher microbial abundance in the rhizosphere than plants grown in greenhouse. Besides, the effect of plant cultivar and plant vegetation stage was more pronounced in field than in greenhouse, indicating the necessity of field experiments to assess the risks of GM plants, as potential changes might be overlooked under controlled conditions.

Despite the lack of GM-dependent effects on gene abundance pattern of selected microbes with specific functions in ecosystem, it cannot be excluded that genetic engineering leads to altered carbon allocation within the whole GM potato, although a tuber-specific promoter is used. Furthermore, this may result in modified root exudation, causing shifts in microbial population structure that cannot be

discovered by quantifying gene copy numbers. Therefore, an additional greenhouse experiment with ^{13}C pulse chase labelled potato plants was conducted, which is described in **Publication Ⅲ**. Using isotope ratio mass spectrometry, the photosynthate partitioning through the plant into WEOC and subsequently into soil microbial community could be tracked. By combination with PLFA analysis, those microbial groups utilizing plant derived carbon were identified.

Our results revealed that the genetic modification had no influence on carbon allocation within plants or on rhizosphere microbial community structure and activity, which was in agreement with the lack of effects on gene abundance pattern investigated previously. In turn, ^{13}C distribution in above-ground plant biomass, WEOC, total microbial biomass carbon and individual PLFA differed significantly among the natural cultivars. Furthermore, shifts in microbial community structure were observed between the non-transgenic potato varieties. Besides the considerable impact of plant cultivar, also plant developmental stage affected carbon partitioning via plant into rhizosphere and, subsequently, microbial community structure.

9. General and summarizing discussion

The present Ph.D. thesis was part of a project funded by the Bayerisches Staatsministerium für Umwelt, Gesundheit und Verbraucherschutz (StMUGV) with the objective to assess potential effects of the GM amylopectin-accumulating potato line #1332, generated by the LfL, on microbial soil communities. As photosynthetically assimilated CO_2 released via root exudates is the primary C source in soil during vegetation period, the plant's influence on soil microorganisms is highest in the rhizosphere. Therefore, potential impacts depending on the growth of GM plants are likely to detect there. However, genetic modification of a plant results in a more targeted modification of the plant genome than obtained by classical breeding techniques (Catchpole *et al.*, 2005). Thus, effects of the genetic modification on microbial rhizosphere populations might be minor compared to the influence of genotype differences among natural cultivars. Consequently, besides the parental cultivar, 'Walli', a second non-transgenic potato variety was included in the present study. This allows the relation of potential GM-caused differences to natural cultivar variability. As root exudation depends not only on plant genotype but also on plant vegetation stage (Jones *et al.*, 2004), rhizosphere communities were additionally investigated at different plant developmental stages. In order to gain insight into the

functional and structural composition as well as the activity of microbial rhizosphere communities, a polyphasic approach including qPCR and PLFA-SIP was chosen. Furthermore, carbon fluxes from plant into soil were investigated by isotope ratio mass spectrometry. Detailed information about the used methods is given in chapter 7.

9.1. Effects of genetic modification on the abundance of microorganisms with specific ecological function in the rhizosphere

For the detection of potential impacts of GM plants on soil microbes a detailed picture of the prevailing microbial community is required. As traditional cultivation-based methods gather less than 1% of the soil microbiota (Amann *et al.*, 1995) which is unlikely to be representative for the entire population (Rondon *et al.*, 1999), numerous molecular techniques have been developed to overcome this problem, including hybridization-based and PCR-based approaches. Whereas the first have detection limits in the order of 10^5 DNA/RNA copies and thus can only be used for environmental samples with relatively high microbial concentrations, PCR techniques are much more sensitive (Zhang & Fang, 2006). To overcome the problems associated with methods based on traditional “end point” PCR discussed in chapter 7.1., in the present Ph.D. thesis abundance pattern of distinct microbial groups in potato rhizosphere were investigated using qPCR.

Our results revealed no differences between the GM potato and its parental cultivar regarding abundance pattern of both phylogenetic genes (16S rRNA, ITS rRNA, *Pseudomonas* specific 16S rRNA, ITS rRNA of *Trichoderma*, IGS rRNA of *Clavibacter michiganensis* and nuclear satellite sequence of *Phytophthora infestans*) and functional genes (*apr*, *npr* and *chi*A). This confirms findings of other studies, where GM-dependent effects on microbial community structure in the rhizosphere were also not found (Heuer *et al.*, 2002; Schmalenberger & Tebbe, 2002). Although most other reports showed significant changes by the growth of GM plants, the modification-dependent effects were described as minor compared to shifts caused by the soil type, plant genotype and plant developmental stage during the monitoring (Lottmann *et al.*, 1999; Gyamfi *et al.*, 2002; Milling *et al.*, 2004; Rasche *et al.*, 2006; Weinert *et al.*, 2009).

In agreement with Starnier *et al*. (1966), who reported *Pseudomonas* spp. as ubiquitous soil bacteria excreting extracellular metalloproteases, we found a significant positive correlation between gene

abundance pattern of *Pseudomonas* specific 16S rRNA and *apr*. This indicates that pseudomonads contribute mainly to *apr*-expressing communities in the rhizosphere. Coinciding, Sakurai *et al*. (2007) observed a close relationship between *apr* varieties isolated from different fertilized soils and *apr* genes derived from *P. fluorescens*. These findings confirmed the previous report of *Pseudomonas* spp. being the most abundant proteolytic species in arable, grassland and forest soils (Bach & Munch, 2000).

In contrast to *apr*, no correlation to *Pseudomonas* abundance was found for *npr* but instead a positive relationship with total 16S rRNA genes. Similarly, Mrkonjic Fuka *et al*. (2008) could also demonstrate that *npr* and 16S rRNA gene copy numbers were positively correlated in different arable soils. Interestingly, in that study *npr* sequences were highly diverse and related to *npr* genes from *Vibrio* spp., *Bacillus* ssp., *Thermoactinomyces* ssp., *Paenibacillus* ssp., *Clostridium* ssp. and *Alicylobacillus* ssp. and largely from unknown proteolytic bacteria (Mrkonjic Fuka *et al.*, 2009). Contrary, Sakurai *et al*. (2007) detected only *npr* genes related to *npr* sequences of *Bacillus* spp., particularly *B. megaterium*. This finding coincides with other reports showing that *Bacillus* spp. were dominant in *npr*-expressing soil communities (Watanabe & Hayano, 1994; Bach & Munch, 2000; Bach *et al.*, 2002).

Furthermore, our results revealed a 1000-fold higher gene copy number for *apr* than for *npr*. This was not surprising when considering that *apr*-expressing community is predominated by *Pseudomonas* spp. (Sakurai *et al.*, 2007), which are generally known as the most abundant soil bacteria (Janssen, 2006). Additionally, it could be demonstrated that *Pseudomonas* spp., mainly various biotypes of *P. fluorescens*, were the dominant species of proteolytic communities in four different soils (Bach & Munch, 2000).

When relating *chi*A gene abundance to phylogenetic genes, we found a positive correlation to 16S rRNA gene abundance but a negative relationship to ITS rRNA copy numbers. As chitin is widely distributed in fungal cell walls, this negative correlation may be due to the active degrading of hyphal walls by chitinolytic bacteria. Such is assumed for actinobacteria (Gomes *et al.*, 2000, 2001) which have an important chitinolytic function in soil ecosystems (Metcalfe *et al.*, 2002).

Although in this study no effects on gene abundance pattern dependent on genetic modification were observed, our results revealed significant differences between the two natural potato cultivars in the rhizosphere. This coincides with previous studies, where shifts in microbial communities were also more pronounced between different plant varieties than between the GM plant and its parental line (Gyamfi *et al.*, 2002; Rasche *et al.*, 2006; Weinert *et al.*, 2009). This cultivar-impact is likely due to

genotype-specific root exudation (Grayston *et al.*, 1998; Marschner *et al.*, 2001; Söderberg *et al.*, 2002). Interestingly, the observed differences in gene abundances among the potato cultivars occurred mainly at the early leaf developmental stage, indicating that root exudation pattern may converge with increasing plant age. As the amount and chemical composition of root exudates is known to be highly dependent on plant vegetation stage (Jones *et al.*, 2004), it can be assumed that gene abundance pattern in the rhizosphere finally are more dependent on plant vegetation stage than on genotype differences. In addition, previous studies reported microbial rhizosphere communities being highly contingent upon plant age (Gyamfi *et al.*, 2002; Milling *et al.*, 2004; Rasche *et al.*, 2006).

9.2. Effects of genetic modification on carbon partitioning within plant, soil and rhizosphere microbial community

Coinciding with non-modified gene abundance pattern, GM line #1332 did also not affect microbial community structure investigated by PLFA analysis. However, significant shifts in rhizosphere population structure between the two natural potato cultivars and also during plant growth were observed. Similar results were obtained in a previous study with another amylopectin-accumulating GM potato line, where GM-dependent impacts were detected but described as minor compared to both natural variation among different potato cultivars and influence of plant developmental stage (Milling *et al.*, 2004).

When examining carbon fluxes by tracking ^{13}C through the plant-soil system, we did not observe significant differences between the GM line and its parental cultivar in addition. Therefore, it can be assumed that the present genetic modification has also no effect on carbon metabolism, resulting highly probably in unmodified root exudation pattern and thus in unaltered microbial communities in the rhizosphere. The absence of any influence of the present genetic modification on carbon fluxes within the plant-soil system and microbial community structure in the rhizosphere might be due to either the controlled environmental conditions in greenhouse or the absence of antibiotic marker genes in the GM plant examined in this Ph.D. thesis, as all previously investigated GM plants contained additional genes enabling transformants selection via application of antibiotics and/or herbicides. The latter assumption is confirmed by Wu *et al*. (2009), who investigated rice genetically engineered to produce *Bacillus thuringiensis* toxin (*Bt* rice). While also using PLFA-SIP, significant

differences to the parental line in carbon partitioning and microbial community structure were observed. The authors supposed this not being a result of the inserted *cry1Ab* gene itself, because it has been shown that *cry1Ab* did not cause changes in exudate quality (Saxena & Stotzky, 2001) and the *cry1Ab* protein did not residue in the rhizosphere of the GM plants during rice growth (Wang *et al.*, 2006). Thus, it seems likely that either the insertion of reporter and marker genes or site effects of the insertion of a foreign gene caused changes in the composition of root exudates, as has been suggested previously (Widmer *et al.*, 1997). However, no second non-transgenic plant cultivar was included in that study to examine if the observed differences between the GM plant and its parental natural variety exceed differences between natural cultivars, as was done in other studies (Milling *et al.*, 2004; Rasche *et al.*, 2006; Weinert *et al.*, 2009).

Altogether, our results revealed that the present genetic modification seems to have no influence on carbon partitioning from plant into soil or on plant associated microbial community structure in the rhizosphere. However, it has to be considered that cultivar- and plant developmental stage-dependent effects on gene abundance pattern determined with qPCR were more pronounced in field than in greenhouse. This finding is probably due to the fluctuating weather conditions and the high complexity of natural soil ecosystem in field compared with the controlled environmental conditions prevailing in greenhouse. Therefore, further experiments should be conducted to evaluate if the GM line might be more influenced in uncontrolled field conditions with additional abiotic and biotic stressors.

9.3. Greenhouse versus field studies

By comparing our results of the greenhouse and field experiments described in **Publication** Ⅰ and **Publication** Ⅱ it became obvious that microbial gene abundance pattern was affected much more in field than in a controlled environment. Interestingly, this difference was more visible for abundance pattern of phylogenetic groups than for functional genes, indicating that phylogenetic groups may be more sensitive towards environmental changes than a whole functional community consisting of different genera. This is in agreement with the theory of functional redundancy, which means that different species can perform the same functional role in ecosystems, so that changes in species diversity does not affect ecosystem functioning (Loreau, 2004). This capacity of sharing metabolic

processes between different members of a microbial community is essentiell for its efficiency, especially under stress conditions. Thereby, when a phylogenetic group is outcompeted due to environmental fluctuations, the community functionality is assured by the presence of other, formerly inferior species capable of performing the same function (Marzorati *et al*., 2008; Fernandez *et al*., 2000). Regarding the fluctuating abiotic and biotic conditions prevailing in open land, the need for functional redundancy might be much higher there than in a controlled greenhouse environment, explaining the more pronounced effects on gene abundance pattern found in field.

A previous study investigated the influence of GM potatoes producing lectins on non-target organisms under greenhouse and field conditions (Griffiths *et al*., 2000a). Coinciding with our results, no significant differences in the microbial rhizosphere population due to genetic modification were observed in the greenhouse. Otherwise, in the field experiment alterations in microbial communities were detected, although these did not persist to the next growing season. Therefore, we state according to Griffiths *et al*. (2000a) that a complete experiment should consist best of two sequential tiers: microcosm or pot studies with the option of previous studies under controlled environmental conditions, followed by field tests. Both experimental setup types have potential advantages: Because in microcosm or pot experiments the environmental conditions like humidity or temperature could be controlled, they are easier to conduct and results are more repeatable and interpretable than in field trials (Donegan *et al.*, 1995). However, such studies are highly artificial and not representative for natural situation. In contrast, field experiments are more realistic but the results are often variable and difficult to interprete due to fluctuating weather conditions and the complexity of natural soil ecosystem (Lottmann *et al.*, 1999; Heuer *et al.*, 2002; Dunfield & Germida, 2003). Furthermore, they are often costly, time consuming and labour intensive. In agreement with Liu *et al*. (2005) we propose that the evaluation of the potential effects of GM plants on soil microorganisms begins with experiments in a controlled environment mimicking natural conditions, followed by field studies designed and conducted due to the results of the previous experiments. Contributing to the high influence of soil type and seasonal changes on microbial community (Milling *et al.*, 2004; Rasche *et al.*, 2006; Weinert *et al.*, 2009), these field releases should best include several sites with different soils and should be conducted over several years.

10. Conclusions

Using ^{13}C stable isotope probing, it is possible to track photosynthetically assimilated carbon fluxes through plants into soil and microbial biomass. As plant growth and health is, among others, dependent on microbial community composition in the rhizosphere, which in turn is strongly determined by quality and quantity of root exudates, this technique provides a powerful tool in risk assessment studies, as it facilitates the evaluation of unintended side effects of plant genome transformation on carbon-partitioning characteristics within the GM plant, probably resulting in altered root exudation pattern. In combination with PLFA analysis, microbial population structure in the rhizosphere could be investigated and those microbial groups utilizing plant derived carbon could be identified.

