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Agronomic Performance and Transcriptional Analysis of Genetically Engineered Zeaxanthin-rich Potato (*Solanum tuberosum* L.)

Xia Dong

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Abstract

Age-related macular degeneration is the primary cause for blindness of aged people. Recent studies have shown that enhanced carotenoid supply can lower the risk for this disease. In order to provide a higher dietary intake, two genetically engineered (GE) zeaxanthin-rich potato clones were derived from the potato (*Solanum tuberosum* L.) cultivar Baltica. Both strategies rely on tuber-specific silencing of the zeaxanthin epoxidase (*zep*) gene, either through co-suppression or anti-sense suppression. Primary evaluation was carried out under greenhouse conditions in a previous study. However, further evaluation including their agronomic performance, stability and tuber-specific expression of the inserted *zep* gene and any potential unexpected changes at the transcriptional level compared with their conventional counterpart cultivar Baltica needs to be carried out under open-field conditions.

In this work, a three-year open-field trial was conducted for the two GE potato clones, their conventional counterpart cultivar Baltica and the four other conventional potato cultivars Désirée, Ditta, Selma and Sibu from 2005 to 2007 in two fields located in Bavaria, Germany. Additionally, a two-year greenhouse experiment was also carried out with the two GE potato clones and Baltica in 2005 and 2006 as control. The aims of this work were to evaluate whether the inserted *zep* gene affects the general morphology, agronomic performance and gene expression profiles of the two zeaxanthin-rich GE potato clones in comparison to their conventional counterpart cultivar. In order to answer these questions, multiple comparison analyses at agronomic, biochemical and transcriptional levels were applied to different tissues at different growth stages of the two GE potato clones and the conventional cultivars grown under greenhouse and open-field conditions.

General morphological and agricultural data showed no significant differences in growth, development and tuber yield of the two GE potato clones compared with Baltica under greenhouse and open-field conditions. Zeaxanthin concentration in tuber tissue as a biochemical index was detected by using HPLC and results showed high zeaxanthin

content in mature GE tubers as the desired trait was consistently inherited under open-field conditions. Furthermore, extremely low out-crossing rate (0.17‰) of conventional potato cultivars with GE potato clones was also determined under open-field conditions.

At the transcriptional level, targeted comparison of the *zep* gene was carried out by using quantitative real time PCR method. In leaves and roots, expression analysis of *zep* showed no significant differences between the two GE potato clones and Baltica at three growth stages under both greenhouse and open-field conditions. Significant *zep* expression changes were only detected in tuber tissue of the two GE potato clones. Untargeted comparison of the transcriptome was performed by employing cDNA macro- and microarray techniques. Expression profiling through cDNA macroarrays derived from subtracted cDNA libraries potentially enriched in differentially expressed genes showed that eight genes in leaves and fifteen in tubers of GE potato clones genes were found significantly up- or down regulated with moderate fold-change (< 2.5) compared with Baltica. Results from cDNA microarray analysis conducted for 11,412 cDNAs showed that no differentially expressed genes were found in GE tubers with expression changes greater than two-fold. As a more comprehensive and accurate expression analysis method, cDNA microarray results further indicated that at the transcriptional level, no significant un-expected changes were caused by the insertion of *zep* constructs in potato clones.

In conclusion, the inserted *zep* gene successfully led to tuber-specific zeaxanthin accumulation without affecting the general morphology, agronomic performance and overall non-target gene expression patterns of the two zeaxanthin-rich GE potato clones under both greenhouse and open-field conditions.

Zusammenfassung

Altersbedingte Makuladegeneration ist die Hauptursache für Altersblindheit. Aktuelle Studien zeigten, dass eine verbesserte Carotionoid-Versorgung das Risiko für diese Krankheit senken kann. Um eine höhere Aufnahme schon mit der Ernährung zu erreichen, wurden zwei gentechnisch veränderte zeaxanthinreiche Kartoffelklone aus der Kartoffelsorte (*Solanum tuberosum* L.) Baltica entwickelt. Beide Strategien beruhen auf der knollenspezifischen Inaktivierung des Zeaxanthin-Epoxidase (*zep*)-Gens entweder durch Co-Suppression oder Antisense-Suppression. Eine grundlegende Evaluierung wurde in einer Vorläuferstudie unter Gewächshausbedingungen durchgeführt. Jedoch ist es nötig, eine weiterführende Evaluierung unter Feldbedingungen vorzunehmen, welche agronomische Leistung, Stabilität und knollenspezifische Expression des inserierten *zep*-Gens und mögliche unerwartete Veränderungen auf transkriptioneller Ebene im Vergleich zur Ausgangssorte Baltica umfasst.

Im Rahmen dieser Arbeit wurde zwischen 2005 und 2007 auf zwei Standorten in Bayern ein dreijähriger Feldversuch mit beiden transgenen Kartoffelklonen, ihrer Ausgangssorte Baltica sowie vier weiteren konventionellen Sorten (Désirée, Ditta, Selma und Sibu) durchgeführt. Zusätzlich fanden 2005 und 2006 Gewächshausexperimente mit beiden transgenen Klonen und Baltica als Kontrolle statt. Ziele dieser Arbeit waren es, zu evaluieren, ob das inserierte *zep*-Gen die generelle Morphologie, agronomische Leistung und Genexpressionsprofile der beiden zeaxanthinreichen transgenen Kartoffelklone im Vergleich zu ihrer Ausgangssorte beeinflusst. Zur Beantwortung dieser Fragen wurden unterschiedliche Organe der gentechnisch veränderten und konventionellen Sorten zu verschiedenen Wachstumsstadien unter Freiland- und Gewächshausbedingungen einer multiplen Vergleichsanalyse auf agronomischer, biochemischer und transkriptioneller Ebene unterzogen.

Morphologische wie agronomische Daten erbrachten bezüglich Wachstum, Entwicklung und Knollenertrag sowohl im Gewächshaus als auch im Feld keine statistisch signifikanten Unterschiede zwischen den gentechnisch veränderten

Kartoffelklonen und Baltica. Die Zeaxanthin-Konzentration der Knollen als biochemische Messgröße wurde mittels HPLC bestimmt und zeigte, dass der Zeaxanthin-Gehalt als das gewünschte Merkmal in reifen transgenen Knollen unter Feldbedingungen stabil vererbt war. Zudem wurde im Feld eine äußerst geringe Auskreuzungsrate (0,17 ‰) von transgenen Klonen in konventionelle Kartoffelsorten beobachtet.

Auf transkriptioneller Ebene wurde ein zielgerichteter Vergleich des *zep*-Gens anhand der quantitativen Real-Time-PCR-Methode durchgeführt. In Blättern und Wurzeln zeigte eine *zep*-Expressionsanalyse in drei Entwicklungsstadien sowohl im Gewächshaus als auch im Feld keine signifikanten Unterschiede zwischen beiden transgenen Kartoffelklonen und Baltica. Signifikante Expressionsunterschiede des *zep*-Gens wurden ausschließlich in den Knollen der zwei gentechnisch veränderten Klone festgestellt. Vergleichsstudien zu Nicht-Ziel-Genen des Transkriptoms wurden anhand von cDNA-Makro- und Mikroarrays angestellt. Expressionsprofilierung mittels cDNA-Makroarrays, die aus subtrahierten, mit potenziell differenziell exprimierten Genen angereicherten cDNA-Banken erstellt wurden, zeigte, dass acht Gene in Blättern und 15 in Knollen der transgenen Kartoffelklone im Vergleich zu Baltica signifikant, jedoch mit mäßigen Expressionsfaktoren (< 2,5) hoch- oder herunterreguliert waren. Die Ergebnisse der cDNA-Mikroarray-Analyse für 11 412 cDNAs wiesen jedoch darauf hin, dass über einem Schwellenwert von zweifacher Änderung keine differenziell exprimierten Gene vorhanden waren. Aufgrund der größeren Abdeckung des Transkriptoms und höheren methodischen Genauigkeit zeigten Mikroarray-Untersuchungen, dass durch die Insertion der *zep*-Konstrukte in Kartoffelklone auch auf transkriptioneller Ebene keine signifikanten unbeabsichtigten Änderungen verursacht wurden.

Schlussfolgernd lässt sich feststellen, dass das inserierte *zep*-Gen erfolgreich zu einer knollenspezifischen Zeaxanthin-Anreicherung führte, ohne dass sowohl unter Gewächshaus- als auch unter Feldbedingungen die generelle Morphologie, agronomische Leistung und Expressionsmuster von Nicht-Ziel-Genen der beiden zeaxanthinreichen transgenen Kartoffelklone betroffen waren.

List of Abbreviations

BLAST	Basic Local Alignment Search Tool
bp	Base pair
cDNA	Complementary deoxyribonucleic acid
Ci Curie	A unit of radioactivity equal to the amount of a radioactive isotope that decays at the rate of 37,000,000,000 disintegrations per second)
Ct	Threshold cycle in RT-PCR
Cy3	Cyanine 3
Cy5	Cyanine 5
DEPC	Diethyl Pyrocarbonate
DW	Dry weight
dNTP	Deoxyribonucleotriphosphate
DMSO	Dimethyl Sulfoxide
dATP	Deoxyadenosinetriphosphate
dCTP	Deoxycytosinetriphosphate
dGTP	Deoxyguanosinetriphosphate
dTTP	Deoxythymidinetriphosphate
ddNTP(s)	Didesoxynucleosidetriphosphate, didesoxynucleotide
dpm	decays per minute
EDTA	Ethylenediamine tetraacetic acid
EST	Expressed sequence tag
FW	Fresh weight
HPLC	High performance liquid chromatography
IPTG	Isopropyl-1-thio-β-D-galaktopyranoside
Lowess	Locally weighted scatter plot smoothing
LB_{Amp} medium	Luria-Bertani-Ampicillin medium
mRNA	Messenger RNA

MOPS	3-(N-morpholino)-propansulfonic acid
OD_{260}	Optical density at 260 nm
PCR	Polymerase chain reaction
p.a.	pro analysi = for analytical use
QTL	Quantitative trait loci
qRT-PCR	Quantitative reverse transcript polymerase chain reaction
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Rotations per minute
SDS	Sodium dodecyl sulfate
SNP	Single nucleotide polymorphism
SOC medium	Bacterial growth medium
SSC	Saline sodium citrate
SSTE	Saline sodium dodecyl sulfate Tris-EDTA
TAE	Tris-Acetate-EDTA
TBE	Tris-Borate-EDTA
TIFF	Tagged image file format
TE	Tris-EDTA
Tris	Tris-(hydroxymethyle)-aminomethane
X-gal	5-bromo-4chloro-3-indolyl-ß -D-galactopyranoside

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1. Introduction

1.1 Zeaxanthin

1.1.1. General introduction

Carotenoids are lipophylic isoprenoid compounds synthesized by all photosynthetic organisms (including plants, algae, and cyanobacteria) but also by some non-photosynthetic bacteria (e.g. *Mycobacterium spp*. and *Flavobacterium sp*.) and fungi (e.g. *Rhodotorula rubra* and *Rhizophylctis rosea*) (Jensen, 1965; Goodwin, 1972; Botella-Pavía and Rodríguez-Concepción, 2006). They are the most widespread group of pigments found in nature, with over 600 characterized structures and an estimated yield of 100 million tones per year (Fraser and Bramley, 2004). The detailed elucidation of the carotenoid biosynthetic pathway has been achieved over the past decades. Carotenoid biosynthesis in plants has been reviewed in detail by several authors (Cunningham and Gantt, 1998; Hirschberg, 2001; Fraser and Bramley, 2004; Giuliano *et al.*, 2008).