Our results revealed that the genetic modification present in potato line #1332 does not affect carbon fluxes through plant into WEOC and rhizosphere microorganisms. Furthermore, no differences in microbial community structure were observed between the GM plant and its parental cultivar (**Publication III**). Similar results were found when investigating abundance pattern of phylogenetic and functional genes of rhizosphere microorganisms by qPCR (**Publication I, II**). Moreover, it could be shown that carbon-partitioning characteristics, microbial community structure and abundance pattern differed significantly between natural potato varieties and were also dependent on plant vegetation stage (**Publication I–III**), which is in agreement with previous studies.

Although our results may indicate that soil microorganisms are not impacted by the growth of GM potato #1332, it has to be considered that the presented data were based on greenhouse studies under optimal growth conditions (**Publication I–III**) and a 1-year-1-site field trial (**Publication I, II**). Therefore, a generalization of the obtained results with regard to risk assessment of potato line #1332 is not possible. If the growth of this GM potato may cause long-time effects, has to be considered in further field studies, which should be conducted continuously over several years and, due to the recognized influence of soil type (Heuer *et al.*, 2002; Weinert *et al.*, 2009), on different field sites.

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List of abbreviations

ACC	1-aminocyclopropane-1-carboxylic acid
AMF	arbuscular mycorrhizal fungi
apr	gene encoding the bacterial alkaline metalloprotease
BCA	biological control agents
bp	base pair(s)
Bt	genetically modified to produce *Bacillus thuringiensis* toxin
BVL	Federal Office of Consumer Protection and Food Safety
C	carbon
*chi*A	gene encoding the bacterial chitinase group A
CO_2	carbon dioxide
cry1Ab	gene encoding the *Bacillus thuringiensis* toxin
C_t	threshold cycle
DAPG	2,4-diacethylphloroglucinol
DGGE	denaturing gradient gel electrophoresis
DNA	desoxyribonucleic acid
dsDNA	double stranded desoxyribonucleic acid
dsRNA	double stranded ribonucleic acid
e.g.	for example, *exempli gratia*
EFSA	European Food Safety Authority
EPS	exopolysaccharides
EU	European Union
gbss	gene encoding the granule-bound starch synthase
GBSS	granule-bound starch synthase
GC/C/IRMS	gas chromatography-combustion-isotope ratio mass spectrometry
GM	genetically modified
GV	gentechnisch verändert
IAA	indole-3-acetic acid
IGS	intergenic spacer
ISR	induced systemic resistance

ITS	internal transcribed spacer
k-strategists	organisms with high competitive ability that are able to use diverse, complex substrates
LfL	Bayerische Landesanstalt für Landwirtschaft
mRNA	messenger ribonucleic acid
MUFA	monounsaturated fatty acid(s)
N	nitrogen
NEL-PLFA	non ester-linked phospholipid fatty acid(s)
npr	gene encoding the bacterial neutral metalloprotease
PCR	polymerase chain reaction
PGPR	plant growth promoting rhizobacteria
PLFA	phospholipid fatty acid(s)
PLFA-SIP	phospholipid fatty acid analysis combined with stable isotope probing
PLOH	hydroxyl-substituted fatty acid(s)
PUFA	polyunsaturated fatty acid(s)
qPCR	quantitative real-time PCR
RNA	ribonucleic acid
rRNA	ribosomal ribonucleic acid
r-strategists	organisms with high reproduction rate that require simple, readily available substrates
SATFA	saturated fatty acid(s)
SIP	stable isotope probing
spp.	species
ssp.	subspecies
StMUGV	Bayerisches Staatsministerium für Umwelt, Gesundheit und Verbraucherschutz
TGGE	temperature gradient gel electrophoresis
TILLING	targeting induced local lesions in genomes
T-RFLP	terminal restriction fragment length polymorphism
WEOC	water extractable organic carbon

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Publication I

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SHORT COMMUNICATION

Abundance of bacterial genes encoding for proteases and chitinases in the rhizosphere of three different potato cultivars

Silvia Gschwendtner · Michael Reichmann · Martin Müller · Viviane Radl · Jean Charles Munch · Michael Schloter

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Abstract In terrestrial ecosystems, the majority of soil N is present in organic macromolecules like proteins, nucleic acids and chitin. Because these compounds cannot be assimilated by plants, plant growth is often N-limited. Therefore, the hydrolysis of proteins and chitin is particularly important in making N available for plants. In this study, we investigated the influence of different potato cultivars and different plant developmental stages on the abundance of genes encoding for alkaline and neutral metalloproteases (*apr*, *npr*) as well as bacterial chitinases (*chi*A) in the rhizosphere using quantitative real-time PCR in a greenhouse trial and a field study. It could be clearly demonstrated that abundance pattern was mainly affected by the plant vegetation stage, whilst the used plant genotype had only a minor influence on the development of the two functional populations.

Keywords Potato · Real-time PCR · Gene abundance · *apr* · *npr* · *chi*A

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Introduction

Plants have been considered as drivers for microbial diversity in the rhizosphere (Kowalchuk et al. 2002). Whereas this has been verified in many studies for microbial community structure based on the analysis of ribosomal genes (Bremer et al. 2007; Houlden et al. 2008; Kielak et al. 2008), there is still a lack of knowledge about how different functional groups of bacteria, fungi and archaea are influenced by the plant. However, the degradation of organic compounds by microbial enzymes is of central importance for soil fertility as carbon (C) and nitrogen (N) are mobilised from dead biomass and returned to the global nutrient cycles (Ladd and Butler 1972; Paul and Clark 1996). In this respect, the hydrolysis of proteins and chitin is particularly relevant in making N available for plants (Gooday 1990; Jones et al. 2005). To link enzyme activity and community structure, it is necessary to describe abundance pattern of genes coding for functional enzymes involved in nutrient cycling (Nannipieri and Paul 2009).

The cultivation of potatoes is becoming increasingly important in most cropping rotations in Central Europe as potatoes are not only used as food but also for industrial production of papers and other goods. Therefore, many new potato cultivars have entered the market in the last years. Amflora® is the first commercially available genetically modified (GM) potato line which accumulates amylopectin in its tubers. However, the consequences of increased cropping of potato varieties for industrial purpose on soil quality have not been considered so far. Our study focussed on the effects of three different potato cultivars (including one GM line) on proteolytic and chitinolytic rhizosphere microorganisms at different plant developmental stages using real-time PCR to quantify the abundance of alkaline

metalloprotease genes (*apr*), neutral metalloprotease genes (*npr*) and bacterial chitinase group A genes (*chi*A). To estimate the use of greenhouse studies as a model for field trials, data of greenhouse and field were compared.

Materials and methods

Two natural potato cultivars (*Solanum tuberosum* L.), 'Jumbo' and 'Walli', which are used for industrial starch production, as well as the amylopectin-accumulating GM line #1332 constructed from the parental line 'Walli' using RNA silencing strategy (Kuipers et al. 1994; Visser et al. 1991), were planted in April 2007 at a field site in Freising, Germany (48°24′ N/11°43′ E) with soil characterised as: 24% clay, 61% silt, 15% sand, pH 6.6 (measured in 0.01 M $CaCl_2$), 0.6% total nitrogen content, 5.1% total carbon content and a maximum water holding capacity of 38%. The experiment was conducted in a randomised block design consisting of five replicate plots (1.5 × 3 m) per 'Walli', #1332 and 'Jumbo'. In April and May, respectively, the herbicide Artist® (2.5 kg ha^{-1}) and the fungicide 'Epok' (0.5 l ha^{-1} combined with 'Dantop', 35 g ha^{-1}) were applied. Rhizosphere samples were collected according to Huic Babic et al. (2008) at early leaf development (EC_{30}) and flowering stage (EC_{60}) by mixing the roots of four plants per plot to form one composite sample. The five replicate plots were sampled separately and used as independent replicates.

For the greenhouse study, soil from the same site has been collected before the field experiment started, sieved (4 mm mesh), filled into 72 plastic pots (20 × 20 × 20 cm) and adjusted to 60% of the maximum water holding capacity. One plant was cropped per pot. Similarly to the field site, NPK fertiliser (850 kg ha^{-1}) was applied 1 week before planting. In contrast, no herbicide or pesticide was added. To keep the soil water content constant during potato growth, irrigation was performed by hand. Plants were grown at 25/15°C day/night temperature, relative humidity 50% and daylight. Rhizosphere samples were

Table 1 Primer sets and thermal profiles used for the absolute quantification of the respective genes

Target gene	Primer set	Amplicon size (bp)	Reference	Thermal cycling profile	No. of cycles
apr gene	FP aprI RP aprII	194	Bach et al (2002)	95°C–10 min 95°C–20 s/53°C–30 s/72°C–60 s	1 40
npr gene	FP nprI RP nprII	234	Bach et al (2002)	95°C–10 min 95°C–20 s/53°C–30 s/72°C–60 s	1 40
*chi*A gene	chiF2 chiR	406	Xiao et al (2005)	95°C–5 min 95°C–30 s/60°C–30 s/72°C–60 s	1 40

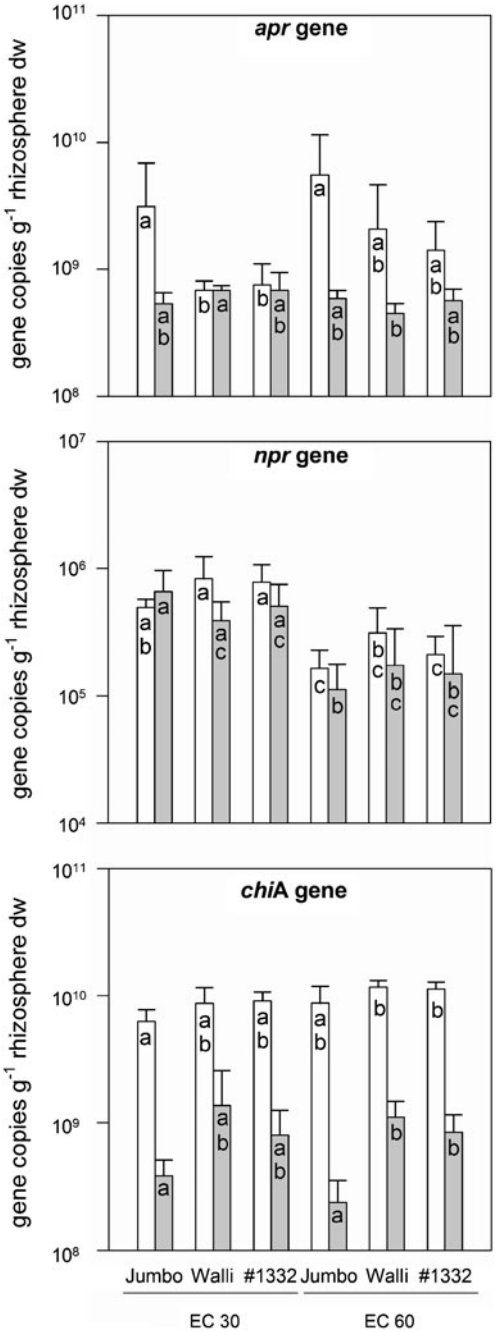

Fig. 1 Gene copy numbers of *apr*, *npr* and bacterial *chi*A per gram dry weight of the rhizosphere soil/root complex (rhizosphere dw) for field-grown plants (*white bars*, $n=5$) and greenhouse-grown plants (*gray bars*, $n=4$) for different cultivars ('Jumbo', 'Walli' and #1332) at two plant developmental stages (EC_{30}, early leaf development, and EC_{60}, florescence). *Error bars* represent standard deviations. *Different letters* indicate significance at $p<0.05$

collected in four independent replicates at EC_{30} and EC_{60} by mixing the roots of three plants to form one composite sample.

The samples of the rhizosphere soil/root complex were immediately frozen on dry ice and afterwards stored at −80°C for nucleic acid extraction.

DNA was extracted as described by Hai et al. (2009). Quantitative real-time PCR was performed on the ABI 7300 Cycler (Applied Biosystems, Germany) using the following assay reagents: dimethyl sulfoxide (DMSO) and betaine (Sigma, Germany), primers (Metabion, Germany) and 2× Power SYBR Green master mix (Applied Biosystems). The reaction mixtures for quantification of the genes listed in Table 1 contained 12.5 µl of master mix, 20 pmol (*apr*, *npr*) or 10 pmol (*chi*A) of each primer and 2 µl of DNA template. For quantification of the *chi*A gene, 0.5 µl DMSO and 2.5 µl betaine were additionally added. All reaction mixtures were adjusted with MilliQ water to a final volume of 25 µl. Calculation of standard curve and amplifications were performed as described by Hai et al. (2009). Amplification efficiency was calculated using the formula $Eff=[10^{(-1/slope)} - 1]$ and resulted in the following average efficiencies (standard deviations <2% of mean): *apr* gene, 92.3%, *npr* gene, 88.9% and *chi*A gene, 94.5%. The quantified gene copy numbers were normalised to 1 g dry weight (dw) of the rhizosphere soil/root complex.

Data were analysed by analysis of variance at a significance level of $p<0.05$ using SPSS 11.5 (SPSS, Inc.).

Results and discussion

In the field experiment, the abundance of *apr* genes was not affected by plant developmental stage but by cultivar as *apr* gene copy numbers in the rhizosphere of 'Jumbo' at EC_{30} were four times higher than those of 'Walli' and #1332 (3.1×10^9 copies per gram dw). In contrast, *npr* gene copy numbers were highly influenced by plant vegetation stage as genes decreased significantly from 7.0×10^5 copies per gram dw at EC_{30} to 2.3×10^5 copies per gram dw at EC_{60}. The *chi*A gene abundance was affected neither by cultivar nor by plant developmental stage, revealing 9.3×10^9 copies per gram dw.

Whereas results between the field trial and the greenhouse study were comparable for factors influencing *npr* gene copy numbers in the rhizosphere, for abundance of *apr* genes in the greenhouse experiment, only plant developmental stage depending effects were visible, revealing 6.3×10^8 copies per gram dw at EC_{30} and 5.3×10^8 copies per gram dw at EC_{60} for all cultivars. Interestingly, *chi*A abundance of greenhouse plants showed a significant cultivar effect at EC_{60} as gene copy numbers in the rhizosphere of 'Jumbo' (2.3×10^8 copies per gram dw) were significantly lower than those of 'Walli' and #1332 (average 9.7×10^8 copies per gram dw). All data are summarised in Fig. 1.