Two main classes of naturally occurring carotenoids are distinguishable: (1) carotenes such as β-carotene and α-carotene, which are either linear or cyclized at one or both ends of the molecule hydrocarbons, and (2) xanthophylls, the oxygenated derivatives of carotenes such as violaxanthin, antheraxanthin, zeaxanthin, neoxanthin, and lutein (Yeum and Russell, 2002).

Zeaxanthin is isomeric with lutein (Fig. 1.1), and the difference between them is the shift of one double bond. In zeaxanthin, all double bonds are conjugated. Lutein is structurally related to α-carotene and zeaxanthin to β-carotene (Whitehead *et al.*, 2006). Its Chemical Abstract Service (CAS) number is 144-68-3, chemical formula is $C_{40}H_{56}O_2$, and molecular weight is 568.87 g/mol (Kotíková *et al.*, 2007).

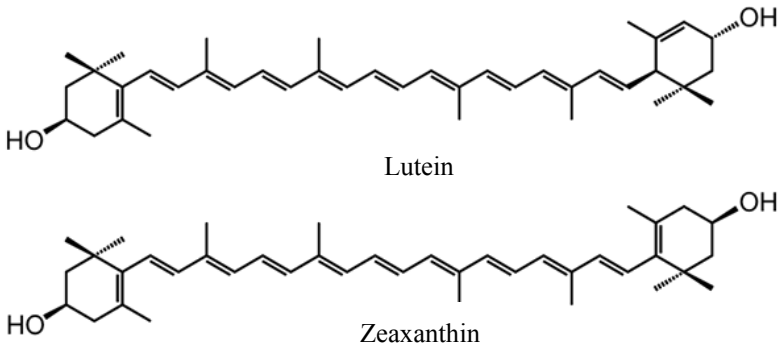


Fig. 1.1Chemical structures of lutein and zeaxanthin.

The color of zeaxanthin is yellow and it occurs naturally in corn, egg yolks, oranges, yellow fruits and vegetables (Humphries and Khachik, 2003). More details about biochemical and structural characteristics of zeaxanthin were given in a recent review of Sajilata *et al.* (2008).

Zeaxanthin can be synthesized in two different ways, either by hydroxylation of ß-carotene or by de-epoxidation of violaxanthin. In the first case, zeaxanthin formation is catalyzed by ß-carotene hydroxylase, using ß-carotene as a substrate. In the second case, zeaxanthin is converted to violaxanthin by zeaxanthin epoxidase under low-light conditions, whereas high-light conditions lead to the production of zeaxanthin via violaxanthin de-epoxidase due to xanthophyll cycle activity (Woitsch and Römer, 2003).

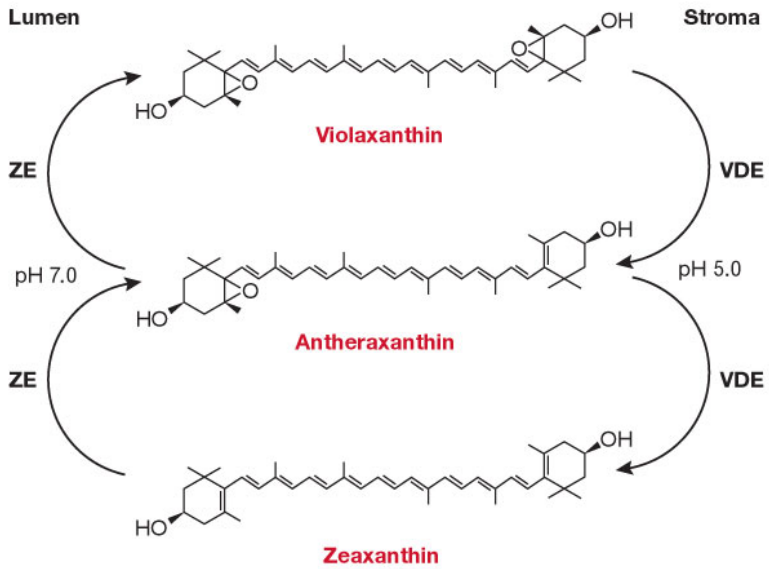


Fig. 1.2 The xanthophyll cycle. VDE, violaxanthin de-epoxidase; ZE, zeaxanthin epoxidase (Szabó *et al.*, 2005).

Xanthophyll cycle is found throughout the plant kingdom and plays a vital role in protecting photosynthetic apparatus as well as a range of other essential cell components (Fig. 1.2) (Demmig-Adams and Adams, 1996). Under excess light, violaxanthin is converted rapidly via the intermediate antheraxanthin to zeaxanthin, and this reaction is reversed under low light levels. The carotenoids participating in this cycle are the only carotenoids present in the photosynthetic membrane that undergo very rapid, light-triggered concentration changes (Demmig-Adams and Adams, 1992). More details of the protection mechanism of xanthophyll cycle can be found in several recent reviews (Morosinotto *et al.*, 2003; Demmig-Adams and Adams, 2006; Garcia-Plazaola *et al.*, 2007).

Up-to-date, majority of the genes encoding enzymes involved in carotenoid biosynthesis pathways, including xanthophyll biosynthetic genes has been cloned in higher plants and green algae (Bouvier *et al.*, 1996; Bugos *et al.*, 1998; Marin *et al.*, 1996; Sun *et al.*, 1996). However, the knowledge of the complex regulation of both xanthophyll biosynthesis and carotenoid biosynthesis is still vastly limited.

1.1.2. Zeaxanthin and eye health

Age related macular degeneration (AMD) is a cause of vision loss of more than 25 million people world-wide and is a leading cause of blindness of people over 55 years of age in most industrial countries (Bone *et al.*, 2003). As the proportion of the elderly in the world's population increases, the public health impact of AMD will become even more severe.

Zeaxanthin and lutein are the most important macular pigments present in the human retina (Bone *et al.*, 1997). These yellow pigments are postulated to participate in photo protection, and a diminished macular pigment might be related to retinal damage (Whitehead *et al.*, 2006; Khachik *et al.*, 2006). Bone *et al.* (1997) discovered unresolved distribution of zeaxanthin and lutein in the human retina, with twice-higher concentration of zeaxanthin in the inner macula, as compared to lutein. Gale *et al.* (2003) reported that

eyes are highly selective and preferentially places dietary zeaxanthin in the very center of the macula, the most critical area for central vision with the greatest need for protection. Zeaxanthin and lutein protect the eyes through (1) absorbing blue light that initiates the production of oxyradicals that place photo-oxidative stress on the retina, and (2) quenching single oxygen and reactive radicals that damage photoreceptor cells (Thomson *et al.*, 2002a). A series of research gave strong evidences that diets rich in lutein and zeaxanthin are associated with decreased prevalence of AMD and cataract in elder people (Thomson *et al.*, 2002b; Tan *et al.*, 2008; O'Connell *et al.*, 2008).

1.1.3. Dietary gap of zeaxanthin

Humans cannot synthesize zeaxanthin in vivo, therefore, must rely on dietary sources. Lutein and zeaxanthin occur in nature in many vegetables, fruits and flowers, such as kale, spinach, broccoli, sweet corn, yellow peppers, oranges, peaches, mango and Marigold flowers (Humphries and Khachik, 2003). The content of lutein varies from 0.1-8.2 mg/100 g FW in peppers to 14.7-39.6 mg/100 g FW in kale. However, the content of zeaxanthin has a relative low value, which varies from zero in kale, broccoli and carrots to 1.5-16.8 mg/100 g FW in yellow peppers (Humphries and Khachik, 2003). Zeaxanthin intake in human has been reported to be 0.1-0.2 mg/day, approximately 10% of the lutein intake (Garcia-Closas *at al.*, 2004). The daily average lutein/zeaxanthin intake in some European countries is 3.25 mg (Spain), 2.50 mg (France), 1.59 mg (UK), and 2.01 mg (The Netherlands) (O'Neill *et al.*, 2001). No dietary intake reference has yet been set for lutein/zeaxanthin, but some epidemiological studies showed that intake of approximately 6 mg/day seem desirable for healthy people (Seddon *et al.*, 1994). However, the amount of lutein/zeaxanthin from average daily fruit/vegetable consumption could not meet the recommended level to prevent against AMD.

One way to fill the gap of carotenoid nutrition is to fall back on commercial supplements obtained from microorganism cultures and chemical synthesis. However, the relatively high production costs and accessibility may limit their wide consumption,

especially in developing countries (Hampton, 2005). Another, more efficient and economical way is direct consumption of plants and foods with higher carotenoid contents. The utilization of biotechnology approaches for the improvement of nutritional value of staple foods is particularly desirable. Recent research on the bioavailability of zeaxanthin from transgenic potatoes showed that the consumption of the zeaxanthin-rich potatoes significantly increases chylomicron zeaxanthin concentrations and this result provide concrete evidence that such potatoes could be potentially used as an important dietary source of zeaxanthin (Bub *et al*., 2008).

1.2 Genetically engineered (GE) plants

1.2.1. GE plants with enhanced agronomic performance

Plants are attractive biological resources as they synthesize and accumulate around 200,000 natural products that are being utilized as foods, pharmaceuticals, dyes, flavors and other materials for every day human needs (Fiehn, 2002). Beside their already attractive native form, transgenic technologies may add new and valuable characteristics for plant cultivation and post-harvest handling.

Agronomic traits such as herbicide tolerance, virus resistance, insect resistance, disease-tolerance and delayed ripening were the initial focus of genetic engineering in plants (Wenzel, 2006), for instance oilseed rape with herbicide tolerance, maize with pest-resistance and tomato with delayed ripening character (Herrera-Estrella *et al.*, 2004). Genetic engineered high performance crops have a particular value for agricultural production in developing countries, while they not only deliver higher yields but also possess the ability to grow under different biotic and abiotic challenges without more chemical inputs, such as fungicides and pesticides. Furthermore, GE crops with increased tolerance to drought, salinity and other adverse circumstances provide a valuable solution for the countries with lower-potential lands (Conway, 1997). It is evident that GE crops will make a vital contribution in reducing poverty and hunger in poor countries in the

upcoming years (Robinson, 1999; Thomson, 2006). In 2007, the number of small and resource-poor farmers benefiting form GE crops in developing countries exceeded 10 million. The majority of 7.1 million was located in China (Bt cotton), 3.8 million in India (Bt cotton), and the rest in the Philippines (GE maize) and South Africa (GE cotton, maize and soybean) (James, 2007).

1.2.2. GE plants with enhanced nutritional value

Nutrients in the human diet come directly or indirectly from plants. However, human can obtain adequate nutrients only through consumption of variable food sources. For people of developing countries, who rely mostly on staple crops, nutrition deficiency is still a significant public health issue (Christou and Twyman, 2004). However, improper diet composition and eating habits can also lead to deficiency in certain nutrients (Zhu *et al.*, 2007).

GE crops, especially the GE staple crops with increased essential nutrient contents provide an efficient and economical means to resolve the malnutrition and unbalanced-nutrition status. Some GE crops have already been developed with this purpose, for instance Golden Rice with high vitamin A content (Ye *et al.*, 2000), lysine-rich maize (Torrent *et al.*, 1997), or ferritin-rich lettuce (Goto *et al.*, 2000).