Comparing both proteolytic genes, we observed 1,000-fold higher gene copy numbers for *apr* than for *npr*. This is not surprising as *apr*-harbouring communities are dominated by *Pseudomonas* spp. (Sakurai et al. 2007) which are generally known as highly abundant rhizosphere bacteria (Janssen 2006). Furthermore, Bach and Munch (2000) examined the composition of proteolytic bacteria in four different soils and demonstrated that *Pseudomonas* spp., mainly various biotypes of *Pseudomonas fluorescens*, were the most abundant species.

Overall, our results indicate that for the abundance of the measured functional genes, the plant developmental stage is a more important driver than the genotype of the used plant cultivars. However, for the three functional genes quantified, no clear rhizosphere effect (with increased gene copy numbers at the flowering stage due to enhanced exudation) was visible (Bürgmann et al. 2005). This might be related to the fact that the analysed genes are mainly involved in the degradation of highly polymeric substances, which are not increased at the florescence of the plant in the rhizosphere. No differences in proteolytic and chitinolytic gene abundance pattern between the GM potato line and its non-transgenic parental cultivar were observed. Although this is the first study investigating the potential effects of GM plants on the abundance pattern of functional genes codifying enzymes involved in the N mineralisation, a lot of data on the impact of GM plants on microbial community structure have already been published in the last two decades. Those studies observed either no GM plant-related effects (Heuer et al. 2002; Schmalenberger and Tebbe 2002) or described the GM-dependent influence as negligible compared to shifts caused by soil type, plant genotype and plant vegetation stage during monitoring (Milling et al. 2004; Rasche et al. 2006; Weinert et al. 2009).

By comparing data of greenhouse and field, no relevant differences in the investigated gene abundance patterns were found. Although field-grown plants showed significantly higher *chi*A gene copies in the rhizosphere than greenhouse plants, this may be due to preventive fungicide application in the field, which increases dead fungal biomass and thus chitinolytic microbial community. Whereas we rate our greenhouse design as good model for field studies, we state according to Griffiths et al. (2000) that a complete experimental setup should consist of both trials to overcome the limitations of greenhouse experiments.

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REGULAR ARTICLE

Effects of genetically modified amylopectin-accumulating potato plants on the abundance of beneficial and pathogenic microorganisms in the rhizosphere

Silvia Gschwendtner · Michael Reichmann · Martin Müller · Viviane Radl · Jean Charles Munch · Michael Schloter

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Abstract In this study, the potential effects of a genetically modified (GM) amylopectin-accumulating potato line (*Solanum tuberosum* L.) on plant beneficial bacteria and fungi as well as on phytopathogens in the rhizosphere were investigated in a greenhouse experiment and a field trial. For comparison, the non-transgenic parental cultivar of the GM line and a second non-transgenic cultivar were included in the study. Rhizospheres were sampled during young leaf development (EC30) and at florescence (EC60). The microbial community composition was analysed by real-time PCR to quantify the abundances of *Pseudomonas* spp., *Clavibacter michiganensis*, *Trichoderma* spp. and *Phytophthora infestans*. Additionally, total bacterial and fungal abundances were measured. None of the examined gene abundance patterns were affected by the genetic modification when wild type and GM line were compared. However, significant differences were observed between the two natural potato cultivars, especially during the early leaf development of the plants. Furthermore, gene abundance patterns were also influenced by the plant developmental stage. Interestingly, the impact of the cultivar and the plant vegetation stage on the microbial community structure was more pronounced in field than in greenhouse. Overall, field-grown plants showed a higher abundance of microorganisms in the rhizosphere than plants grown under greenhouse conditions.

Keywords Genetically modified potato plants · Real-time PCR · Gene abundance · Plant beneficial microorganisms · Phytopathogens · Rhizosphere

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Introduction

Interactions between plants and microorganisms in the rhizosphere are of central importance for plant performance (Soerensen 1997). Rhizosphere microorganisms strongly influence plant growth and health via nutrient mobilization, production of plant growth hormones, induction of systemic resistance in the host plant and play a role as biological control agents (Raaijmakers et al. 2009). In turn, they benefit from nutrients provided

by the root exudates and consequently, their abundance and community composition is highly affected by the plant species, different plant genotypes and plant vegetation stage (Grayston et al. 1998; Gyamfi et al. 2002; Kowalchuk et al. 2002; Marschner et al. 2006; Smalla et al. 2001). Contrary to plant beneficial microorganisms, soil-borne pathogens can severely impact plant growth and health and cause great economic losses (Raaijmakers et al. 2009). Despite the importance of plant–microbe interactions in the rhizosphere for crop quality and yield, there is still a lack of knowledge which factors drive abundance and activity pattern of selected functional groups of microorganisms in this compartment.

The discussion how genetically modified (GM) plants influence microbial rhizosphere communities in comparison to the wild type and other non-transgenic cultivars of the same species is still continuing and considerably affects the acceptance of these plants for commercial field release. Among other concerns, this is related to previous studies revealing shifts in diversity patterns of the rhizosphere microflora associated with GM plants, although the modification-dependent effects were described as minor compared to shifts caused by the soil type, plant genotype and plant developmental stage (Gyamfi et al. 2002; Heuer et al. 2002; Liu et al. 2005; Lottmann et al. 1999; Milling et al. 2004; Rasche et al. 2006; Weinert et al. 2009).

However, mainly in the field of renewable resources, GM plants may carry a huge potential for sustainable development, considering not only economical but also ecological aspects. For industrial processing of selected GM crops, e.g. less fossil energy is necessary compared to conventional cultivars. The most discussed GM constructs in this area are potatoes with modified starch composition. Starch consists of the two polysaccharides, amylose and amylopectin. Because of their different physico-chemical properties, amylose and amylopectin have to be separated for industrial use (Kraak 1993). To avoid the high production and energy costs of this separation process (Visser et al. 1991; Young 1984), the production of a potato tuber (*Solanum tuberosum* L.) containing starch composed purely from amylopectin is an important aim in plant breeding, mainly for the paper and textile industries.

Since a modified starch metabolism may also result in altered root exudates and thus influence microbial rhizosphere community (Milling et al. 2004), the aim of this study was to investigate the effect of a GM amylopectin-accumulating starch potato line on representative beneficial and pathogenic microorganisms in the rhizosphere. *Pseudomonas* spp. and *Trichoderma* spp. were selected as bacterial and fungal plant beneficial model microorganisms, respectively. They were chosen because both genera are ubiquitous in soil, are known to be excellent root colonisers (Cordier and Alabouvette 2009; Lugtenberg et al. 2001) and are most widely studied biocontrol agents (Alabouvette et al. 2009). Moreover, they can inhibit soil-borne pathogens by a variety of mechanisms, such as competition for resources, the synthesis of antibiotics and biosurfactants, the production of cell wall-degrading enzymes and the induction of systemic resistance in the host plants (De Meyer et al. 1998; Ghisalberti and Rowland 1993; Haran et al. 1996; Walsh et al. 2001; Winding et al. 2004). Furthermore, *Pseudomonas* spp. and *Trichoderma* spp. are commercially available bioinoculants and are used in agriculture to substitute fungicide application (Berg 2009).

The most important potato pathogen in temperate zones is the oomycete *Phytophthora infestans*, the causal agent of late blight disease (Tooley et al. 1997). Nevertheless, among all potato diseases, the bacterial ring rot of potatoes, caused by the actinomycete *Clavibacter michiganensis* subsp. *sepedonicus*, is probably the most regulated potato disease worldwide with a strict zero-tolerance quarantine of the pathogen in Canada, the USA and Europe (Bach et al. 2003; De Boer and Slack 1984). Due to the high economic losses caused by these two microorganisms, they were selected as representatives for potato pathogens.

To measure the abundance patterns of the plant beneficial and pathogenic indicator organisms mentioned above, real-time PCR assays were performed. The obtained data were linked to total bacterial and fungal abundance quantified via 16S rRNA gene respectively ITS rRNA gene copy numbers. The potential impact of genetic modification on microbial gene abundance was related to natural variation among potato cultivars and the effect of the plant vegetation stage. Furthermore, data from greenhouse and field studies were compared to visualise the importance of field experiments for genetically modified plants (Griffiths et al. 2000a; Kowalchuk et al. 2003; Liu et al. 2005). We postulated that the

effects of the genetic modification were less pronounced than those of genotype differences among cultivars resulting from conventional breeding or of plant vegetation stage.

Materials and methods

Potato cultivars and the GM line

The GM potato line #1332 with increased amylopectin levels in its tubers, its parental cultivar 'Walli' and one additional potato cultivar used for industrial starch production, 'Jumbo', were used in this study. Both natural cultivars were provided by the Bavarian State Research Center for Agriculture (LfL), where also the GM line was developed. The GM line was genetically modified by the insertion of a gene fragment of the granule-bound starch synthase gene (*gbss*), which encodes one of the key enzymes for amylose formation, in antisense direction. Consequently, transcription leads to the formation of *gbss* dsRNA and thus inhibition of *gbss* expression and amylose production (Kuipers et al. 1994; Visser et al. 1991). Hence, the tuber starch of the GM line consists of more than 99% amylopectin, while the starch granules of 'Walli' and 'Jumbo' contain only 75–80% amylopectin (Reichmann, personal communication). A new method without using antibiotic- or herbicide-resistance marker genes for selection was used for transformation (De Vetten et al. 2003), resulting in marker-free GM line #1332.

Field experiment

The experimental field site was located in Freising, approximately 30 km north of Munich, Germany (48°24′N/11°43′E) and had been cultivated under conventional farming management with crop rotation (oilseed rape—winter barley—maize) before the experiment started in April 2007. The soil was characterised as follows: 24% clay, 61% silt, 15% sand, pH 6.6 (measured in 0.01 M $CaCl_2$), 0.6% total nitrogen content, 5.1% total carbon content and a maximum water-holding capacity of 38%. At the beginning of April, one week before potatoes were planted, NPK fertiliser (850 kg ha^{-1}, which was equivalent to 100 kg N ha^{-1}) was applied. The experiment was conducted in a randomised block design consisting of five replicate plots (1.5×3 m) per block with 20 plants per plot. During the vegetation period, the herbicide Artist® (2.5 kg ha^{-1}) and the fungicide 'Epok' (0.5 l ha^{-1} combined with 'Dantop', 35 g ha^{-1}) were applied once in April and May 2007, respectively.

Rhizosphere samples were collected at early leaf development (EC30; May) and the flowering stage (EC60; July) by mixing the roots of four plants per plot to form one composite sample. Individual plots were sampled separately and used as true replicates in this study. The roots were shaken vigorously to seperate loosely adhering soil. The soil layer still attaching to the roots can be defined as rhizosphere soil (Yanai et al. 2003). To standardize the amount of root relative to soil mass, the rhizospheric soil layer was reduced to 1 mm by careful brushing. Afterwards, the rhizosphere soil/root samples were immediately frozen on dry ice and stored at −80°C for nucleic acid extraction.

Greenhouse experiment

The greenhouse experiment started in April 2007 and was performed with soil collected from the field site five weeks before the potatoes were planted. Soil was sieved through a 4 mm mesh, filled into plastic pots (20 cm×20 cm×20 cm) and adjusted to 60% of the maximum water-holding capacity one week before planting the potato tubers. As was done for the field site, the same amount of NPK fertiliser (850 kg ha^{-1}) was applied one week before planting. One plant was cultivated per pot. Overall, 24 pots of each cultivar were used. During potato growth, irrigation was performed by hand to keep the water content of the soil constant. No herbicides or pesticides were applied. Rhizosphere samples were collected at early leaf development (EC30) and the flowering stage (EC60) in four independent replicates by mixing the roots of three plants to form one composite sample. Afterwards the samples were treated as described above.

Nucleic acid extraction

DNA was extracted from 0.5 g of rhizosphere soil/root complex according to the protocol described by Griffiths et al. (2000b). The extraction was performed using Precellys-Keramik kit lysing tubes

(PeqLab Biotechnology GmbH, Germany) in combination with the Precellys 24 bead-beating system (Bertin Technologies, France). The DNA yield and purity were checked with a spectrophotometer (Nanodrop, PeqLab, Germany). The extracts were stored at −80°C until use.

Real-time PCR assay

Quantitative real-time PCR was performed using an ABI 7300 Cycler (Applied Biosystems, Germany) with the following assay reagents: dimethyl sulfoxide (DMSO) and betaine (Sigma, Germany), primers (Metabion, Germany) and 2× Power SYBR Green PCR master mix (Applied Biosystems, Germany). The respective reaction mixtures (25 µl volume) for quantification of the genes given in Table 1 were composed as follows: 12.5 µl master mix, 10 pmol of each primer and 2 µl DNA template. For the amplification of *Pseudomonas* specific 16S rRNA, 2.5 µl betaine and 0.5 µl DMSO were added. Because of the small size of the amplicon (see Table 1), the quantification of *P. infestans* was conducted with a target-specific probe (Metabion, Germany) combined with the TaqMan Universal PCR master mix (Applied Biosystems, Germany) instead of SYBR Green. Therefore, the PCR reaction mixture contained 12.5 µl master mix, 8 pmol of each primer, 5 pmol probe and 2 µl DNA template. For the quantification, serial dilutions of plasmid DNA containing the PCR products of the respective genes listed in Table 1 were used to calculate standard curves. Data showing the quality of the standard curves are presented in Table 2. The PCR detection limit was assessed to 10 gene copies according to manufacturer's instruction. To avoid the inhibition of PCR caused by co-extracted humic substances, the optimal uniform dilution for each amplification assay was determined by a pre-experimental dilution series of randomly chosen DNA extracts (data not shown). Amplifications were performed in 96-well plates (Applied Biosystems, Germany) for all target genes as described in Table 1, conducting in triplicates for all standards, non-template controls and samples. To confirm the specificity of the SYBR Green-quantified amplicons, a melting curve analysis and a 1.5% agarose gel were performed after each PCR run. The amplification efficiency was calculated from the formula $\mathrm{Eff} = \left[10^{(-1/\mathrm{slope})} - 1\right]$ and resulted in the following average efficiencies (standard deviation less than 2% of mean): bacterial 16S rRNA, 86.7%, *Pseudomonas* specific 16S rRNA, 92.3%, intergenic spacer (IGS) rRNA of *C. michiganensis* ssp., 83.6%, fungal internal transcribed spacer (ITS) rRNA, 85.4%, *Trichoderma* specific ITS rRNA, 85.5%, and nuclear satellite DNA of *P. infestans*, 95.6%.