The identification of carotenoid biosynthetic genes in plants and other organisms has opened the gate to biotechnological overproduction of carotenoids in crops (Botella-Pavía and Rodríguez-Concepción, 2006). In recent years, the number of successful examples about metabolically engineering of carotenoid biosynthesis pathways in higher plants has grown with an impressive rate. Examples of GE crop plants with increased carotenoid levels are given in Table 1.1 (Giuliano *et al.*, 2008; Lopez *et al.*, 2008).

Table 1.1 GE plants with enhanced carotenoids level (Giuliano *et al.*, 2008; Lopez *et al.*, 2008)

Target plant/Tissue	Strategy	Traits
Canola seed	*CrtB, CrtI, CrtY* overexpression	Beta-, α-carotene accumulation
Potato plant, tuber	*CrtO* overexpression	Ketocarotenoid accumulation in leaves, tubers
Potato tuber	*CrtB, I, Y* overexpression	Beta-, α-carotene accumulation
Potato tuber	*DXS* overexpression	Enhanced zeatin levels, early sprouting
Potato tuber	*ZEP* silencing	Zeaxanthin accumulation
Potato tuber	*LCY-e* silencing	Enhanced β-carotene, total carotenoids
Potato tuber	*CHY* silencing	Enhanced β-carotene
Potato tuber	*Or* overexpression	Beta-carotene, total carotenoid accumulation
Rice seed	*PSY*, *CrtI* overexpression	Beta-carotene, zeaxanthin accumulation
Lotus japonicus flower	*CrtW* overexpression	Ketocarotenoid accumulation
Tomato plant	*PSY* overexpression	Gibberellin depletion, dwarfism
Tomato plant	*CHRD* silencing	Decreased flower carotenoids, decreased photosynthetic electron flow
Tomato plant	*DXS* overexpression	Enhanced fruit carotenoids
Tomato plant	*CRY2* overexpression	Enhanced carotenoids, flavonoids
Tomato plant, fruit	*DET1, DDB1, COP1-like* silencing	Enhanced carotenoids, flavonoids
Tomato fruit	*CrtB* overexpression	Enhanced lycopene
Tomato fruit	*FIBRILLIN* overexpression	Enhanced carotenoids, carotenoid-derived volatiles, delayed thylakoid loss
Tomato fruit	*LCY-b B* silencing	Enhanced lycopene
Tomato fruit	*CrtI* overexpression	Beta-carotene accumulation
Tomato fruit	*LCY-b*, *CHY* overexpression	Beta-carotene, zeaxanthin accumulation
Tomato fruit	*CrtY* overexpression in plastids	Beta-carotene accumulation
Tobacco, tomato plant	*CrtZ*, *W* polyprotein overexpression	Ketocarotenoid accumulation in leaves and nectary (flower)
Tobacco plant, flower	*CrtO* overexpression	Ketocarotenoid accumulation in leaves, nectary, petals
Tobacco leaf	*CaCCS* virus-mediated overexpression	Capsanthin accumulation
Carrot plant	*CrtO* overexpression	Ketocarotenoid accumulation in leaves, roots
Arabidopsis plant	nt*LCY-e* overexpression	Enhanced leaf lutein, enhanced NPQ
Arabidopsis plant	*CrtZ*, *CHY* overexpression	Enhanced leaf β-xanthophyll pool, resistance to UV and to temperature + light stress
Arabidopsis plant	*HDR* overexpression	Enhanced leaf carotenoids, seed dormancy
Arabidopsis seed	*CrtO* overexpression	Ketocarotenoid accumulation

1.2.3. Regulation of gene expression in GE plants

Regulation of expression of specific genes by antisense RNA is a naturally occurring mechanism in bacteria (Simons *et al.*, 1983). Antisense refers to short DNA or RNA sequences, termed oligonucleotides, which are designed to be complementary to known genes of interest. The aim of the oligonucleotides is to alter gene expression resulting in prevention or reduction of protein production from a targeted gene (Kuipers *et al.*, 1998).

Co-suppression effect was discovered in 1990, in the attempts to genetically modify petunia plants (Napoli *et al.*, 1990; van der Krol *et al.*, 1990). Co-suppression is one of the forms of post-transcriptional gene silencing (PTGS). The accumulation of homologous sense-oriented transcripts results in a co-suppression of both transgene and homologous endogene. It usually leads to inactivation of certain enzymes in a given metabolic pathway (Jorgensen *et al.*, 1996).

Two zeaxanthin-rich GE potato clones used throughout this work represent successful applications of co-suppression and antisense technologies (Römer *et al.*, 2002). The common ground of these two GE potato clones are (1) both were derived from the same conventional potato cultivar; (2) both contained higher zeaxanthin and total carotenoid amounts, and (3) the activity of zeaxanthin epoxidase (ZEP) in both GE potato tubers was inactivated allowing zeaxanthin accumulation (Gerjets and Sandmann, 2006).

1.3 Assessment of GE plants

1.3.1 Legal background

In accordance with the general food law Regulation (EC) 178/2002, the legal framework in the EU for Genetically Modified Organism (GMO) risk assessment is composed of two different sets of legislation: Regulation (EC) 1829/2003 and Directive 2001/18/EC. Moreover, the European Food Safety Authority (EFSA) Guidance Document

(EFSA, 2006) provides guidance for applications within the framework of the two legislations. The general concept of these laws can be explained as following: the general food law Regulation (EC) 178/2002 lays down the general principles of food law and procedures in food safety. It defines food law broadly, including animal feed and other agricultural inputs at the level of primary production. The general food law defines 'hazard', 'risk', 'risk analysis', 'risk assessment', 'risk management' and 'risk communication' (EC, 2002). GE food and feed Regulation (EC) 1829/2003 provides the basis for ensuring a high level of protection of human life and health, animal health and welfare, environment and consumer interests in relation to GE food and feed, whilst ensuring the effective functioning of the internal market. It lays down community procedures for the authorization and supervision of genetically engineered food and feed the provisions for the labeling of GE food and feed (EC, 2003). Directive 2001/18/EC regulates the deliberate release of GMO into the environment (EC, 2001). This directive ensures a step-by-step approval process based on a case-by-case assessment of the risk to human health and the environment before any GMO can be released into the environment or placed on the market.

Under EU legislation, all GMO and derived products must be evaluated by EFSA before they can be authorized in the EU. For any GMO and GMO-derived food or feed to be authorized, a company must submit an authorization application in line with European legislation. The European Commission forwards the application to EFSA and requests a scientific risk assessment. EFSA's GMO Panel carries out a detailed risk assessment to evaluate the safety of the GMO and GMO-derived food or feed. The Panel's independent scientific advice is then used by the European Commission and EU Member States when taking a decision on market approval (EFSA, 2006; EFSA, 2007). The European Commission shall submit a draft decision to the Standing Committee on the Food Chain and Animal Health within three months of receipt of the EFSA's opinion. A final decision shall be adopted in accordance with the Committee procedure. The authorization is valid throughout the Community for 10 years. More details and cases can be found under www.efsa.europa.eu.

1.3.2 Strategies

1.3.2.1 Concept of substantial equivalence

The concept of *substantial equivalence*, which is also mentioned as the comparative safety approach, has been recommended by FAO/WHO and OECD (Organization for Economic Cooperation and Development) as a guiding tool for safety assessment of foods and food ingredients derived from GE plants intended for human consumption (OECD, 1993; FAO, 1996). The concept is based on the idea that an existing organism, which is used as food or feed and proved to be safe by consumption over a long period, can serve as a comparator when assessing the safety of the GE food/feed (OECD, 1993; EFSA, 2007). Moreover, it has been recognized that the application of the concept of *substantial equivalence* contributes to a robust safety assessment framework and it has been adopted in the EU guidelines as well as in Codex guidelines[1].

The concept of *substantial equivalence* was developed as a practical approach to the safety assessment of GE foods. It is used to structure the safety assessment of a GE food relative to its conventional counterpart plant (Fig. 1.4). It follows a stepwise process aided by a series of structured questions. The first step of the approach is the comparative analysis of the molecular, agronomic and morphological characteristics of the organisms questioned, as well as their chemical composition. The outcome of this comparative analysis will further structure the second part of the assessment procedure, which may include further specific safety and nutritional testing. This approach should provide evidence on whether or not the GE crop-derived food/feed is as safe as the traditional counterpart (FAO/WHO, 2000; EFSA, 2007).

[1] Codex Alimentarius Commission: joint commission of the United Nations Food and Agriculture Organization (FAO) and World Health Organization (WHO) established in 1963 to develop an international code of food quality standards (http://www.codexalimentarius.net).

1.3.2.2 *Intended effects*

Intended effects of genetic engineering are those that are targeted to occur from the introduction of the gene(s) in question, and that fulfill the original objectives of the genetic transformation process. Alterations in the phenotype may be identified through a comparative analysis of growth performance, yield, disease resistance, etc. Intended alterations in the composition of a GE plant compared to their conventional counterpart plant, *e.g.* the parent, may be identified by measurements of single compounds, *e.g.* newly expressed proteins, macro- and micro-nutrients (targeted approach). Analytical detection methods used must meet specific quality and validation criteria (EFSA, 2007).

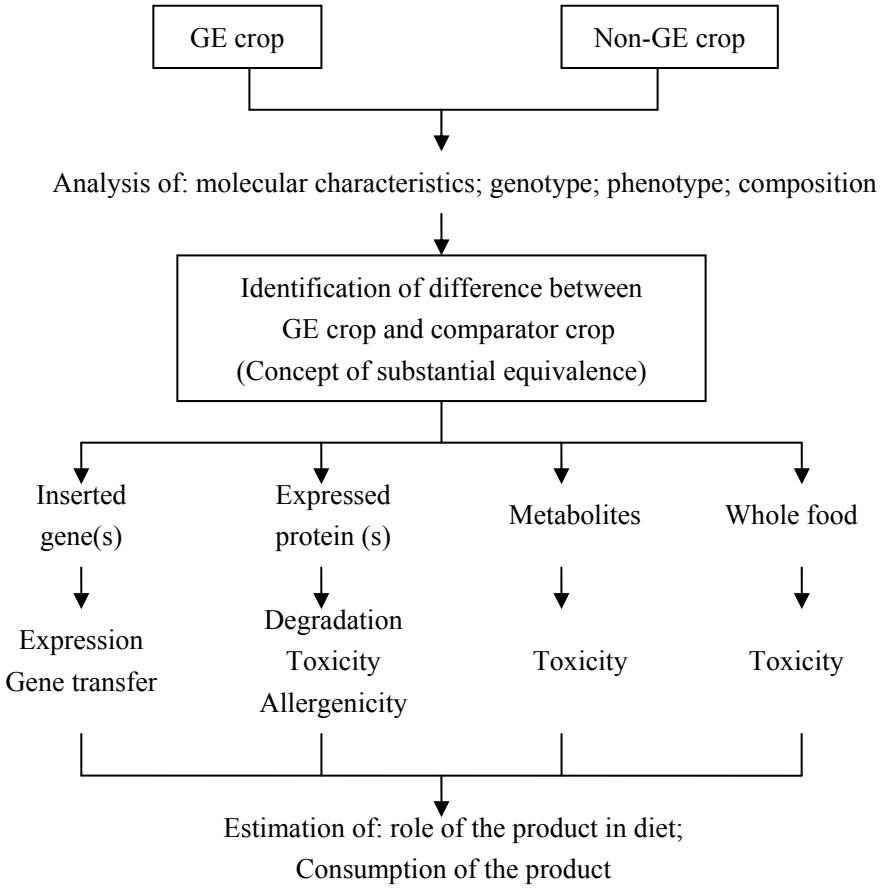


Fig. 1.3 Concept of substantial equivalence in the assessment of GE crops: Safety assessment strategy in crops (Kuiper and Kleter, 2003).

1.3.2.3 *Unintended effects*

Unintended effects are defined as consistent differences between the GE plant and its counterpart, that go beyond the primary expected effect of introducing the target gene. *Unintended effect* could potentially be linked to genetic rearrangements or metabolic perturbations (EFSA, 2007).

In order to identify any potential *unintended effects* in the GE plant, a targeted analysis should be carried out first on the compounds, which represent the important metabolic pathways in the GE plant and its conventional counterpart plant (Fig. 1.5). These pathways may include macronutrients, micronutrients and/or secondary metabolites, as well as known anti-nutrients and toxins (EFSA, 2007). For a further and comprehensive detection of potential *unintended effects* in GMO, profiling techniques are considered as a very powerful alternative approach. More details regarding the methods to detect *unintended effects* of GMO will be presented in the next sections.

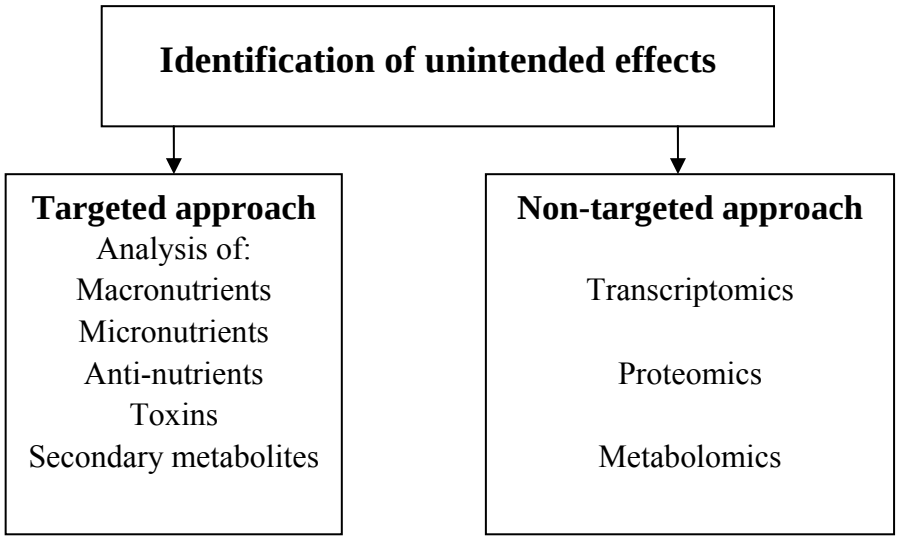


Fig. 1.4 Identification of unintended affects resulting from genetic modification (Kuiper *et al.*, 2003).

1.3.3 Methods

1.3.3.1 Field testing

High correlations between greenhouse and field performances of the transgenic traits of GE plants were frequently reported (Tu *et al.*, 2000; Bashir *et al.*, 2004; Datta *et al.*, 2007). However, no guarantee could be given that the greenhouse performance of any specific GE plant would be translated into field condition (Conner, 2007). Therefore, it is critical to verify the transgenic phenotypes under field conditions for each GE plant.

Protocols for field trials performed with GE plants and their control plants must be specified and documented according to the guidance in the following main aspects: (1) number of locations, growing seasons, geographical spreading and replicates; (2) statistical models for analysis, confidence and intervals and (3) baseline used for consideration of natural variations (EFSA, 2007).

1.3.3.2 Expression profiling

One of the safety concerns regarding GE crops and GE-derived foods is the possibility of plant inherent gene expression alteration, particularly with respect to levels of endogenous toxicants and anti-nutrients (e.g., glycoalkaloids in potato). In order to detect such alterations at the gene expression level mRNA fingerprinting methods, such as Northern blotting, S1 nuclease protection, serial analysis of gene expression (SAGE), or cDNA array technology can be applied. Among these methods, microarray technology has revolutionized global gene expression profiling by allowing the study of the entire genome in a single experiment (Cellini *et al.*, 2004). It has the capacity of parallel screening of a large number of gene sequences for differences in gene expression in cells, tissues or organs of variable origin (van Hal *et al.*, 2000).

The application of microarray technology for the detection of *unintended effects* in GE plants is unfolding promptly. Several array technologies comprising large numbers of expressed genes in specific organisms are already commercially available. For example,

the microarray-based comparative analysis of gene expression profiles during grain development in transgenic and wild type wheat plants was carried out with the wheat unigene cDNA microarrays containing more than nine thousand selected genes. Gene expression profiles of the transgenic versus counterpart lines revealed only slight differences at the various developmental stages, with no subsequent confirmation of any of the identified significantly differentially expressed gene by qRT-PCR. These results proved that no significant *unintended effects* of the modified gene on the overall gene expression level were discovered in GE wheat (Gregersen *et al.*, 2005).

cDNA microarrays were also used for comparison of gene expression profiles of transgenic fungus-resistant barley with its non-transgenic counterpart (www.gmo-safety.eu). Moreover, cDNA microarrays with up to 10,000 elements were developed for comparison of different potato tissues to investigate disease resistances (Cellini *et al.*, 2004). Research projects to develop informative arrays for tomato and potato, as model systems for food plants are on the way, e.g., the GMOCARE (www.ENTRANSFOOD.com) and the UK Foods Standards Agency FSA projects, where transcriptome, proteome and metabolome analyses are being introduced in order to detect *unintended effects* in genetically modified potatoes (www.foodstandards.gov.uk).

Microarray technology is developing very fast, especially with the support of powerful bioinformatics tools up-to-date. Except the application in plant gene expression analysis, more applications of microarrays can be found in functional genomic research, drug target identification, and disease diagnostics (Rinaldis, 2007). We have the reason to believe that microarray technology will continue to contribute to various new, highly significant research areas and as a result, will benefit the human society.

1.3.3.3 Protein profiling

DNA microarrays have been successfully used for the analysis of RNA transcript level in cellular extracts. Although the expression of proteins can be inferred by assessing the levels of mRNA, there is no concrete evidence for proportional relationship between

the changes at protein and mRNA levels (Gygi *et al.*, 1999). Moreover, nucleotide screens cannot provide information on the post-translational modifications of proteins, therefore a reliable and high-throughout approach for measuring the corresponding proteins becomes critical essential (Tomlinson and Holt, 2001).

One most widely used approach for protein profiling is two-dimensional gel electrophoresis (2DGE), coupled with mass spectrometry (MS) and subsequent computer assisted identification of the fragments using online databases (Anderson and Mann, 2000). The application of such proteomics approaches for the safety assessment of GE crops was adopted in several projects, e.g. GMOCARE 2002 and 2008 (more information is available at www.entransfood.nl/RTDprojects/GMOCARE. In GMOCARE project, 2DGE protein profiling is being tested on GE potato and tomato lines to compare the GE plants with their conventional counterpart plant at a protein level. However, the limitation of 2DGE is the quantification methods at protein level. It requires both a high degree of technical skill and sophisticated computational analysis. Nevertheless, improvements in separation techniques, protein preparations, and detection methods are evolving rapidly in this area (Thomas *et al.*, 2008).

Another powerful approach for protein profiling, which was established over the past few years, is the protein microarray technology (also mentioned as biochip or protein-chip). As a complementary approach to cDNA microarrays, protein microarrays allow simultaneous and rapid analysis of thousands of proteins in a high-throughput manner. Currently, three main types of protein microarrays are used to explore biochemical activities of the proteins. These involve analytical microarrays, functional microarrays and reverse phase microarrays. Antibody microarrays are the most commonly used analytical protein microarrays (Bertone and Snyder, 2005). Protein microarrays are widely used in characterizing function of proteins, discovering new functions of known proteins, and in studying protein-protein and other interactions (Hall *et al.*, 2004). Their application in plant sciences has been strongly supported by the completion of genome sequencing projects of *Arabidopsis thaliana* and rice (Kersten and Feilner, 2007). More details about protein microarrays can be found in a recent review of Hall *et al.* (2007).

1.3.3.4 Metabolite profiling

A comprehensive food safety assessment is necessarily required before release of any GE crop. According to the principle of *substantial equivalence*, which is a key safety evaluation strategy agreed by most international bodies, the comparison of the chemical composition of GE plants and their conventional counterpart plant is a crucial step (Frenzel *et al.*, 2003).

Metabolite profiling is an analytical method for relative quantification of a number of metabolites from biological samples (Fiehn, 2002). The approach is based on two basic methods, nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS). In the MS analysis, a pre-separation of metabolic components requires the usage of either gas chromatography (GC) or high-performance liquid chromatography (HPLC). HPLC is a form of column chromatography used frequently in biochemistry and analytical chemistry to separate, identify and quantify compounds (Snyder and Dolan, 2006). Analysis of plant carotenoids utilizing HPLC approach offers the advantage of rapidity, relative simplicity, ease of automation, sensitivity, precision, proper sample preservation, and on-line detection over the full spectral range (Long *et al.*, 2006). Other more specialized methods include FTIR (Fourier-Transform (near) Infrared) spectroscopy and arrayed electrochemical detection (Nicholson *et al.*, 2007).

All these approaches are capable of detecting, identifying, resolving and quantifying a wide range of compounds in a single sample, making it possible to obtain unbiased and targeted information on the metabolites in metabolic pathways (Fraser *et al.*, 2007). In this light, metabolite profiling increases the chance of detecting potential *unintended effects* caused by genetic manipulation.

Several reviews have illustrated the application of metabolite profiling in functional genomics, including studies on bio-safety evaluation of GE crops (Fiehn *et al.*, 2000; Catchpole *et al.*, 2005; Poulsen *et al.*, 2007).

1.4 Aims of the work

The primary goal of this work was to evaluate general morphology, agronomic performance and gene expression profiles of the two GE zeaxanthin-rich potato clones in comparison to their conventional counterpart plant. In order to fulfill this objective, the following sub-goals were set to: (1) evaluate the general morphology of two GE zeaxanthin-rich potatoe clones in comparison to their conventional counterpart cultivar Baltica and four other conventional cultivars under both greenhouse and open-field conditions, (2) verify the steady and tuber-specific expression of the inserted *zep* gene by using qRT-PCR method and compare the differences of *zep* expression between GE and non-GE plants with the natural variation in other conventional cultivars under the same agricultural field conditions, and (3) detect any potential unexpected changes in transcriptome of GE potato clones except the inserted gene in comparison to Baltica through cDNA macro- and microarray techniques.

2. Materials and Methods

2.1 Materials

2.1.1 Plant materials

2.1.1.1. Conventional potatoes

The *Solanum tuberosum* L. cv. Baltica was used in the experiments as reference to the two GE potato clones SR47 and SR48. Further conventional genotypes, cvs. Sibu (SaKa-Ragis Pflanzenzucht GbR, Windeby, Germany), Ditta (EUROPLANT Pflanzenzucht GmbH, Lüneburg, Germany), Désirée (Saatzucht Fritz Lange KG, Bad Schwartau, Germany) and Selma (Bavaria-Saat Vertriebs GmbH, Schrobenhausen, Germany) were used as references for assessing natural trait variation. All the conventional potato clones used for the field trials were purchased from breeding stations mentioned above.

2.1.1.2. GE potatoes

Two GE potato clones SR47/00#18 and SR48/00#17 (SaKa-Ragis Pflanzenzucht GbR, Windeby, Germany) were used in this study in a three-year field trial. To simplify matters, clone SR47/00#18 will be referred throughout this work as SR47, and SR48/00#17 as SR48. SR47 and SR48 were both derived from the potato cultivar Baltica that was modified with co-suppression and anti-sense technologies, respectively (Sandman *et al.*, 2002; Römer *et al.*, 2002; Lübeck *et al.*, 2006).