Table 1 Primer sets and thermal profiles used for the absolute quantification of the respective genes

Target gene	Primer set	Amplicon size	Reference	Thermal cycling profile	No. of cycles
Bacterial 16S rRNA	FP 16S	264 bp	Bach et al. (2002)	95°C–10 min	1
	RP 16S			95°C–20 s/62°C–60 s	40
16S rRNA of *Pseudomonas* spp.	9-27F	441 bp	Johnsen et al. (1999)	94°C–6 min	1
	PSM_G			92°C–30 s/52.5°C–30 s/68°C–60 s	40
IGS rRNA[a] of *C. michiganensis* ssp.	FP Cm	222 bp	Bach et al. (2003)	95°C–10 min	1
	RP Cm			95°C–30 s/64°C–30 s/72°C–30 s	40
Fungal ITS rRNA[b]	ITS1	545 bp	White et al. (1990)	95°C–10 min	1
	ITS4			94°C–30 s/50°C–30 s/72°C–60 s	40
ITS rRNA[b] of *Trichoderma* spp.	uTf	525 bp	Hagn et al. (2007)	95°C–5 min	1
	uTr			95°C–30 s/55.5°C–30 s/72°C–60 s	40
Nuclear satellite DNA of *P. infestans*	P3f	73 bp	Boehm et al. (1999)	50°C–2 min/ 95°C–10 min	1
	P4r			95°C–30 s/56°C–30 s/72°C–30 s	45
	FAM-labelled probe F2				

[a] IGS rRNA: intergenic spacer rRNA

[b] ITS rRNA: internal transcribed spacer rRNA

Table 2 Quality of standard curves (n=4 ± standard deviations)

Target gene	Slope[a]	Intercept[c]	$R^{2,\ d}$	Copy no.[e]
Bacterial 16S rRNA	−3.69±0.07	39.20±0.76	0.997±0.002	10^3–10^8
16S rRNA of *Pseudomonas* spp.	−3.52±0.05	41.55±0.63	0.998±0.002	10^2–10^7
IGS rRNA of *C. michiganensis* ssp.	−3.79±0.04	40.01±0.26	0.998±0.002	10^1–10^6
Fungal ITS rRNA	−3.73±0.06	38.35±1.39	0.997±0.004	10^2–10^8
ITS rRNA of *Trichoderma* spp.	−3.73±0.06	42.56±1.10	0.996±0.004	10^1–10^6
Nuclear satellite DNA of *P. infestans*	−3.43±0.03	38.92±0.23	0.997±0.003	10^1–10^6

[a] Slope obtained for the standard curve

[b] Intercept obtained for the standard curve

[c] Correlation coefficient obtained for the standard curve

[d] The copy number represents the number of target genes added to the PCR reaction mix

Statistical analyses

The data were analysed by analysis of variance (ANOVA) at the significance level $p<0.05$ using SPSS 11.5 (SPSS, Inc.). The normal distribution of the data was checked by the Kolmogorov-Smirnov test and histograms. If necessary, the data were log-transformed prior to analysis. The homogeneity of the variances was checked by the Levene test. For the pairwise comparison of means with the ANOVA, either the Tukey test or, if the homogeneity of the variances was not given, the Games-Howell test was used.

Results

To contribute to the problem of different DNA extraction efficiencies among environmental samples, the quantified gene copy numbers were normalised to one gram dry weight (dw) of the rhizosphere soil/root complex as well as to nanogram extracted DNA (data not shown). Because both give comparable abundance patterns, similar extraction efficiencies for all samples could be assumed.

Abundance pattern of the indicator microorganisms in the greenhouse experiment

Neither the cultivar nor plant developmental stage influenced the abundance pattern of total 16S rRNA genes significantly (average 3.5×10^{10} copies g^{-1} dw); the *Pseudomonas*-specific 16S rRNA gene copy numbers also did not vary between the treatments analysed (average 2.3×10^{7} copies g^{-1} dw). In contrast, the IGS rRNA gene of *C. michiganensis* showed at the young leaf developmental stage a significantly higher gene copy number for 'Jumbo' (1.3×10^{6} copies g^{-1} dw) compared to 'Walli' (7.5×10^{5} copies g^{-1} dw) and #1332 (8.3×10^{5} copies g^{-1} dw) at the same plant age. As the abundance of *C. michiganensis* for 'Jumbo' decreased during plant development, no cultivar effect was observed at the flowering stage (average 8.9×10^{5} copies g^{-1} dw).

The abundances of fungal ITS rRNA genes were highest at EC30 for 'Jumbo' (7.0×10^{11} copies g^{-1} dw) and differed clearly from 'Walli' (2.1×10^{11} copies g^{-1} dw) and #1332 (2.6×10^{11} copies g^{-1} dw). As the ITS rRNA gene copy number of 'Walli' increased with increasing plant age, again no differences for the ITS rRNA genes were found among the cultivars at the flowering stage (average 4.3×10^{11} copies g^{-1} dw). In contrast, for the abundance pattern of the *Trichoderma*-specific ITS rRNA genes, no cultivar effect at early leaf development was observed and similar copy numbers were found among all cultivars within one plant development stage. However, the abundance pattern of the ITS rRNA genes of *Trichoderma* spp. showed a clear dependence on plant age and increased significantly with increasing plant age from 1.3×10^{6} copies g^{-1} dw at EC30 up to average 6.2×10^{6} copies g^{-1} dw at EC60. Interestingly, the abundance of the nuclear satellite DNA of *P. infestans* was influenced by both the plant developmental stage and cultivar, as gene copies were only detected at the flowering stage and only for 'Walli' and #1332 but not for 'Jumbo'. However, no significant differences for abundance of

P. infestans DNA were observed between 'Walli' and #1332 at EC60, revealing an average of 2.0×10^5 copies g^{-1} dw. All of the data from the greenhouse study are summarised in Fig. 1.

Abundance pattern of the indicator microorganisms in the field experiment

In the rhizosphere of 'Jumbo', a significantly lower total 16S rRNA gene copy number was detected (7.0×10^{10} copies g^{-1} dw) compared to 'Walli' (8.2×10^{10} copies g^{-1} dw) or #1332 (8.4×10^{10} copies g^{-1} dw) at young leaf development. In contrast, the abundance of the *Pseudomonas*-specific 16S rRNA genes was significantly higher for 'Jumbo' (1.9×10^8 copies g^{-1} dw) than for 'Walli' (1.4×10^7 copies g^{-1} dw) or #1332 (1.6×10^7 copies g^{-1} dw) at EC30. At the flowering stage, differences in abundance pattern among the cultivars were not observed for either the total 16S rRNA genes or for the *Pseudomonas*-specific 16S rRNA genes. Overall, universal 16S rRNA gene copy numbers decreased with increasing plant age to 5.5×10^{10} copies g^{-1} dw, whereas the abundance of *Pseudomonas*-specific 16S rRNA genes increased with increasing plant age to 3.5×10^8 copies g^{-1} dw. The abundance pattern of *C. michiganensis* was influenced neither by the cultivars under investigation nor by the plant developmental stage in the field trial (3.4×10^6 copies g^{-1} dw).

For fungal ITS rRNA genes, the highest gene copy numbers were detected in the rhizosphere samples of 'Jumbo' at both sampling time points (2.2×10^{12} g^{-1} dw and 1.5×10^{12} copies g^{-1} dw, respectively), while the gene abundances of 'Walli' and #1332 amounted to an average of 9.0×10^{11} copies g^{-1} dw and remained constant during plant development. In contrast to the overall fungal ITS rRNA genes, no cultivar effect was observed for the abundance of *Trichoderma*-specific ITS rRNA genes as similar copy numbers were found among all cultivars at one plant developmental stage (average 2.1×10^6 copies g^{-1} dw at EC30 and 1.2×10^6 copies g^{-1} dw at EC60). Similarly to the greenhouse study, the nuclear satellite DNA of *P. infestans* was only detected at the flowering stage. In contrast, the gene abundances were not affected by cultivar as no significant difference was observed among 'Jumbo', Walli' and #1332 (average of 1.1×10^5 copies g^{-1} dw). All of the data from the field study are summarised in Fig. 2.

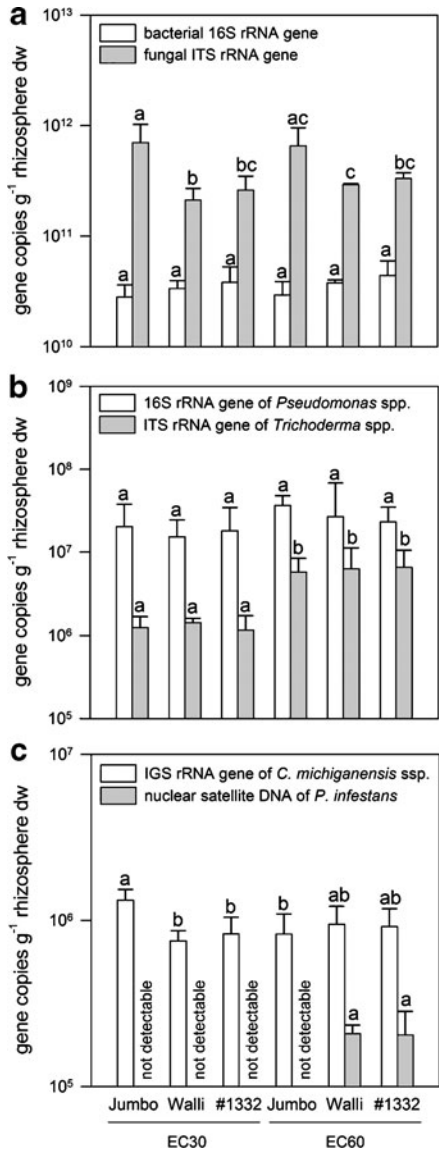

Fig. 1 Copy numbers of (**a**) bacterial 16S rRNA gene and fungal ITS rRNA gene, (**b**) 16S rRNA gene of *Pseudomonas* spp. and ITS rRNA gene of *Trichoderma* spp. and (**c**) IGS rRNA gene of *C. michiganensis* ssp. and nuclear satellite DNA of *P. infestans* per gram of rhizosphere soil/root complex dry weight (rhizosphere dw) for different cultivars ('Jumbo', 'Walli' and #1332) at two plant developmental stages (EC30: early leaf development and EC60: florescence) for greenhouse-grown potato plants (n=4); error bars represent standard deviations. For each panel different letters indicate significance at $p<0.05$

Discussion

Because the plant rhizosphere is a dynamic environment, the activity and composition of microbial

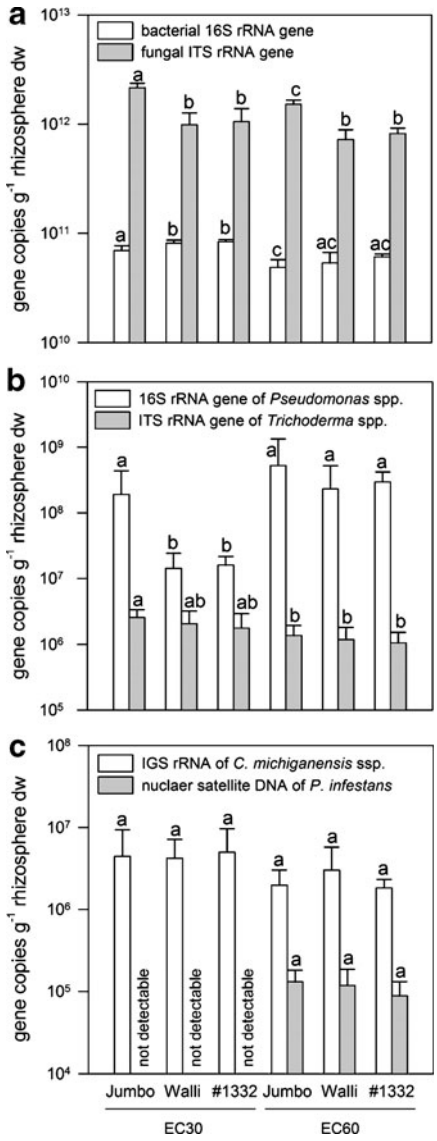

Fig. 2 Copy numbers of (**a**) bacterial 16S rRNA gene and fungal ITS rRNA gene, (**b**) 16S rRNA gene of *Pseudomonas* spp. and ITS rRNA gene of *Trichoderma* spp. and (**c**) IGS rRNA gene of *C. michiganensis* ssp. and nuclear satellite DNA of *P. infestans* per gram of rhizosphere soil/root complex dry weight (rhizosphere dw) for different cultivars ('Jumbo', 'Walli' and #1332) at two plant developmental stages (EC30: early leaf development and EC60: florescence) for field-grown potato plants (n=5); error bars represent standard deviations. For each panel different letters indicate significance at $p<0.05$

communities could be influenced by many parameters. The aim of this study was to investigate the potential effects of a GM marker-free amylopectin-accumulating potato line on the abundance of plant beneficial microbes as well as phytopathogens and to compare the modification-dependent impact with variations caused by plant cultivars obtained by classical breeding and plant developmental stage.

Our results revealed that the examined gene abundance patterns in the rhizosphere of the potato plants were not affected by the genetic modification in both the greenhouse and field study, as the data did not vary between the GM line and the non-transgenic parental cultivar. This confirms the findings of other studies, where GM plant-related effects on the microbial community structure were also not found (Heuer et al. 2002; Saxena and Stotzky 2001; Schmalenberger and Tebbe 2002). Although other reports showed significant changes in the rhizosphere microbial community caused by GM plants, the modification-dependent effects were described as minor compared to shifts caused by the soil type, plant genotype and plant developmental stage during the monitoring (Gyamfi et al. 2002; Lottmann et al. 1999; Milling et al. 2004; Rasche et al. 2006; Weinert et al. 2009). However, these differences in the data clearly indicate the need for a case-by-case study of GM plant-related effects because a generalisation of effects is not possible.