Details of the plasmid constructs used for engineering zeaxanthin-rich GE potato clones are shown in Fig. 2.1. cDNA fragment encoding the potato derived *zep* gene was cloned into the pBin19-related transformation vector pPGB121S in the sense (SR47), or anti-sense orientation (SR48). Fragments were inserted between the granule-bound starch

synthase (GBSS) promoter and the nopaline synthase (NOS) terminator sequences in order to drive tuber-specific expression. The resulting constructs were used to transform Baltica, via *Agrobacterium* mediation. SR47 and SR48 were selected due to their unaltered phenotype and relative high contents of zeaxanthin and total carotenoids of 40.1 and 60.8, and 16.5 and 44.2 µg/g DW, respectively (Römer *et al.*, 2002).

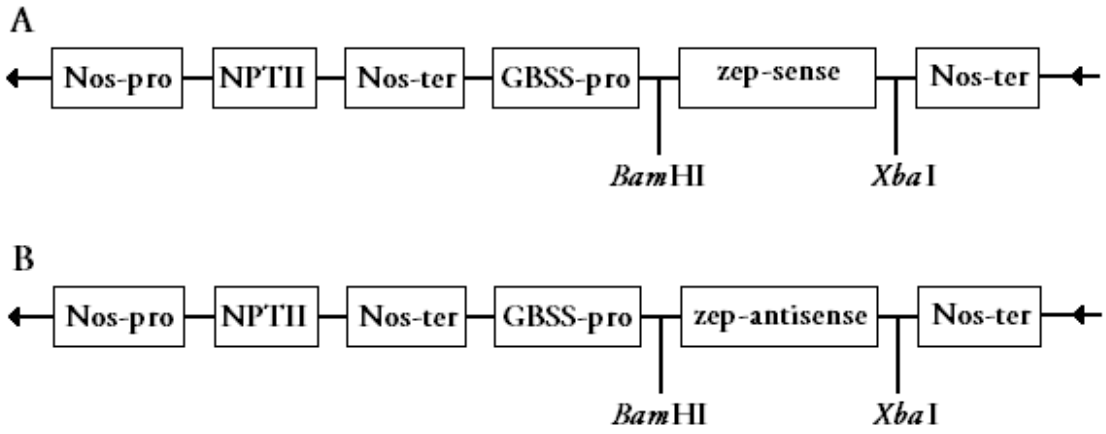


Fig. 2.1 Plasmid constructs used for engineering zeaxanthin-rich potato clones used in this work. A: SR47; B: SR48 (Römer *et al.*, 2002).

2.1.2 Chemicals and reagents

Detailed list of chemicals and their manufacturers used in this work is given in Appendix A. Appendix B includes commercial kits, enzymes and oligonucleotides. All media and buffers were prepared from distilled, deionized Millipore water. When required, media and buffers were autoclaved at 120°C for 20 min. Solutions used for RNA isolation were dissolved in diethylpyrocarbonate (DEPC)-treated water. DEPC-treated water was prepared by addition of 0.1 % DEPC to the distilled water, shaken vigorously and incubated for 12 hours at room temperature (RT) under the fume hood. Subsequently, the solution was autoclaved for 15 minutes at 120°C to remove any trace of the toxic DEPC. Detailed description of media and buffers used is given in Appendix C.

2.2 Methods

2.2.1 Growth conditions and sampling

2.2.1.1. Greenhouse experiment

Potato cultivar Baltica and two GE potato clones were grown from tubers in 2 L pots in the greenhouse at 18 ± 4°C, under a 16-h day length. Samples were taken from leaves, root and tubers at principle growth stage (PGS) 30, 60 and 90, according to BBCH monograph for growth stage of mono- and dicotyledonous plants (Meier, 2001). For gene expression study leaf, root and tuber samples were pooled from three plants of Baltica, SR47 and SR48 harvested at PGS 30, 60 and 90, respectively. Tissues were subsequently frozen in liquid nitrogen and stored at -80°C for further processing. Further conventional potato cultivars Ditta, Désirée, Selma and Sibu used as indirect controls for GE potatoes were grown only in field trials.

GE potato seed tubers were propagated from mini-tubers (ca. 20-35 mm diameter) in the greenhouse and grown in 2 L pots at 18 ± 4°C for approximately 90 days. Supplementary artificial light of minimum of 250 μmol photons m^{-2} s^{-1} was used. Mini-tubers were harvested after maturation and stored at 4°C in darkness.

2.2.1.2. Field trials and sampling

Field trials were conducted in Roggenstein (82223 Roggenstein, Olching, Bavaria, Germany) in 2005, 2007 and Oberviehhausen (94574 Wallerfing, Deggendorf, Bavaria, Germany) in 2006, 2007 (Fig. 2.2). Growing season was from April to September.

At both experimental field sites, seed tubers ranging from 40-60 g were pre-sprouted two weeks prior to planting. A randomized complete block design with six replications was used. Each plot measured 9 × 3 m (L × W) and consisted of four rows with thirty plants each (Fig. 2.3). The distance between two rows was 0.75 m and between plants in the same row 0.3 m. Each plot measured 27 m^2 and contained 120 plants. All

experiments were conducted following standard agronomical procedures. A single harvest was carried out at the end of the growing season according to the growth stages. Samples were taken from leaves, roots and tubers at PGS 30, 60 and 90 in 2005 and 2007 (Roggenstein), and 2006 and 2007 (Oberviehhausen).

Roggenstein, Munich **B**

Fig. 2.2 Experiment fields used for GE potatoes in south Germany. A: Roggenstein (Roggenstein); B: Oberviehhausen (Oberviehhausen).

The field was additionally labelled and monitored for re-emergence of volunteer GE potato plants for the next two seasons. In order to prevent pollen transfer, nylon net was used to cover the GE plants in 2005 (Roggenstein). The size of the net was 3 × 1.5 × 1 m, and the nylon net covered 20-25 GE plants located in the centre of the plots. Flowers of the GE plants outside the net were removed before flowering. Except for the field trial in 2005 (Roggenstein), flowers of the GE plants in field trials in 2006 and 2007 were removed before flowering. Potato tubers were harvested with a mechanical lifter. Tubers were handpicked and weighed for yield determination.

Sampling of leaves, roots and tubers from field was as described in greenhouse sampling. The only difference was the number of plant in each pooled sample. In field sampling, five plants (randomly distributed from the two middle rows as shown in Fig. 2.3B) were harvested. Six replicates for each potato clone at each sampling point were taken. Three of them were analyzed and the other three were kept at -80°C as a reserve. Berries were collected and counted at PGS 90. All berries on the plants and on the ground within a plot were collected.

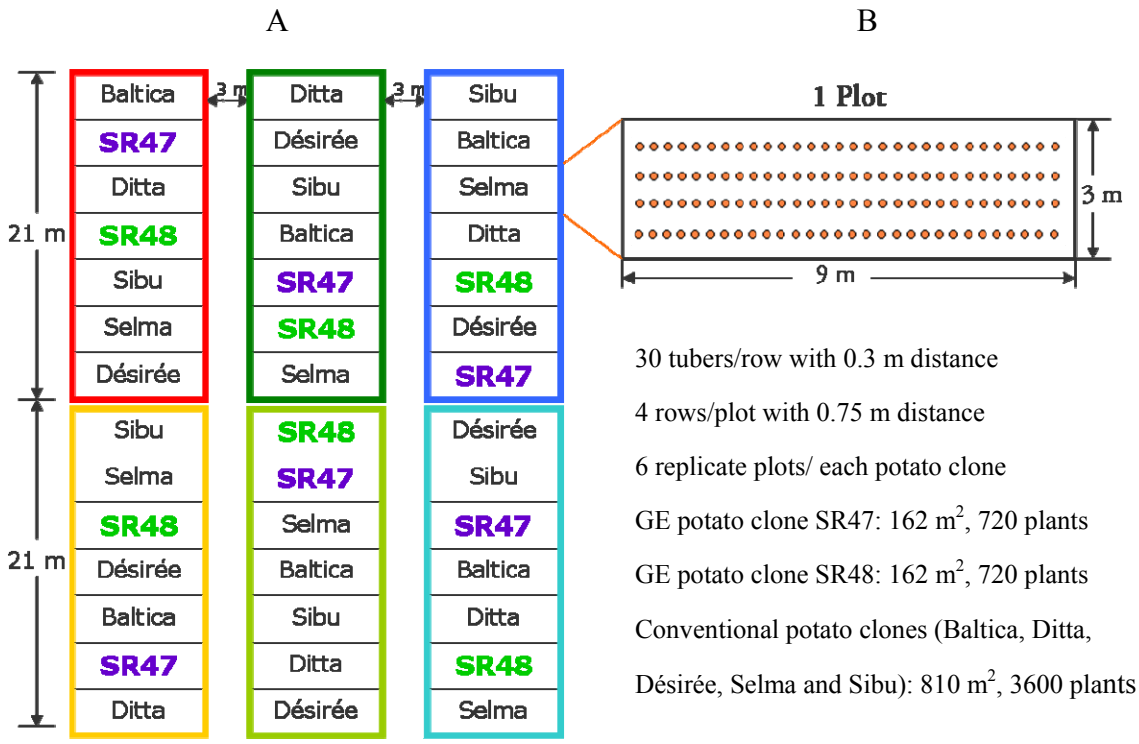


Fig. 2.3 Layout of field trials in Roggenstein and Oberviehhausen. A: overview of the field trial layout; B: layout details inside one plot.

2.2.1.3. Out-crossing rate

Natural out-crossing rate between GE and non-GE potato plants was calculated based on plant performance in Roggenstein 2005. For this reason, seeds from potato berries were collected and dried on filter paper at RT. Around twenty seeds per berry were placed on a filter paper in Petri dish and kept moist with 2500 ppm gibberellic acid solution. Germination rates were recorded and twelve seedlings from each Petri dish were transferred to multi-pot plate for further growth. At plant height of 15 cm leaflet samples were collected from each seedling of the dish and pooled. Genomic DNA was isolated by using CTAB method (Ausubel *et al.*, 1991) and PCR (Chapter 2.2.4.1) was performed with transgenic-specific primer (5'-GACCGGCAACAGGATTCAATC-3'and 5'-CGCTATCAGGACATAGCGTTG-3'; annealing temperature: 45°C). In the event of a positive PCR result, each seedling from the pooled sample was tested separately again, and the exact number of transgenic seeds per berry was recorded.

2.2.2 Isolation and preparation of RNA

2.2.2.1. RNA isolation for RT-PCR

Total RNA from leaves was isolated using TRIzol@Reagent (Invitrogen GmbH, Karlsruhe, Germany), according to manufacturer's instructions. Total RNA from potato tubers and roots was isolated using modified RNA isolation method, originally described by Chang *et al.* (1993). The extraction buffer (20 g/l CTAB, 20 g/l PVP 40, 100mM Tris-HCl pH 8.0, 25mM EDTA, 2M NaCl) was warmed up to 65°C in a water bath and mixed well before applying 13 ml of it into a 50 ml tube. After supplementing the buffer with 260 µl ß-mercaptoethanol and 7.2 µl spermidine, 2-3 g grinded tissue was added and mixed by inverting and vortexing the tube for 1 min. The mixture was transferred into a 50 ml Corex centrifuge tube (Krackeler Scientific, Inc. Albany, USA) and extracted two times with an equal amount of chloroform:isoamyl alcohol (24:1). The phases were separated by centrifugation at 12,000 × g for 10 min at room temperature (RT). The supernatant was collected in a fresh 50 ml tube and 1/3 volume 8 M LiCl was added. RNA was precipitated overnight at 4°C and collected by centrifugation at 12,000 × g for 20 min at 4°C. Total RNA was dissolved in 500 µl of SSTE buffer (1M NaCl, 0.5% SDS, 10mM Tris pH 8.0, 1mM EDTA pH 8.0), transferred into a 1.5 ml reaction tube and extracted once with 500 µl of chloroform:isoamyl alcohol (24:1). Phases were separated by short centrifugation at RT and the supernatant was transferred into a 2 ml tube with addition of two volumes of 100% ethanol and precipitated at -20°C for 2 h. After centrifugation at 13,000 rpm at 4°C and supernatant removal, the pellet was air-dried and dissolved in 50 µl DEPC-treated water. Total RNA was quantified by measuring absorbance at 260 nm using spectrophotometer (Genesys 10 Bio, Thermo Electron Corporation, Madison, USA) and stored at -80°C for later use.

2.2.2.2. mRNA isolation and labelling for microarray

mRNA was directly isolated using DynaBeads oligo (dT) 25 (Dynal Biotech, Oslo, Norway). 1.5 ml of lysis / binding buffer was applied into a 2 ml tube containing ~200 mg grinded raw material, homogenized with a microtube pestle and centrifuged at full speed for 5 min at 4°C. A 2 ml reaction tube with 250 µl paramagnetic beads was placed for 30 s into magnetic particle concentrator (MPC) (all steps including supernatant removal were performed in MPC) to remove beads storage solution and treated with 500 µl lysis/binding buffer. The buffer was subsequently discarded and paramagnetic beads were ready to use. The crude extract (supernatant) from the centrifuged sample was applied to the beads, avoiding floating particles. These samples were well-mixed with the pipette and left for 3-5 min for incubation at RT, with frequent inversion of the tube for beads suspension. The supernatant was discarded and paramagnetic beads were washed with washing buffer A. At this step the next crude extract from the same genotype was ready to be applied to the beads and the procedure was repeated 3-4 times, for the purpose of obtaining sufficient amounts of mRNA. Four to six tubes were handled in parallel following the protocol. After the last sample was applied to the beads, they were washed twice with washing buffer B and stored at 4°C until performing reverse transcription reaction.

Reverse transcription was performed with Superscript II (Invitrogen GmbH, Karlsruhe, Germany) directly on the paramagnetic beads by using the covalently linked oligo (dT)25 sequence as primer. Paramagnetic beads with immobilized mRNA were washed three times in 250 µl of ice-cold first strand buffer and transferred into 1.5 ml reaction tubes between washing steps. Thirty µl of RT-mix per sample was applied and samples were incubated 1 hour in a rotary oven at 42°C. Thereafter, samples were washed twice with 300 µl TE buffer, resuspended in 300 µl TE buffer and incubated 6 min at 95°C in order to remove rest of the RNA annealed to first strand cDNA. For storage at 4°C samples were resuspended in 200 µl TE buffer.

Second strand synthesis was conducted by using Klenow DNA polymerase I (Fermentas Life Sciences, St. Leon-Rot, Germany) on DynaBeads with incorporation of aa-dUTPs. TE buffer was discarded and the sample was resuspended in 23.5 µl water and

4 µl 50 µM random nanomer primer mix (N9B). Incubation was carried out for 3 min at 95°C, followed by primer annealing for 5 min at room-temperature, briefly suspending the beads by rolling. Klenow reaction mix was applied and the probe was incubated 1.5 hours in a rotary oven at 37°C. After second strand synthesis the probe was washed twice with 300 µl washing buffer BX and resuspended in 40 µl water, followed by 5 min incubation at 95°C. After denaturation the supernatant was quickly eluted to a new tube and stored on ice. Second strand synthesis was repeated 2-4 times for each sample and the second strand cDNAs from each genotype were pooled and measured on the spectrophotometer (Genesys 10 Bio, Thermo Electron Corporation, Madison, USA). The absorption was measured at 260 nm in a 50 µl quartz cuvette and the concentrations of second strand cDNAs were calculated as follows:

$$\text{pmol nucleotides} = \frac{OD_{260} \times volume(\mu l) \times 37 ng/\mu l \times 1000 pg/ng)}{324.5 pg/pmol}$$

Note: 1 OD_{260} = 37 ng/µl for cDNA; 324.5 pg/pmol is the average molecular weight of a dNTP.

Second strand cDNA probes were labelled with Cy3 and Cy5 (Amersham Pharmacia, Piscataway, USA), depending on the hybridization design (Fig. 2.4). The same amounts of Cy3 and Cy5 labelled samples were Vacufuge dried (45°C) and carefully resuspended in 10 µl 0.1 M sodium bicarbonate (pH 9.0). The dried, -20°C stored Cy3 / Cy5 dyes were resuspended in 2 µl DMSO in parallel. Samples were mixed and incubated at 28-30°C in a rotary oven in the darkness. The labelling reaction was stopped after 1 hour by adding 38 µl 100 mM NaOAc (pH 5.2) and unincorporated dyes were removed with QiaQuick PCR purification kit (Qiagen AG, Hilden, Germany) according to manufacturer's recommendations. The amount of labelled product was measured spectrophotometrically in a 50 µl quartz cuvette for the wavelengths 260 nm (DNA), 550 nm (Cy3), and 650 nm (Cy5). The incorporation of Cy3 and Cy5 was calculated like following:

$$\text{Cy3 (pmol)} = \frac{OD_{550} \times volume(\mu l)}{0.15},$$

$$\text{Cy5 (pmol)} = \frac{OD_{650} \times volume(\mu l)}{0.25}$$

Note: the absorption coefficient of Cy3 is 0.15 and the absorption coefficient of Cy5 = 0.25.

2.2.2.3. RNA cleanup

RNA clean-up of stock RNA samples was performed on RNeasy Mini Kit columns (Qiagen AG, Hilden, Germany) following manufacturer's instructions with previous DNA digestion. The digestion for qRT-PCR samples preparation was performed using RNase-free DNase (1500 Kunitz units) in microcentrifuge tubes (Qiagen AG, Hilden, Germany). Fifty ng RNA was mixed with 10 µl RDD buffer, 2.5 µl DNase, and filled up to 100 µl with RNase-free water and incubated at 25°C for 15 min. After purification, samples were quantified spectrophotometrically (see section 2.2.3) (Genesys 10 Bio, Thermo Electron Corporation, Madison, USA).

2.2.3 Qualification and quantification of RNA

Formaldehyde agarose gel was used for RNA qualification. A volume of DEPC-treated water was mixed and heated with 1.9% (w/v) agarose. The agarose solution was cooled down to 50°C and 5× electrophoresis buffer and formaldehyde were added to a final concentration of 0.22 M. The solution was then mixed and poured into a horizontal gel chamber to solidify. Thereafter, samples were diluted in RNA loading buffer and denatured at 65°C for 5 min, immediately cooled down on ice for 2 min, loaded on the gel and run at 70 Volt in 1× running buffer for 2 hours. Agarose gel electrophoresis of the purified RNA aimed to distinguish 18S and 28S ribosomal bands.

RNA concentration was measured by using spectrophotometer (Genesys 10 Bio, Thermo electron corporation, Madison, USA). 1 µl of purified RNA was diluted in 99 µl of DEPC-treated water. The 50 µl quartz cuvette was subsequently placed in the

spectrophotometer and the measurements of OD_{260} and OD_{280} performed. Based on the obtained values, RNA concentration was calculated using following equation:

$$\text{Total RNA } (\mu g/\mu l) = OD_{260} \times 40\ \mu g \times \text{dilution factor}$$

Note: $0.1 < A_{260} < 1.0$ represents normal concentration range of measurements. $A_{260} = 1$ indicates 40 µg/ml of RNA. A ratio of A_{260} / A_{280} between 1.8 and 2.0 indicates proper purity of RNA.

2.2.4 PCR

2.2.4.1. Standard PCR

Amplification of DNA fragments was performed in a 30 µl reaction mixture in thin-walled PCR tubes (BRAND, BrandTech Scienctific, Inc, Germany), using Perkin-Elmer GeneAmp PCR System 9600 Thermocycler (PerkinElmer, Massachusetts, USA). The following reaction mixture was used:

Template	2 - 10 ng
Primer 1 (10 mM)	0.5 µl
Primer 2 (10 mM)	0.5 µl
Nucleotides (dNTPs; 10 mM)	0.25 µl
PCR buffer (10 x)	3 µl
Taq polymerase	0.25 U
H_2O	Add to 30 µl

The PCR was performed as following:

Steps		
	Initial denaturation	94°C for 2 min
1	Denaturation	94°C for 0.5 min
2	Annealing	Tm-4°C for 0.5 min
3	Synthesis	72°C for approx. 1 min/1kb DNA
4	Termination	72°C for 5 min
5	Cooling	to 4°C

The amplification procedure (steps 2 - 4) was repeated 30 times.

Melting temperature of the primers was designed based on the GC content, and can be calculated as follows:

$$Tm= n (G + C) \times 4°C + n (A + T) \times 2°C$$

If a pair of designed primers had different melting temperatures, the lower one was chosen for PCR. The quality of PCR products was additionally monitored by gel electrophoresis.

2.2.4.2. Quantitative RT-PCR

Absolute and relative quantification of *zep* were determined by qRT-PCR analysis, utilizing the method described by Pfaffl and Hageleit (2001). A *zep* cRNA fragment used as a standard in absolute quantification was prepared as following procedures (Pfaffl *et al.* 1998). In brief, a 652 bp DNA fragment encoding part of ZEP was amplified by PCR using zep1 primers (5'-CCAAGTCCGACGCCAAGATAA-3' and 5'-TTGGTGCTGATGGCATAAGGTCT-3'; annealing temperature: 62.5°C). After cloning and transforming the 652 bp DNA fragment, linear plasmid DNA which contains target DNA fragment was transcribed by using zep2 primers (5'-AAGTGCCGAGTCAGGAAGCC-3' and 5'-AGTCCGACGCCAAGATAAGC-3'; annealing temperature: 55°C). The transcript cRNA was purified by RNeasy Mini Kit columns (Qiagen AG, Hilden, Germany) following manufacturer's instructions. Ten-fold serial dilutions (5 pg, 50 pg, 500 pg, 5 ng, 50 ng/µl) of the quantified *zep* cRNA stock solution were kept as aliquots at -80°C and used throughout the study as external standards of known concentration for the *zep* qRT-PCR reaction. Calibration curve was created by plotting the threshold cycle number (Ct-value) against its corresponding log number of RNA concentration.

qRT-PCR amplification was performed by using one tube QuantiTect SYBR® Green RT-PCR Kit (Qiagen, Hilden, Germany) in 96-well optical reaction plates with fitted optical caps (Applied Biosystems, California, USA). A 25 µl-reaction volume with 250 ng of total RNA was used. Each sample was run in triplicate, in addition to

non-template controls containing water instead of RNA. The reactions were performed in an automated ABI Prism 7700 sequence detector (Applied Biosystems, California, USA) under following conditions: 50°C for 30 min, 95°C for 15 min, 35 cycles of 94°C for 15 s, 60°C for 30 s and 72°C for 30 s (during which the fluorescence data were collected) and a final extension of 5 min at 72°C. zep2 Primers were used as stated above. For each sample, a specific Ct-value was created, which defined the cycle number at which a statistically significant increase in the reporter fluorescence was first detectable. Average values were used for all calculations. No signal above the level of the non-template control was observed. PCR products were subsequently verified by gel electrophoresis in order to confirm that the detected signal was a result of product amplification and not primer dimer formation. For relative *zep* expression analysis, elongation factor (EF) was chosen as housekeeping gene (Nicot *et al.* 2005). The conditions of qRT-PCR for EF were the same as for ZEP. The sequences of the EF primers were 5'-ATTGGAAACGGATATGCTCCA-3' and 5'-TCCTTACCTGAACGCCTGTCA-3'. Relative expression levels of *zep* were calculated first relative to a housekeeping gene EF, then relative to *zep* expression in Baltica. Data are presented as means ± SD from three biological repetitions consisting of three technical replicates and were averaged over the two environments.