Although in this study the gene abundances of rhizosphere microorganisms of the GM potato line #1332 did not vary from those of the non-transgenic parental cultivar 'Walli', significant differences to the second non-transgenic cultivar 'Jumbo' were observed for most of the investigated genes. The abundance of bacteria and *Pseudomonas* spp. in the microbial rhizosphere community differed between 'Jumbo' and 'Walli' or GM line #1332, but only in the field study at the young leaf developmental stage. Interestingly, 'Jumbo' showed a lower copy number for total 16S rRNA genes but a higher abundance of *Pseudomonas* specific 16S rRNA genes compared to the other two potato cultivars. This might be related to the genotype-specific root exudation patterns (Grayston et al. 1998; Marschner et al. 2001; Söderberg et al. 2002), As many *Pseudomonas* strains produce antimicrobial compounds (Walsh et al. 2001; Winding et al. 2004), we suppose this may be the reason why the abundance of bacteria is reduced in the rhizosphere of 'Jumbo'.

The effects of the plant cultivar on fungi were more pronounced than on bacteria, as the abundance of ITS rRNA genes in the rhizosphere of 'Jumbo' was significantly higher compared to the other potato lines

in both the greenhouse and field-grown plants. This finding was surprising because a previous study by Costa et al. (2006) reported that bacteria were more influenced by different plant species (strawberry and oilseed rape) than fungi. The contrary observations might result from the different plant species examined. Furthermore, Costa et al. (2006) looked more for changes in fungal diversity whereas the fungal abundance patterns were compared in this study. There are only few studies that investigated the potential impact of GM plants on fungal rhizosphere communities. Similarly to our results, Weinert et al. (2009) obtained significant differences in the fungal communities associated with different plant cultivars. In contrast, Milling et al. (2004) found no cultivar-dependent alteration in the fungal communities, but in that study, the NS1/NS2 primer system was used to amplify the fungal community, which may be biased as it lacks the amplification of major fungal groups (Hagn et al. 2003). However, the need for case-by-case studies is also clear for fungal communities.

Jones et al. (2004) stated that the amount and chemical composition of root exudates change during plant development, depending on the different plant stages. This may explain the clear influence of plant age on the microbial gene abundance patterns in the plant rhizospheres monitored in this study. The abundances of total 16S rRNA genes in the rhizosphere of all three field-grown potato lines decreased from EC30 to EC60. This finding was surprising and in contrast to other studies, where the strongest enrichment of bacterial populations in the rhizosphere of potatoes was found at flowering stage (Lottmann et al. 2000; Smalla et al. 2001). We suggest that the reduced abundance of total bacteria at EC60 may be due to the increased *Pseudomonas* abundance because many *Pseudomonas* strains are known to produce antimicrobial compounds (Walsh et al. 2001; Winding et al. 2004). This assumption is confirmed by the fact that in the greenhouse no alteration in the abundance of total 16S rRNA genes or 16S rRNA genes of *Pseudomonas* spp. during plant growth was observed. The abundance of both fungal ITS rRNA genes and *Trichoderma* spp. in the rhizosphere of field-grown plants also decreased from EC30 to EC60, while in the greenhouse the abundances remained stable or increased. This was not surprising because the fungicide 'Epok' was only applied in the field before the flowering stage. Although 'Epok' was developed for plant protection against *P. infestans*, the effects on non-target fungi cannot be excluded. Such unintended negative influences on fungi, especially biocontrol fungi like *Trichoderma* strains, were demonstrated for different fungicides (De Schutter et al. 2002; Dłużniewska 2003). Nevertheless, *P. infestans* was detected in the rhizosphere of both the greenhouse and field plants at the flowering stage, but due to the application of 'Epok' with lower abundance in field-grown plants. The observation that variations in the functional and structural characteristics of the associated microbial rhizosphere community during plant growing occurred was obtained in several other reports, also (Gyamfi et al. 2002; Heuer et al. 2002; Milling et al. 2004; Rasche et al. 2006; Schmalenberger and Tebbe 2002; Sessitsch et al. 2003).

By comparing the results of the greenhouse and field studies, it became obvious that the microbial community structure was affected in the greenhouse and field with very different intensities. This was particularly visible for the abundance pattern of bacteria, *Pseudomonas* spp. and fungi, which were strongly influenced by the cultivar and plant vegetation stage in the field but not in the greenhouse study. Similarly, Griffiths et al. (2000a) compared the effect of GM potatoes that produce lectins on non-target soil organisms in the greenhouse and field. No significant differences in the microbial rhizosphere population due to the genetic modification were observed in the greenhouse, but the field experiment showed alterations in microbial communities, although these did not persist to the next growing season. In our study, besides climatic conditions, the main difference between greenhouse study and field trial is related to the fungicide application in the field, which may influence microbial communties and thus explain at least a part of the differences observed in both studies.

In conclusion, no effects caused by the genetic modification of the marker-free amylopectin-accumulating potatoes on the investigated gene abundance patterns of plant beneficial microbes and phytopathogens were measured in this study. Because cultivar- and plant developmental stage-dependent effects were observed, this indicates the sensitivity of the used methods. However, these results cannot be generalised to other GM plants with different events, and the transfer of the results to other soil types or climatic regions must also be made with care.

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Publication III

Title: Effects of a genetically modified starch metabolism in potato plants on photosynthate fluxes into the rhizosphere and on microbial degraders of root exudates

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Key words: genetically modified potato plants, ^{13}C labelling, phospholipid fatty acid (PLFA) analysis, rhizosphere, microbial biomass, carbon partitioning, photosynthate flux

Abstract

A high percentage of photosynthetically assimilated carbon (C) is released into the soil via root exudates, which are acknowledged as the most important factor for the development of a typical rhizosphere microflora. As quality and quantity of root exudates is, among others, dependent on plant genotype, the genetic engineering of plants might also influence carbon partitioning within the plant and thus microbial rhizosphere community structure. In this study, carbon allocation pattern within the plant-rhizosphere system of a genetically modified (GM) amylopectin-accumulating potato line (*Solanum tuberosum* L.) were linked to microbial degraders of root exudates under greenhouse conditions, using ^{13}C-CO_2 pulse-chase labelling in combination with phospholipid fatty acid (PLFA) analysis. Furthermore, GM plants were compared to the parental cultivar as well as a second potato cultivar obtained by classical breeding. Rhizosphere samples were obtained during young leaf developmental (EC_{30}) and flowering (EC_{60}) stage. ^{13}C allocation in above ground plant biomass, water extractable organic carbon (WEOC), microbial biomass carbon (C_{mic}) and PLFA as well as microbial community structure in the rhizosphere varied significantly among the two natural potato cultivars. However, differences between the GM line and its parental cultivar were not observed. Besides the considerable impact of plant cultivar, also the plant developmental stage affected carbon partitioning via plant into rhizosphere and, subsequently, microbial communities involved in the transformation of root exudates.

Introduction

As photosynthetically assimilated CO_2 released via rhizodeposition is the primary carbon (C) source in soil during vegetation period, the composition and the amount of organic compounds in root exudates are very important factors for the development of a typical rhizosphere microflora (Lynch & Whipps, 1990). By governing quality and quantity of root exudates, plants are able to influence beneficial microorganisms which promote plant growth and plant health (Lynch & Whipps, 1990, Raaijmakers *et al.*, 2009). Due to this close interaction between plants and their microbial rhizosphere populations and its importance for plant performance, there is a need to understand if carbon partitioning in genetically modified (GM) crops is affected by the genetic modification.

It was shown that carbon allocation within plants is highly dependent on plant species, plant genotype and plant vegetation stage (Percival *et al.*, 2001, Carvalho *et al.*, 2006, Shinano *et al.*, 2006). Similar results were obtained for microbial community structure in the rhizosphere (Duineveld *et al.*, 1998, 2001, Smalla *et al.*, 2001, Gyamfi *et al.*, 2002, Marschner *et al.*, 2006). Besides, many studies reported shifts in diversity pattern of the rhizosphere microflora associated with GM plants, although the modification-dependent effects were described as minor compared to the impact of soil type, plant variety and plant development (Milling *et al.*, 2004, Rasche *et al.*, 2006, Weinert *et al.*, 2009). Nevertheless, so far almost nothing is known about the influence of genetic modification on photosynthate allocation, or if GM-related changes in C partitioning in fact lead to shifts in microbial rhizosphere community.

Phospholipid fatty acid (PLFA) analysis in combination with stable isotope probing (SIP) is a suitable approach to link carbon assimilation within plants directly to the microbial community structure in soil if a atmosphere enriched with ^{13}C labelled CO_2 is used for plant growth (Lu *et al.*, 2002, 2007, Esperschütz *et al.*, 2009, Wu *et al.*, 2009). Although this method cannot be linked to the species composition of microbial communities because many phospholipids are not related to one single taxonomic group, it offers several advantages compared to nucleic acid-based SIP approaches, like the possibility of a quantitative evaluation of microbial groups due to the lack of an amplification steps and the very sensitive detection of microorganisms metabolizing plant derived carbon due to analysis by gas chromatography-combustion-isotope ratio mass spectrometry. Therefore, potential cross-feeding problems by the need of long incubation time and/or high labelled substrate concentrations not reflecting environmental conditions can be avoided.

In the present study, a GM potato (*Solanum tuberosum* L.) with modified starch metabolism was used to investigate the influence of genetic modification on photosynthate allocation from plant to soil to microorganisms and on rhizosphere microbial community structure, using ^{13}C pulse-chase labelling combined with PLFA analysis under greenhouse conditions. The GM potato contains an antisense gene fragment of the granule-bound starch synthase gene (*gbss*), which encodes one of the key enzymes for amylose formation. Consequently, transcription leads to an effective inhibition of *gbss* expression and amylose production (Visser *et al.*, 1991, Kuipers *et al.*, 1994). Therefore, tuber starch consists of almost pure amylopectin, which is appropriate for commercial use in paper, textile and food industry. A different engineered transgenic potato variety, 'Amflora' (BASF Plant Science), was already accepted for placing on the market in the European Union in March 2010. Since a GM starch composition can influence microbial community structure in potato rhizosphere (Milling *et al.*, 2004), we suppose that this may be a result of altered carbon allocation in the GM plant. Hence, the objectives of this study were: (1) to assess whether amylopectin-accumulating GM potato line #1332 (Bavarian State Research Center for Agriculture) differs from its non-transgenic parental variety in photosynthate partitioning using ^{13}C pulse-chase labelling, (2) to estimate the effects of GM photosynthate inputs on the microbial rhizosphere community via PLFA-SIP analysis, and (3) to relate the potential GM-caused impact to variation among natural potato cultivars and the effect of plant developmental stage.

Materials and Methods

Potato cultivars and the GM line

The GM potato line #1332 with increased amylopectin levels in its tubers, its parental cultivar 'Walli' and one additional potato variety used for industrial starch production, 'Ponto', were examined in this study. Both natural cultivars were provided by the Bavarian State Research Center for Agriculture, where also the GM line was developed. The GM line was genetically modified by the insertion of a gene fragment of the granule-bound starch synthase gene (*gbss*) in antisense direction. Consequently, transcription leads to the formation of *gbss* dsRNA and thus inhibition of *gbss* expression and amylose production (Visser *et al.*, 1991, Kuipers *et al.*, 1994). Hence, the tuber starch of the GM line consists of more than 99% amylopectin, while the starch granules of 'Walli' and 'Ponto'

contain only 75-80% amylopectin (Reichmann, personal communication). A newly developed method based on PCR screening was used for transformation without antibiotic- or herbicide-resistance selection (http://gmoinfo.jrc.ec.europa.eu/gmp_report.aspx?CurNot=B/DE/03/155), resulting in marker-free GM line #1332.

Experimental setup

The soil used in this experiment was taken from the plow layer (0-30 cm) of a field with potato - winter wheat – maize - winter wheat rotation history from the agroecological research station in Scheyern, approximately 40 km north of Munich, Germany (48°30' N, 11°26' E). It was characterized as sandy Cambisol with 22% clay, 10% silt and 68% sand, pH (0.01 M $CaCl_2$) 5.6, maximum water-holding capacity of 29%, and contained 1.0% total organic carbon and 0.1% total nitrogen. To remove plant residues and gravel, the soil was sieved (< 4 mm), filled into plastic pots (5 l soil per pot) and adjusted to 60% of the water holding capacity (Linn & Doran, 1984) one week before the potato tubers were implanted at the beginning of September 2008. In total, 120 pots were prepared (one plant per pot, 40 pots each plant cultivar), whereof the half was used for pulse-chase labelling and the others as unlabelled controls. The plants were grown in greenhouse at 25/15°C day/night temperature, relative humidity 50% and a photoperiod of 16 hours daylight (additional light by sodium vapour discharge lamps, SON-T Agro 400, Philips, NL). Irrigation was performed by hand with 60-100 ml deionized water every 24 hours to keep the water content of the soil between 50-60% of the water holding capacity. When the plants reached the young leaf developmental stage in mid-October, 10 pots of each cultivar were placed into a tent built of transparent plastic foil (ethylene-tetrafluorethylene ETFE, film thickness 80 µm, Koch Membranen GmbH, Germany) to separate the plants from the outer greenhouse atmosphere. To estimate the amount of soil autotrophic CO_2 fixation, two unplanted pots were also placed within the tent. The CO_2 concentration of the tent atmosphere was measured continuously with a photo acoustic CO_2 controller (7MB1300, Siemens, Germany) and was automatically enriched with ^{13}C-CO_2 (99 atom% ^{13}C, Air Liquide, Düsseldorf, Germany) when dropping below 300 µmol mol^{-1} to keep it within 300 to 400 µmol mol^{-1}. Using this experimental setup, an atmosphere with a $^{13}CO_2$ concentration of 45-50% of the total CO_2 concentration was established in the tent. To reduce the CO_2 produced by plant respiration at night, the tent air was pumped through vials containing soda lime (sodium and calcium hydroxide) using a membrane pump (N 0135.3 AN.18, KNF Neuberger, Freiburg, Germany) with a flow of 200 l min^{-1}. The CO_2 depleted air was re-conducted

into the closed tent to ensure a stable CO_2 concentration of approximately 350 µmol mol^{-1} over night. This labelling occurred for 6 h at two consecutive days.

Afterwards, plant material and rhizosphere soil of 10 labelled and 10 unlabelled control pots were collected by mixing the respective material of two pots to form one composite sample. Rhizosphere soil was defined as the soil still attaching to the roots after vigorous shaking (Yanai *et al.*, 2003) and was sieved through a 2 mm mesh before storing at 4°C for analysis of water extractable organic carbon (WEOC) and microbial biomass carbon (C_{mic}), and at -20°C for PLFA analysis. The soil of the labelled unplanted pots was treated similarly. The plants were cleaned with deionised water and their roots, stems and leaves were dried separately at 65°C for 48 h for determination of carbon content.

At florescence of the remaining potato plants (end of November 2008), labelling and sampling was repeated similarly.

Carbon analysis of plant material

The dried plant material was ball-milled (Retsch MM2, Retsch GmbH, Haan, Germany), weighed into tin capsules and analyzed using an Elemental Analyzer coupled with an isotopic ratio mass spectrometer (EA-IRMS; Eurovector, Milan, Italy coupled with a MAT 253, Thermo Electron, Bremen, Germany) for determination of C content and $\delta^{13}C$. The $\delta^{13}C$ values were related to the international Vienna-Pee Dee Bee Belemnite (V-PDB) standard and were calculated as follows (Werner & Brand, 2001):

$$\delta^{13}C\ (‰) = [(R_{Sample} / R_V) - 1] \times 1000 \qquad [1]$$

where R_{Sample} and R_V represent the ^{13}C to ^{12}C ratios of sample and international standard V-PDB (0.0111802), respectively.

Water extractable organic carbon (WEOC) and microbial biomass carbon (C_{mic})

Within 3 days after harvest, WEOC of the samples was determined by shaking 7.5 g rhizosphere soil (fresh weight) in 0.01 M $CaCl_2$ solution (1:4; w/v) on a rotary shaker for 30 min. After subsequent centrifugation, the supernatant was filtered through 0.45 µm pore-sized polycarbonate filters (Whatman Nucleopore Track-Etch Membrane filters). Simultaneously, C_{mic} was determined with the same amount of soil by the chloroform-fumigation method according to Vance *et al*. (1987), using 0.01 M $CaCl_2$ solution (1:4; w/v) for extraction (Joergensen, 1995). All extractions were done in triplicates and the extracts were stored at -20°C until measurement.

Total organic carbon (C_{total}) contents of the extracts were determined on a Total Carbon Analyzer (Shimadzu TOC 5050, Tokyo, Japan) by catalytic high temperature oxidation. C_{mic} was calculated as the difference between C_{total} in fumigated and non-fumigated samples using a k_{EC}-value of 0.45 (Wu *et al.*, 1990, Joergensen, 1995). Measurement of $\delta^{13}C$ in the extracts was done via on-line coupling of liquid chromatography and stable isotope ratio mass spectrometry (LC-IRMS, Thermo Electron, Bremen, Germany) according to Krummen *et al*. (2004). The $\delta^{13}C$ in microbial biomass ($\delta^{13}C_{mic}$) was computed as described by Marx *et al*. (2007a):

$$\delta^{13}C_{mic}\ (‰) = [(\delta^{13}C_{fum} \times C_{fum}) - (\delta^{13}C_{n\text{-}fum} \times C_{n\text{-}fum})] / C_{mic} \quad [2]$$

where $\delta^{13}C_{fum}$ and $\delta^{13}C_{n\text{-}fum}$ are $\delta^{13}C$ values of the fumigated (fum) and non-fumigated (n-fum) extracts, respectively, while C_{fum} and $C_{n\text{-}fum}$ are C concentrations (in mg l^{-1}) of the fumigated and non-fumigated extracts.

PLFA analysis

PLFA were extracted and analysed as described in detail by Zelles *et al*. (1995) and Esperschütz *et al*. (2009). Briefly, lipids of 25 g rhizosphere soil (dry weight) were extracted by two-phase extraction procedure with chloroform/methanol and water. The total lipid extract was subdivided into neutral, glycol- and phospholipids on a silica-bonded phase column (SPE-SI 2 g / 12 ml; Bond Elut, Analytical Chem International, CA, USA). After mild alkaline hydrolysis of the phospholipid fraction, the unsubstituted fatty acid methyl esters (FAME) were extracted with hexane/dichlormethane on an aminopropyl column. FAME were further separated into saturated (SATFA), monounsaturated (MUFA) and polyunsaturated (PUFA) fatty acids using an Ag^{+} - impregnated SCX column (Bond Elut, Analytical Chem International, CA, USA). Afterwards, the fractions were stored at 4°C until measurement.

Analysis was performed on a gas chromatograph/mass spectrometry system (5973MSD GC/MS Agilent Technologies, Palo Alto, USA) linked to an isotope ratio mass spectrometer (GC/MS-C-IRMS, Delta Plus^{Advantage}, Thermo Electron Cooperation, Bremen, Germany) via a combustion unit. FAME were separated and detected via GC/MS, while isotopic composition of fatty acids was analysed after combustion (GC Combustion III, Thermo Electron Cooperation, Bremen, Germany) in the IRMS. The separation of the FAME was done as described by Esperschütz *et al.* (2009). MUFA have been measured underivatised to receive the correct isotopic signature. Subsequently, disulphide derivatisation and a second measurement were carried out to determine the double bond position

(Zelles *et al.*, 1995). Sample analysis was done in duplicates and repeated when variation of $\delta^{13}C_{V\text{-}PDB}$ in the internal standard exceeds 0.5‰. According to Werner & Brand (2001), the $\delta^{13}C$ ratios of the individual FAME were corrected related to the measurement difference between the $\delta^{13}C_{V\text{-}PDB}$ ratio of the internal standard in the sample and the known $\delta^{13}C_{V\text{-}PDB}$ ratio of the standard (nonadecanoic acid methyl ester, $\delta^{13}C_{V\text{-}PDB}$ = -30.5‰ and myristic acid methyl ester, $\delta^{13}C_{V\text{-}PDB}$ = -28.7‰). To obtain the actual PLFA ratio ($\delta^{13}C_{PLFA}$), the C-atom in the methyl group added into the C isotope ratios of the FAME ($\delta^{13}C_{FAME}$) during derivatisation was computed:

$$\delta^{13}C_{PLFA} = [(n + 1) \times \delta^{13}C_{FAME} - (1 \times \delta^{13}C_{MeOH})] / n \quad [3]$$

where n is the number of C-atoms in the PLFA and $\delta^{13}C_{MeOH}$ is the $\delta^{13}C_{V\text{-}PDB}$ ratio of methanol used for derivatisation (-38.5‰, determined by LC-IRMS).

The percentage of newly incorporated C into the individual PLFA-biomarker relative to the labelling atmosphere was calculated according to Farquhar *et al*. (1989), using a fractionation factor α:

$$\alpha = [(\delta^{13}C_{CO2} / 1000) + 1] / [\delta^{13}C_{plant} / 1000) + 1] \quad [4]$$

where $\delta^{13}C_{CO2}$ means $\delta^{13}C_{V\text{-}PDB}$ of the regular atmosphere (-11‰) and $\delta^{13}C_{plant}$ is the computed $\delta^{13}C_{PLFA}$ ratio of the non-labelled plants.

Using α, the newly incorporated C (C_{new}) was calculated as follows:

$$\delta^{13}C_{max} = \{[(\delta^{13}C_{tent} / 1000) + 1] \times \alpha - 1\} \times 1000 \quad [5]$$

$$C_{new} = (\delta^{13}C_{new} - \delta^{13}C_{plant}) / (\delta^{13}C_{max} - \delta^{13}C_{plant}) \times 100 \quad [6]$$

where $\delta^{13}C_{max}$ is the maximum possible label incorporation with regard to the individual α and the $\delta^{13}C$ ratio of the tent atmosphere ($\delta^{13}C_{tent}$), and $\delta^{13}C_{new}$ means the δ value of the individual PLFA measured under $\delta^{13}C_{tent}$, respectively.

The mass spectra of the individual FAME were identified by comparison with established fatty acid libraries (Solvit, CH 6500 – Luzern, Switzerland), using MSD Chemstation (Version D.02.00.237). The PLFA nomenclature used in the following based on Frostegård *et al*. (1993): fatty acids are designated as the total number of C-atoms : number of double bounds, followed by the position of the double bound from the methyl end of the molecule (ω). The prefixes “cy”, “i” and “a” indicate cyclopropyl-groups, iso- and anteiso-branching, respectively. “10Me” denotes a methyl-branching at the 10th C-atom from the carboxyl end of the molecule, whereas “br” indicates an unknown methyl-branching position.

According to Zelles (1999), iso- and anteiso-branched fatty acids were used as indicator for Gram-positive bacteria, while monounsaturated and cyclopropyl PLFA represent Gram-negative bacteria. “10Me” fatty acids could be taken as marker for actinomycetes (Frostegård & Bååth, 1996), whereas PLFA 18:2ω6.9 indicates fungal biomass (Bååth, 2003). Furthermore, PLFA 16:1ω5 can be mainly found in arbuscular mycorrhizal fungi (AMF) (Olsson *et al.*, 1999, Joergensen & Wichern, 2008). The ratios of Gram-positives-to-Gram-negatives, Gram-positives-to-actinomycetes and fungi-to-bacteria were used to show shifts in microbial community structure (Fierer *et al.*, 2003, Frostegård & Bååth, 1996).

Statistical analysis

Data were analysed by analysis of variance (ANOVA) at the significance level $p < 0.05$ using SPSS 11.5 (SPSS, Inc.). The normal distribution of the data was checked by the Kolmogorov-Smirnov test and histograms. If necessary, the data were log-transformed prior to analysis. The homogeneity of the variances was checked by the Levene test. For the pairwise comparison of means with the ANOVA, either the Tukey test or, if the homogeneity of the variances was not given, the Games-Howell test was used.

Results

Incorporation of ^{13}C labelled carbon into plant biomass

The majority of the assimilated ^{13}C was retained in the above ground plant biomass (Fig. 1). Interestingly, the amount of above ground incorporated ^{13}C decreased with increasing plant age, ranging from maximum 3.6 x 10^3‰ V-PDB at the young leaf developmental stage to minimum 1.7 x 10^3‰ V-PDB at the flowering stage, while the above ground plant biomass increased (supplemental material). Whereas for the GM line #1332 and its non-transgenic parental cultivar ‘Walli’ significantly higher ^{13}C incorporation into leaves than into stem was observed at both sampling times (2.1 x 10^3 vs. 1.3 x 10^3‰ V-PDB at EC_{30}; 1.0 x 10^3 vs. 7.2 x 10^2‰ V-PDB at EC_{60}), no such partitioning was found for the second natural cultivar ‘Ponto’. Furthermore, the ^{13}C amount of ‘Ponto’ stems was considerably higher than that of the other cultivars at the flowering stage. In contrast, neither temporal nor cultivar differences were found for the ^{13}C incorporation into roots.

Incorporation of ^{13}C labelled carbon into WEOC and C_{mic}

To assess cultivar differences related to the carbon flux via plant into soil, the ^{13}C amount of WEOC and C_{mic}, respectively, was determined (Fig. 2). As microbial biomass of labelled and unlabelled unplanted soil samples showed no difference in ^{13}C enrichment (data not shown), microbial autotrophic CO_2 fixation could be neglected. Although the fraction of WEOC showed a significant increase in ^{13}C incorporation compared to the non labelled control pots (Fig. 2), its amount of ^{13}C was obviously lower than that of rhizosphere soil microbial biomass (-18.8 vs. 125.2‰ V-PDB average). Furthermore, no temporal differences were found for ^{13}C enrichment of WEOC. In contrast, the ^{13}C incorporation into C_{mic} among the cultivar 'Ponto' doubled with increasing plant age up to 205‰ V-PDB at florescence. Consequently, 'Ponto' showed the highest ^{13}C amount at EC_{60}.

Microbial community structure in the rhizosphere

In total, 29 individual PLFA were identified (Fig. 3). The most common fatty acids were br16:0, 18:1ω9, 18:1ω7 and 18:2ω6.9, representing 43% of the total PLFA. When considering the fatty acids separately, only differences related to plant vegetation stage were found (br16:0, 18:1ω9, n20:0, 20:3ω6.9.12, 20:4ω6.9.12.15). However, by grouping the individual PLFA into marker selective for Gram-positive bacteria (br14:0, i15:0, a15:0, br15:0, i16:0, br16:0, 10Me17:0, i17:0, a17:0, 10Me18:0, i18:0, br19:0), Gram-negative bacteria (16:1ω9, 16:1ω7, cy17:0, 18:1ω7, 18:1ω5, cy19:0), actinomycetes (10Me17:0, 10Me18:0) and fungi (18:2ω6.9), respectively, cultivar-dependent shifts in microbial community structure appeared. The ratio of Gram-positive to Gram-negative bacteria, total Gram-positive bacteria to actinomycetes and fungi to bacteria is illustrated in Fig. 4. In the rhizosphere of 'Walli' at early leaf developmental stage, the ratio of Gram-positives-to-Gram-negatives amounted 1.6 and was therefore twice as high as that of the second natural cultivar 'Ponto'. Because it declined significantly towards EC_{60}, no shifts among the cultivars were found at the flowering stage. Contrary, the Gram-positives-to-actinomycetes ratio at the first sampling time point was clearly lower in the rhizosphere of 'Walli' and #1332 (7.3 and 7.6, respectively) compared to 'Ponto' (9.1), but increased significantly with increasing plant age. Consequently, again no cultivar-dependent differences were observed at flowering stage. Similar shifts were found when relating fungal to bacterial PLFA: The ratio in the rhizosphere of 'Ponto' was in the range of 0.1 and hence twice as high as that of 'Walli'.

Incorporation of ^{13}C labelled carbon into rhizosphere microorganisms

The relative increase of the ^{13}C label in the individual PLFA normalized to the unlabelled control plants is listed in Table 1. All of the detected fatty acids, except the two cyclopropyl SATFA (cy17:0 and cy19:0) showed significant ^{13}C enrichment at least for one cultivar. Interestingly, ^{13}C incorporation into PLFA br15:0 was significant for rhizosphere microorganisms of 'Walli' and #1332 at young leaf developmental stage, whereas at EC_{60} only for 'Ponto' enrichment was observed. However, the relative increase of ^{13}C in br15:0 was at the maximum 0.02% (which represents 0.4% of the total ^{13}C labelling) and therefore very low. The PLFA 20:3ω6.9.12, 18:3, 18:2ω6.9 and br16:0, indicating protozoa, fungi and Gram-positives, respectively, showed the highest relative ^{13}C incorporation, ranging from 0.2 to 1.7% and contained altogether 63% of the total ^{13}C incorporated into the fatty acids. Furthermore, also PLFA 16:1ω5, representing mainly AMF, revealed with 6.3% of the total ^{13}C included in fatty acids a major ^{13}C sink in the rhizosphere. Considering the fatty acids separately, cultivar-dependent differences in ^{13}C enrichment were observed: At flowering stage, for Gram-positive bacterial (i15:0, i17:0), Gram-negative bacterial (16:1ω7, 18:1ω7, 18:1ω5), fungal (18:2ω6.9), AMF (16:1ω5) and protozoa (20:3ω6.9.12) fatty acids a significantly higher incorporation of ^{13}C was found in the rhizosphere of 'Ponto' compared to the other two potato varieties. Furthermore, temporal shifts in ^{13}C enrichment occurred for PLFA representing actinomycetes (10Me18:1), Gram-negative bacteria (16:1ω7, 17:1ω8), AMF (16:1ω5) and protozoa (18:3, 20:3ω6.9.12).