2.2.5 cDNA macroarrays

2.2.5.1. Construction of cDNA libraries

Four cDNA libraries were constructed by using subtractive hybridization (SH) technique (Table 2.1). Two libraries were produced from leaf material, while other two from tuber materials. Plant materials from field trial in 2005 (Roggenstein) at PGS 60 were utilized. cDNA Libraries were constructed by cooperation with Vertis Biotechnologie AG, Freising, Germany. The construction outline of the cDNA libraries is shown in Fig. 2.4 (Ros *et al.,* 2004). S2 cDNA was subsequently cloned into the pCR®2.1

plasmid using TA Cloning® Kit Vector (Invitrogen, California, USA), and transformed into One Shot® TOP10F' Chemically Competent *Escherichia coli* cells (Invitrogen, California, USA).

Table 2.1 Construction of the differentially expressed cDNA libraries

	Leaf		**Tuber**	
Libraries	SR47-leaf-library	SR48-leaf-library	SR47-tuber-library	SR48-tuber-library
Tester-cDNA	SR47	SR48	SR47	SR48
Driver-cDNA	Baltica		Baltica	
Class of genes expected in the library	differentially expressed genes in leaf of SR47 compared to Baltica	differentially expressed genes in leaf of SR48 compared to Baltica	differentially expressed genes in tuber of SR47 compared to Baltica	differentially expressed genes in tuber of SR48 compared to Baltica

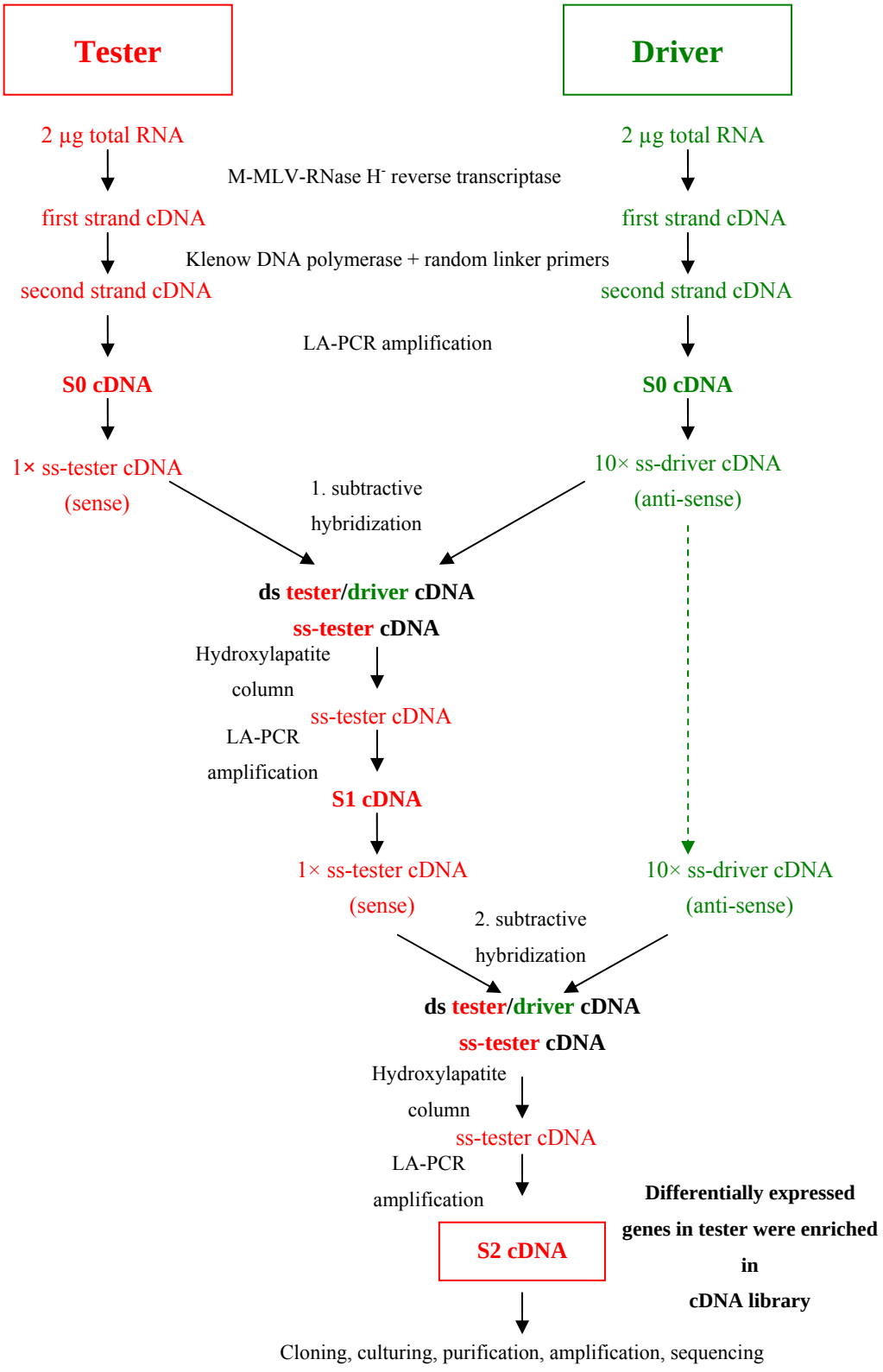


Fig. 2.4 Outline of subtractive hybridization. ss cDNA: single-strand cDNA; ds cDNA: double-strand cDNA. LA-PCR: long and accurate PCR.

2.2.5.2. Sequence analysis of the cDNA inserts

Colonies with cDNA inserts were randomly picked from the plated subtractive cDNA libraries and cultured in 1 ml LB_{Amp} medium in 96-well plates at 37°C. cDNA inserts of the clones were amplified using a Perkin-Elmer GeneAMP PCR System 9600 thermocycler (PerkinElmer, Massachusetts, USA) with pBL2SK primers (Appendix B) flanking the cloning site of the pCR®2.1 Vector (Invitrogen, California, USA). PCR reactions (30 µl) contained 27 µl distilled water, 0.05 µl primers (100 µM each), 3 µl 10× Advantage Buffer (Amersham Biosciences Europe GmbH, Freiburg, Germany), 0.125 U Taq polymerase (Qiagen, Hilden, Germany), and 1 µl bacterial culture. Cycling conditions were 2 min at 95°C and 35× (15 s at 95°C, 30 s at 55°C, 3 min at 72°C). Quality of PCR products was verified by gel electrophoresis according to Ros *et al.* (2004).

M13 reverse/M13(−20) forward, and T7/T13 primers (Appendix B) were used to generate partial 5' sequence information of the cDNA clones with the ABI PRISM® BIG DYE Terminator Cycle Sequencing Ready Reaction Kit version 1.0 (Applied Biosystems, California, USA). PCR reactions were carried out using Perkin-Elmer GeneAMP PCR System 9600 (PerkinElmer, Massachusetts, USA), and fragment detection was performed using ABI PRISM® 377 DNA sequencer (Applied Biosystems, California, USA). Sequence data were analyzed by Sequencing Analysis Software version 3.2, ABI PRISM® (Applied Biosystems, California, USA). Sequences were further evaluated and assembled using Sequencher 3.0 (Gene Codes Corporation, Ann Arbor, USA), and aligned using BioEdit sequence alignment editor (North Carolina State University, Charlotte, USA). BLASTn and BLASTx searches were performed for each sequence with the E-value cut-off set as e-10 (Ros *et al.*, 2004). Hits with E-value greater than e-10 were not considered statistically significant, or no similarity was found.

2.2.5.3. Hybridization of cDNA macroarrays and data analysis

All amplified cDNA fragments were concentrated by using MultiScreen®-PCR Plates (Millipore, Massachusetts, USA), to an approximate concentration of 0.4 µg/µl before spotted onto the filter. cDNA macroarrays were prepared by transferring 30 ng of PCR products (in duplicates) onto positively charged nylon membranes (Pall Corporation, New York, USA), using nano plotter NP1c (GeSiM GmbH, Großerkmannsdorf, Germany).

Plasmid pMM14 (Mittag *et al.*, 1994) was printed on each membrane as spiking control. The membranes were treated with 0.2 M NaOH solution and 0.2 M SDS solution for 15 min at 65°C, and in 30 mM Tris with pH 7.5 at RT for 15 min. First-strand cDNA synthesis and concurrent labelling with ^{32}P modified nucleotides were performed on total RNA by oligo dT priming. 10 µg of total RNA was mixed with 40 pg spiking RNA prepared from pMM14 plasmid, and with 2 µg oligo (dT) 12-18 (Roche, Penzberg, Germany) in 8 µl DEPC-treated water. The mixture was then denatured for 2 min at 72°C and subsequently cooled to 42°C. Reverse transcription was performed in a total volume of 22.5 µl using REVERTAID M-MuLV reverse transcriptase (Fermentas GmbH, Leon-Rot, Germany). Samples were further incubated for 1 hour at 42°C in the presence of 0.33 mM of dATP, dGTP and dTTP, 3.3 µM dCTP, and 20 µCi α-[^{32}P]dCTP (3.000 Ci/mmol) (Hartmann Analytics GmbH, Braunschweig, Germany). The reaction was stopped for 30 min at 65°C after addition of 150 mM NaOH and 10 mM EDTA. The unincorporated nucleotides were removed using SEPHADEX G-50 (Amersham Biosciences Europe GmbH, Freiburg, Germany). The incorporation of the label was checked by scintillation counting (Bioscan/QC-4000 XER, Bioscan Inc, USA). Samples were denatured for 5 min at 95°C before hybridization. Macroarray filters were prehybridized for 2 hours in a Church buffer (Church and Gilbert, 1984) containing 0.25 M $NaPO_4$ (pH 7.2), 7% of SDS, 1 mM EDTA, 250 µg tRNA (Roche, Penzberg, Germany), and 2.5 µg oligo (dA) 40-60 (Amersham Biosciences Europe GmbH, Freiburg, Germany). Hybridization was carried out in the same buffer, but containing 40 µg tRNA (Roche, Penzberg, Germany) and 0.4 µg oligo (dA) 40-60 (Amersham Biosciences Europe GmbH,

Freiburg, Germany), overnight at 65°C. Membranes were washed twice in 2× SSC / 0.1% SDS, and twice in 40 mM $NaPO_4$ (pH 7.2)/0.1% SDS, at 65 °C for 20 min. Thereafter, filters were exposed to imaging plates (Kodak) for at least 24 h.