Discussion

^{13}C distribution in plant compartments

The analysis of ^{13}C incorporation into the potato plants showed that the majority of assimilated ^{13}C was retained in the above ground biomass. Surprisingly, despite increasing leaf and stem biomass during plant development (supplemental material), the appropriate ^{13}C-to-^{12}C ratios decreased from young leaf developmental stage to florescence. As in contrast the relative ^{13}C enrichment of roots remained constant during sampling, this suggests an enhanced translocation of photo-assimilates to roots with increasing plant age and might be due to the initiating tuber development shortly before flowering (Dwelle & Love, 1993, Fernie & Willmitzer, 2001). Coinciding, several other studies also observed rising ^{13}C enrichment in roots during plant growth when examining beech trees (Esperschütz

et al., 2009), oats (Yevdokimov *et al.*, 2006) and maize or wheat (Marx *et al.*, 2007b), respectively, reflecting roots as highly active plant compartment requiring large amounts of carbon for growth. In agreement with Lu *et al*. (2002), our results revealed different carbon allocation among the cultivars, as the ^{13}C amount of leaves was significantly higher than that of stems for both the GM line #1332 and its non-transgenic parental cultivar 'Walli', while no such partitioning was found for the third cultivar 'Ponto'. Although we did not observe a GM-related effect on the ^{13}C incorporation into the individual plant parts, other studies investigating different transgenic plants expressing the *Bacillus thuringiensis* (Bt) toxin observed differences related to carbon assimilation between the GM plant and its parental cultivar (Hebbar *et al.*, 2007, Rossi *et al.*, 2007, Wu *et al*., 2009). As in contrast to "Bt plants" the GM line investigated in the present study does not contain any foreign gene and expresses only potato-derived *gbss* sequences, this might explain the lack of transformation effects on carbon partitioning.

^{13}C distribution in WEOC and C_{mic}

Plants release a major part of their photosynthetically fixed carbon as root exudates into the soil (Soerensen, 1997), where it contributes mainly to the pool of WEOC (Hütsch *et al.*, 2002). This root-derived carbon is an important source of readily available C for microorganisms (Paterson *et al.*, 2007) and is thus rapidly metabolized. Following the ^{13}C flux from plant into soil, we observed much lower $\delta^{13}C$ V-PDB values in WEOC than in C_{mic}. This coincides with previous reports (Yevdokimov *et al.*, 2006, Marx *et al.*, 2007b, Esperschütz *et al.*, 2009) and is probably due to the considerably small fraction of labelled rhizodeposits remaining in WEOC after microbial uptake (Yevdokimov *et al.*, 2006, Marx *et al.*, 2007a). Our results revealed a strong correlation between the ^{13}C amounts of WEOC and C_{mic} ($R = 0.8$, $p < 0.01$), reflecting the close relationship and the importance of root exudates for the initialisation of food webs in soil. According to the carbon partitioning in the plant itself, differences in ^{13}C incorporation between the natural cultivars were observed, but no GM-related differences occurred. Similarly, Wu *et al*. (2009) did not monitor changes in the amount of ^{13}C in microbial biomass between Bt rice and the non-transgenic rice cultivar.

Influence of different potato cultivars and plant developmental stage on microbial community structure

The two most abundant fatty acids were 18:1ω7 and br16:0, representing 26% of the total PLFA. This indicates high proportions of Gram-negative and Gram-positive bacteria in the rhizosphere of potato plants, which is in the line with previous reports (Frostegård *et al.*, 1993, Esperschütz *et al.*, 2009, Wu

et al., 2009). Furthermore, by comparing the abundance of bacterial subpopulations in the rhizosphere, our results revealed significant differences in the ratios of Gram-positive to Gram-negative bacteria as well as Gram-positive bacteria to actinomycetes between the potato cultivars. This might be due to plant genotype-specific root exudation pattern, as it is known that root exudates clearly influence microbial community structure (Marschner *et al.*, 2001, Söderberg *et al.*, 2002). No difference in community composition between the GM line and its non-transgenic parental cultivar was observed. Although PLFA analysis cannot give the species composition of microbial communities, this finding coincides with previous studies using nucleic acid-based methods, where GM plant-related impacts on the community structure of microorganisms were also not found (Saxena & Stotzky, 2001, Schmalenberger & Tebbe, 2002, Gschwendtner *et al*., 2010). Therefore, it might be assumed that the genetic modification did not affect microbial diversity in the rhizosphere.

PLFA 18:2ω6.9 is often used as biomarker for fungi (Frostegård & Bååth, 1996, Fierer *et al.*, 2003, Esperschütz *et al.*, 2009, Wu *et al.*, 2009). Although there has been some discussion in the past about its validity to quantify fungal biomass, as this fatty acid also occurs in plant cells (Fierer *et al.*, 2003), Kaiser *et al*. (2010) could prove in a recent article, that the contribution of root-derived PLFA to soil-borne fungal biomarker 18:2ω6,9 can be neglected. Furthermore, also oleic acid 18:1ω9 is an important component of fungal membranes, but can be present in Gram-negative bacteria, too (Zelles, 1999). Thus, for calculation of fungal phospholipids only linoleic acid 18:2ω6.9 was used in this study. By relating 18:2ω6.9 to bacterial PLFA, we observed different abundance ratios between the natural cultivars, but again no shift in microbial community between the GM line and its non-transgenic parental cultivar. Accordingly, also other authors reported no significant impact of GM plants on fungal rhizosphere community (Milling *et al.*, 2004, Wu *et al.*, 2009, Gschwendtner *et al.*, 2010).

Besides the genotype-specific influence on microbial community composition in the rhizosphere, our results revealed also a clear effect of the plant developmental stage. This may be due to the change of the amount and the chemical composition of root exudates during plant growth (Jones *et al.*, 2004). While at EC_{30} microbial rhizosphere community was considerably cultivar specific, no differences between the cultivars were found at flowering stage, suggesting that quantity and quality of rhizodeposits converge between the different cultivars with increasing plant age. Coinciding, Milling *et al.* (2004) and Weinert *et al*. (2009) observed the highest similarity in DGGE pattern of microbial rhizosphere communities at EC_{60} when investigating GM potatoes over the growing season.

^{13}C incorporation into rhizosphere microbial community

All detected PLFA were significantly enriched with ^{13}C compared to those in the rhizosphere of the unlabelled control plants, except the Gram-negative biomarker cy17:0 and cy19:0. These fatty acids are derivates of 16:1ω7 and 18:1ω7, respectively, and are known to be formed under environmental stress conditions like starvation to stabilize cell membrane (Frostegård *et al.*, 1993). The lack of ^{13}C incorporation suggests that these stress-PLFA were not actively formed in microbial community structure throughout the experiment. Coinciding, Esperschütz *et al*. (2009) observed decreasing abundance of cy17:0 and cy19:0 over time when investigating rhizosphere microorganisms of beech trees after dormancy, indicating that growth conditions for Gram-negative bacteria improve during plant development.

Although the relative abundance of individual PLFA revealed only temporal shifts in microbial community pattern, the rhizosphere microbial populations did strongly differ in metabolizing root-derived carbon also between the cultivars. Commonly, ^{13}C incorporation into the PLFA was significantly higher in the rhizosphere of 'Ponto' compared to 'Walli' and #1332, particularly at the flowering stage. This correlates with the increased ^{13}C label detected in total rhizosphere microbial biomass of 'Ponto' and might be a result of either higher microbial activity or enhanced ^{13}C exudation. The latter is indicated by the raised ^{13}C amount of above ground plant biomass compared to the other cultivars and reflects different carbon partitioning in and via the plant cultivars. Coinciding, when considering rhizosphere PLFA of different natural rice lines, variations in ^{13}C enrichment were found and supposed to be a result of genotype-induced difference in the quality and/or quantity of root exudates (Lu *et al.*, 2004). Although ^{13}C incorporation differed between 'Ponto' and the other cultivars, the genetic modification seemed to have no effect on carbon partitioning or activity of microbial populations, as no change in ^{13}C amount of the PLFA was observed between 'Walli' and #1332.

Besides a considerable incorporation of the ^{13}C label into fungal biomarker 18:2ω6.9, which reflects a strong interaction between plant and fungi previously described by other authors (Lu *et al.*, 2004, Esperschütz *et al.*, 2009, Wu *et al.*, 2009), the largest ^{13}C incorporation was detected in PLFA 18:3 and 20:3ω6.9.12, which have been described as marker for Gram-negative bacteria as well as for protozoa. It can be postulated that the high labelling was linked mainly to Gram-negatives, which are known to be most active in assimilating root-derived C (Wu *et al.*, 2009). Although protozoa are not directly influenced by root exudates, they graze on bacteria (Bonkowski *et al.*, 2000) and thus incorporate labelled carbon as secondary consumers.

Furthermore, a major part of total ^{13}C was detected in PLFA 16:1ω5, representing mainly AMF (Olsson *et al.*, 1999, Joergensen & Wichern, 2008). Interestingly, despite the importance of mycorrhizal associations for nutrient uptake, Cesaro et al. (2008) observed only low AMF root colonization for potatoes, suggesting this as a result of high nutrient concentrations in the soil and therefore no need for mycorrhizal symbiosis. Coinciding, in the present study low root mycorrhization was observed microscopically. This was surprising, because PLFA 16:1ω5 contributed substantially to the total amount of fatty acids in the rhizosphere and, furthermore, also showed high ^{13}C incorporation. However, due to the restriction of microscopic approaches for quantification mycorrhizal biomass, further analyses are necessary to evaluate if the considerable ^{13}C incorporation into 16:1ω5 represents active AMF or rather a high turnover rate of root derived carbon by Gram-negative bacteria, which can also form this PLFA (Zelles, 1999).

Conclusions

PLFA analysis in combination with ^{13}C labelling could be a useful tool in risk-assessment studies to analyze the immediate influence of plant genome transformation on carbon-partitioning characteristics within the plant and, subsequently, into the rhizosphere microbial community. Although our results revealed no differences between the GM line and its non-transgenic parental cultivar concerning photosynthate partitioning (objective 1) and microbial population structure and activity (objective 2), the influence of plant genotype and plant developmental stage could be shown (objective 3). However, the presented data do not allow a generalization due to risk assessment as this experiment was performed as a greenhouse study under optimal growth conditions. If the GM plant might be more affected by abiotic and biotic stressors in the field, resulting in changes in carbon allocation compared to the wild type needs to be addressed in further experiments.

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Table 1

Relative increase of the ^{13}C label in individual PLFA in rhizosphere soil related to control plants (n = 5 ± standard deviations) at different plant developmental stages (EC_{30}: young leaf development, EC_{60}: flowering) for different potato cultivars ('Ponto', 'Walli' and #1332). Significance ($p < 0.05$) among rows is indicated by different letters.

increase %	EC_{30}						EC_{60}					
	Ponto	±	Walli	±	#1332	±	Ponto	±	Walli	±	#1332	±
br14:0	0.027 ab	0.016	0.022 b	0.010	0.035 ab	0.011	0.043 a	0.006	0.028 ab	0.006	0.030 ab	0.010
i15:0	0.022 ab	0.010	0.011 ab	0.005	0.022 ab	0.010	0.016 a	0.003	0.008 b	0.002	0.011 ab	0.005
a15:0	0.013 a	0.005	0.007 a	0.005	0.011 a	0.004	0.013 a	0.003	0.007 a	0.002	0.008 a	0.002
br15:0	0.011 ab	0.013	0.014 a	0.005	0.017 ab	0.015	0.021 ab	0.014	0.000 ab	0.007	0.002 b	0.003
n15:0	0.027 ab	0.014	0.023 ab	0.010	0.027 ab	0.009	0.039 a	0.007	0.023 ab	0.004	0.019 b	0.004
15:1ω6	0.015 a	0.009	0.006 a	0.002	0.009 a	0.003	0.011 a	0.005	0.005 a	0.001	0.005 a	0.001
i16:0	0.027 a	0.009	0.023 a	0.009	0.029 a	0.009	0.027 a	0.003	0.016 a	0.004	0.016 a	0.002
br16:0	0.265 ab	0.141	0.271 ab	0.140	0.237 a	0.042	0.623 b	0.179	0.343 ab	0.056	0.326 ab	0.048
16:1ω9	0.134 a	0.113	0.036 a	0.017	0.042 a	0.013	0.070 a	0.013	0.042 a	0.025	0.049 a	0.042
16:1ω7	0.117 a	0.056	0.145 ab	0.073	0.103 a	0.019	0.287 b	0.066	0.133 a	0.017	0.126 a	0.015
16:1ω5	0.127 a	0.091	0.205 ac	0.084	0.115 a	0.052	0.512 b	0.199	0.293 bc	0.084	0.277 c	0.075
10Me17:0	0.026 a	0.007	0.039 a	0.009	0.038 a	0.007	0.081 a	0.034	0.033 a	0.004	0.034 a	0.005
i17:0	0.119 ab	0.078	0.061 ab	0.019	0.142 ab	0.058	0.102 a	0.023	0.045 b	0.006	0.041 b	0.004
a17:0	0.032 a	0.010	0.026 a	0.008	0.037 a	0.010	0.061 a	0.022	0.026 a	0.007	0.027 a	0.004
17:1ω8	0.011 a	0.004	0.005 ac	0.002	0.008 a	0.002	0.003 ab	0.004	0.001 b	0.002	0.003 bc	0.002
cy17:0	0.000 a	0.002	0.001 a	0.002	0.002 a	0.003	0.002 a	0.003	0.000 a	0.000	0.000 a	0.001
10Me18:0	0.002 a	0.002	-0.002 ab	0.002	0.001 ab	0.003	0.007 b	0.005	0.002 ab	0.003	0.002 ab	0.001
i18:0	0.092 a	0.052	0.073 a	0.034	0.081 a	0.026	0.099 a	0.037	0.064 a	0.009	0.046 a	0.008
18:1ω9	0.106 ab	0.062	0.076 ab	0.037	0.065 a	0.007	0.155 b	0.041	0.086 ab	0.011	0.070 ab	0.008
18:1ω7	0.165 ab	0.066	0.148 ab	0.059	0.119 a	0.021	0.286 b	0.080	0.104 a	0.021	0.103 a	0.018
18:1ω5	0.123 ab	0.060	0.102 ab	0.036	0.093 a	0.021	0.227 b	0.063	0.101 a	0.025	0.095 a	0.017
18:2ω6.9	0.295 ab	0.156	0.810 ab	0.440	0.859 ab	0.428	0.733 a	0.232	0.360 ab	0.056	0.239 b	0.112
3un/18:3	0.237a	0.093	1.214 ab	0.700	1.021 ab	0.826	0.511 b	0.072	0.253 ab	0.135	0.578 ab	0.801
br19:0	0.004 a	0.003	0.005 a	0.002	0.008 a	0.004	0.009 a	0.003	0.004 a	0.001	0.004 a	0.001
cy19:0	0.002 a	0.001	0.000 ab	0.000	0.000 bc	0.001	-0.001 bc	0.000	-0.001 c	0.000	0.000 bc	0.001
n20:0	0.030 a	0.024	0.015 a	0.009	0.027 a	0.012	0.042 a	0.015	0.015 a	0.003	0.013 a	0.004
20:3ω6.9.12	0.893 a	0.521	1.120 ac	0.412	0.527 a	0.349	1.727 b	0.341	0.788 a	0.382	0.981 ab	0.235
20:4ω6.9.12.15	0.117 a	0.054	0.108 a	0.046	0.069 a	0.036	0.115 a	0.055	0.070 a	0.014	0.124 a	0.171
n24:0	0.214 a	0.233	0.090 a	0.057	0.176 a	0.084	0.199 a	0.067	0.089 a	0.019	0.104 a	0.038

Figure Legends

Fig. 1

^{13}C incorporation ($\delta^{13}C$‰ V-PDB) into total carbon of potato plant parts (*n* = 5, error bars represent standard deviations) at different plant developmental stages (EC_{30}: young leaf development, EC_{60}: flowering) for different potato cultivars ('Ponto', 'Walli' and #1332). Significance ($p < 0.05$) is indicated by different letters.

Fig. 2

^{13}C incorporation ($\delta^{13}C$‰ V-PDB) into water extractable organic carbon (WEOC) and microbial biomass carbon (C_{mic}) of unlabelled (●) and labelled (○) plants (*n* = 5, error bars represent standard deviations) at different plant developmental stages (EC_{30}: young leaf development, EC_{60}: flowering) for different potato cultivars ('Ponto', 'Walli' and #1332). Significant differences ($p < 0.05$) among the labelled plants are indicated by different letters.

Fig. 3

Relative abundance of PLFA (mol%) in rhizosphere soil (*n* = 5, error bars represent standard deviations) for different potato cultivars ('Ponto', 'Walli' and #1332) at different plant developmental stages (black bars: EC_{30}, grey bars: EC_{60}). Significance ($p < 0.05$) is indicated by asterisks.

Fig. 4

Ratio of the relative abundance of Gram-positives-to-Gram-negatives (●), Gram-positives-to-acinomycetes (○) and fungi-to-bacteria (◍) (*n* = 5, error bars represent standard deviations) at different plant developmental stages (EC_{30}: young leaf development, EC_{60}: flowering) for different potato cultivars ('Ponto', 'Walli' and #1332). Significance ($p < 0.05$) is indicated by different letters.

Fig. 1

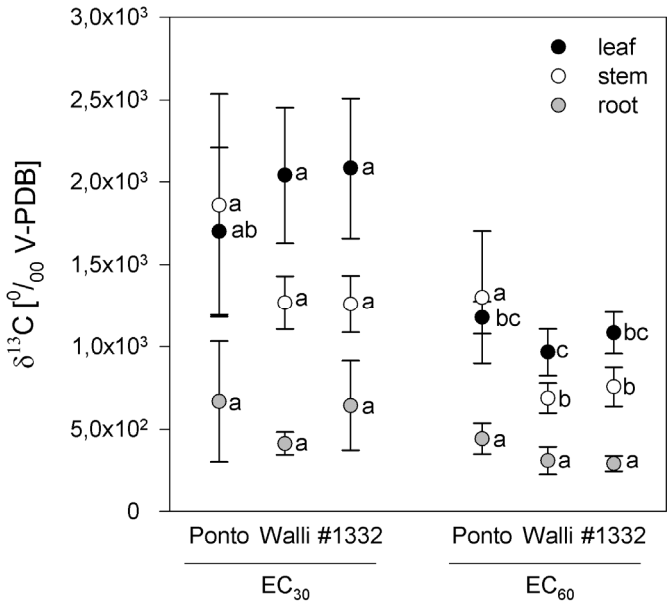
3,0x10³
2,5x10³
2,0x10³
1,5x10³
1,0x10³
5,0x10²
0
δ¹³C [‰ V-PDB]
leaf
stem
root
a
ab
a
a
a
a
a
a
a
a
bc
c
b
a
bc
b
a
Ponto Walli #1332
Ponto Walli #1332
EC30
EC60

Fig. 2

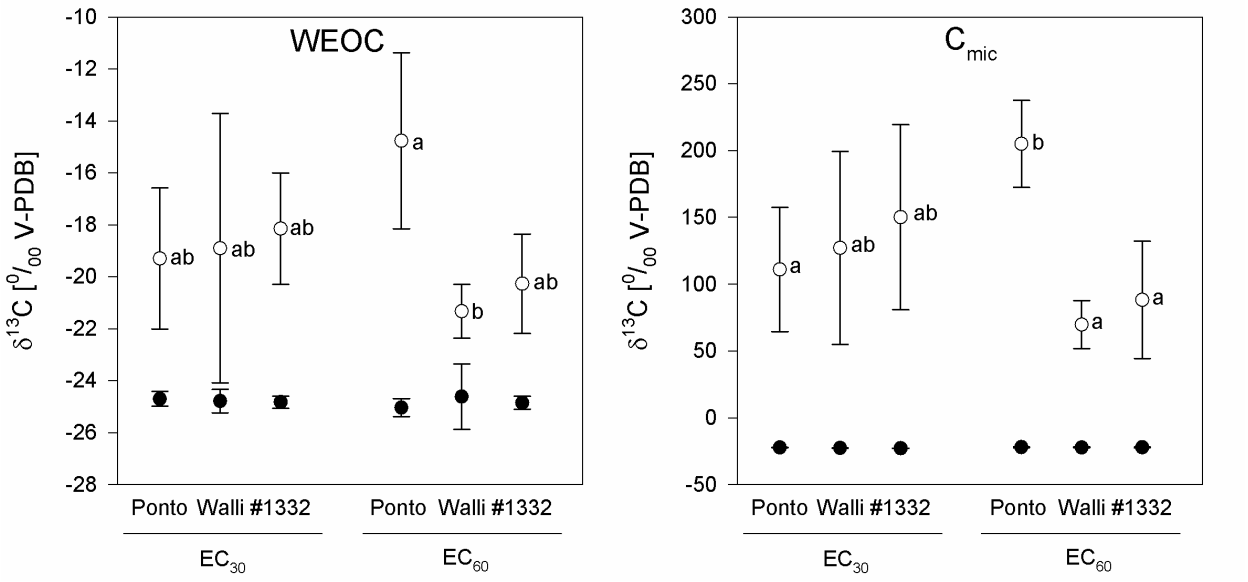
WEOC
-10
-12
-14
-16
-18
-20
-22
-24
-26
-28
δ¹³C [‰ V-PDB]
ab
ab
ab
a
b
ab
Ponto Walli #1332
Ponto Walli #1332
EC30
EC60
Cmic
300
250
200
150
100
50
0
-50
δ¹³C [‰ V-PDB]
a
ab
ab
b
a
a
Ponto Walli #1332
Ponto Walli #1332
EC30
EC60

Fig. 3

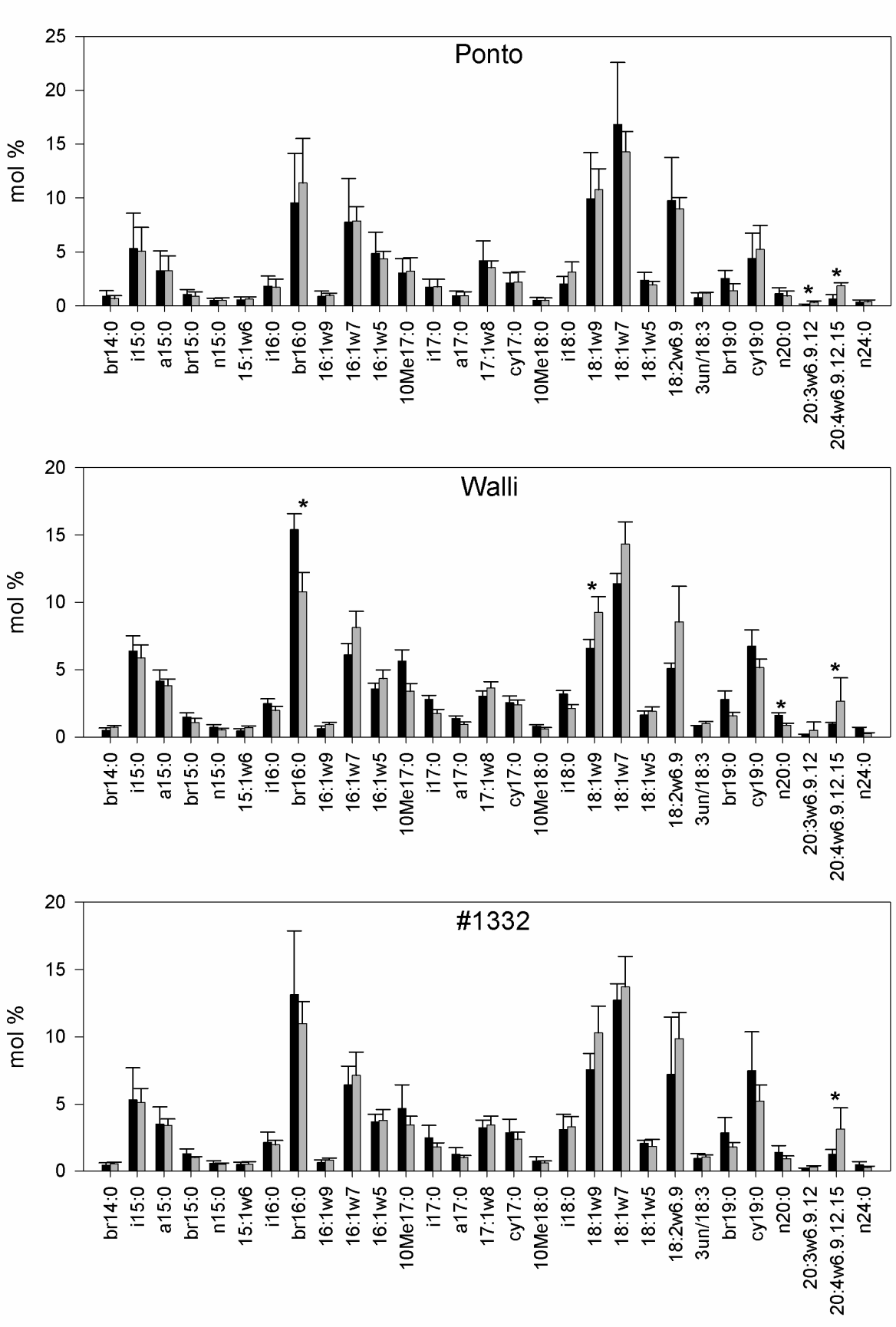
Ponto
Walli
#1332
mol %
br14:0
i15:0
a15:0
br15:0
n15:0
15:1w6
i16:0
br16:0
16:1w9
16:1w7
16:1w5
10Me17:0
i17:0
a17:0
17:1w8
cy17:0
10Me18:0
i18:0
18:1w9
18:1w7
18:1w5
18:2w6.9
3un/18:3
br19:0
cy19:0
n20:0
20:3w6.9.12
20:4w6.9.12.15
n24:0

Fig. 4

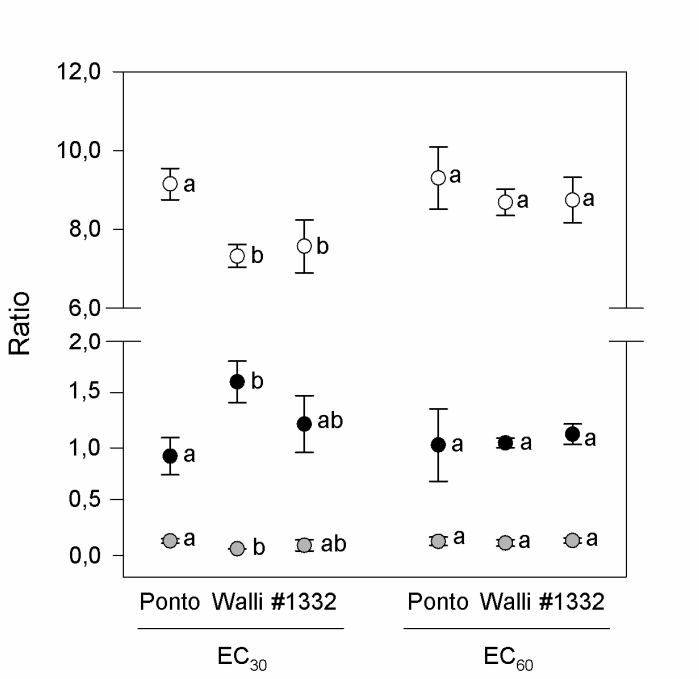

Fig. S5 (Supplemental material)

Biomass of potato plants (n = 5, error bars represent standard deviations) at different plant developmental stages (EC_{30}: young leaf development, EC_{60}: flowering) for different potato cultivars ('Ponto', 'Walli' and #1332). Significance ($p < 0.05$) is indicated by different letters.