Hybridization signals on the imaging plates were detected using STORM 860 scanner (Molecular Dynamics, California, USA) with resolution of 50 μm. The image data obtained were then imported into the Array Vision program package (Amersham Biosciences Europe GmbH, Freiburg, Germany) for spot detection and quantification of hybridization signals. Signals derived from hybridization of spiking RNA with pMM14 plasmid were defined as external reference. For normalization, individual spot intensity was first calculated relative to the external reference and then to the internal control (clone B-G5, encoding a DNA J-like protein from *S. tuberosum*, accession No. X94301), which exhibited equal gene expression levels in all experiments.

2.2.6 cDNA microarrays

2.2.6.1. Potato cDNA Microarray

Potato cDNA microarray (10K, version 4) purchased from The Institute for Genomic Research (TIGR, Rockville, USA) was used in this work. It comprised 15,264 cDNAs duplicated on a slide, thereof 11,412 verified and usable fragments. The 11,412 cDNAs were derived from potato stolons, roots, microtubers, dormant tubers, germinating eyes, and healthy and *Phytophthora infestans*-challenged libraries. More details about the sequences of the genes printed on the slide can be found at www.tigr.org/tdb/potato/microarray_desc.shtml. Controls spotted on the array included potato and tomato cDNAs, potato gDNAs, and human spiking controls. These represent genes involved in housekeeping functions, photosynthesis and defence responses. A detailed list of controls can be found at www.tigr.org/tdb/potato/clones2.shtml. Details on the sequencing of the cDNAs can be found at www.tigr.org/tdb/potato/est2.shtml.

TIGR potato microarrays were printed on Corning Ultra Gaps Slides (Serial No.: #40015) (Corning/Costar Corporation Charlotte, NC, USA). Fig. 2.5 illustrates the design of the printing area. Protocol of microarray printing can be found at http://www.tigr.org/tdb/potato/images/SGED_SOP_2.1.1.pdf. Printed slides were stored in dessicator, in light-protected slide boxes to avoid moisture.

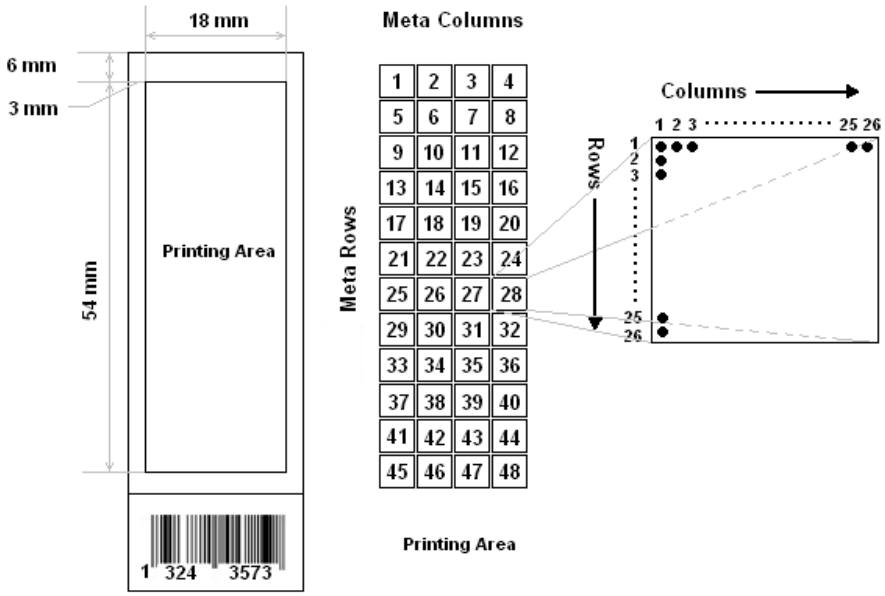


Fig. 2.5 Layout of TIGR Potato cDNA Array (10K, Version 4).

2.2.6.2. Hybridization design

A total number of twelve microarray hybridizations were performed in this work (details given in Fig 2.6). The experimental design included three types of replications (biological, technical and in-slide replicates). The experimental setup was balanced with respect to the use of Cy3/Cy5 in the labelling reactions.

The aim of the microarray hybridization experiment was to compare the expression profiles of tuber materials from GE potato clones SR47 and SR48 and conventional counterpart cultivar Baltica (Fig. 2.4). Tuber materials from field trial Roggenstein 2005 at PGS 60 were utilized. Samples from five plants of the same clone were pooled before mRNA isolation. mRNA was extracted, transcribed, labelled and hybridized to 10K potato microarray slides (TIGR, Rockville, USA) as indicated in the hybridization design (Fig.

2.6). Three biological replications were used for each comparison pair (SR47 vs. Baltica and SR48 vs. Baltica).

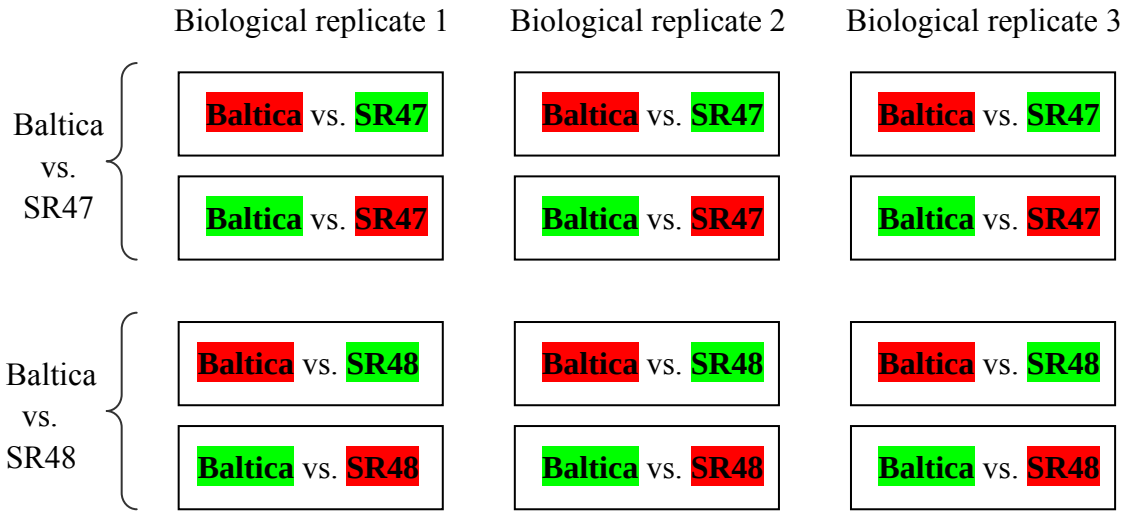


Fig. 2.6 Hybridization design of microarray experiment

2.2.6.3. Hybridization conditions

According to hybridization design, a minimum of 25 and maximum of 60 pmols Cy3 and Cy5 labelled second strand cDNAs were combined, vacuum dried with VacuFuge™ Concentrator (Eppendorf, Wesseling-Berzdorf, Germany) (45°C, ~1.5 hours) and dissolved in 5 µl water and 40 µl hybridization solution (Pronto™ Hybridization Kit, Corning, NC, USA). Dissolved labelled cDNA samples were subsequently denatured at 95°C for 3-4 min, quenched on ice for 30 sec and spun down in a table centrifuge. The lifter slip (25 × 60 mm) was placed onto the slide and samples were applied with a pipette, avoiding bubble formation. Arrays were positioned into a Quantifoil IHC1 hybridization chamber (QUANTIFOIL Instruments GmbH, Jena, Germany). Three ml of 0.5 × SSC were applied to the bottom of the chamber to prevent desiccation. Arrays were hybridized in oven, slightly agitating, both overnight at 42°C.

2.2.6.4. Post hybridization process and scanning

After hybridization, slides were removed from the chamber, lifter slips discarded in washing solution 1 (2 × SSC, 0.1 % SDS), slides placed in a slide-holder and washed with post-hybridization solutions. Washing steps were carried out as follows (Gregersen *et al.*, 2005, with some modifications): 1st washing at 42°C in washing solution 1 for 10 min, 2nd washing 5 min in washing solution 2 (0.2 % × SSC, 0.1 % SDS) at 42°C and in the same solution for another 5 min at room temperature, 3rd washing in washing solution 3 (0.1 × SSC) at room temperature three times for 5 min, and 4th washing in washing solution 4 (0.01 × SSC) in 50 ml tubes per slide for 5-10 sec. Washed slides were immediately spin-dried two times (first for 5 min followed by 3 min) in a centrifuge at 1500 rpm in 50 ml tubes at RT with a piece of dust-free paper on the bottom, and kept covered with aluminium foil to avoid light exposure. Microarray slides were scanned at wavelengths for Cy3 and Cy5 by using Gene TAC UC 4 × 4 microarray scanner (GeneMachines™, Genomic Solutions, Inc., USA) with a resolution of 5 μm/pixel. 16-bit greyscale TIFF files for each channel were scanned separately.

2.2.6.5. Quantification, evaluation and normalization of the signals

Microarray data processing included: data input, pre-processing, exploration, visualization and interpretation (Fig. 2.7). TIFF image files were subsequently converted into *.gpr* files using GenePix Pro 6.0 Microarray Image Analysis (Molecular Devices, CA, USA). Raw data in the *.gpr* format were used for further data evaluation and analysis in R-based software tool LIMMA (Linear Models for Microarray Data) (Smyth 2004; Smyth *et al.*, 2005) downloadable from an open source (www.bioconductor.org), and KTH-package (Wirta, 2005), a collection of tools for microarray data analysis downloadable from http://biobase.biotech.kth.se/~valtteri/R/.

Image plots were used to demonstrate the variation of background values over the array. Separate image plots of the red and green background for each array were generated

in order to check for uneven background, thus to eliminate any unreliable signal area and to give suggestion which normalization methods could be used.

The LIMMA log-ratio is represented by the M-value (M = log2(R) − log2 (G)), and the overall intensity by the A-value (A = (log2(R) +log2 (G))/2)) (Smyth, 2004; Smyth *et al.*, 2006). MA-plots plot the log-ratio of Red/Green (R/G) signal against the overall intensity of each spot. Quality of raw microarray data was evaluated by diagnostic MA-plots that were generated for the whole array and for individual print-tip groups to visualize distribution of spots. MA-plot of the average M and A-values for each gene was created considering dye-swaps and technical replicates by using *lmFit* function of LIMMA. Afterwards, empirical Bayes statistics (B-test) for differential expression was applied to the dataset. The B-test (also known as moderated t-statistics) uses sample standard deviations shrunk towards a pooled standard deviation value (Lonnstedt and Speed, 2002). It is a log posterior odds ratio of differential expression vs. non-differential expression, and allows for gene-specific variances. It also combines information across many genes and thus it is regarded more stable than the *t* statistic (Cui and Churchill, 2003).

Microarray data applied to LIMMA can be visualized by MA-plot, Student Q-Q plot, volcano-plot or Topgene table (Wirta, 2005). Volcano-plot was used to visualize the microarray data in this study due to its efficiency and easy-to-interpret characters. In volcano plot, processed microarray data was visualized by a scatter-plot of Log-odds, which refer to the probabilities that a certain gene is differentially expressed, against the $\log_2$ fold change between the target and the control. Genes with statistically significant differential expression according to B-test should lie above a horizontal threshold line, which in this study was set at B = 4.6 (4.6 of B-value stands for 99% chance of being statistically significant). Genes with large fold-change values should lie outside a pair of vertical threshold lines, in this study chosen as a fold-change greater or equal to two ($\log_2$ fold-change ≥ 1 or ≤ -1). Therefore, significant genes identified by B-test should tend to be located in the upper left or upper right parts of the plot.

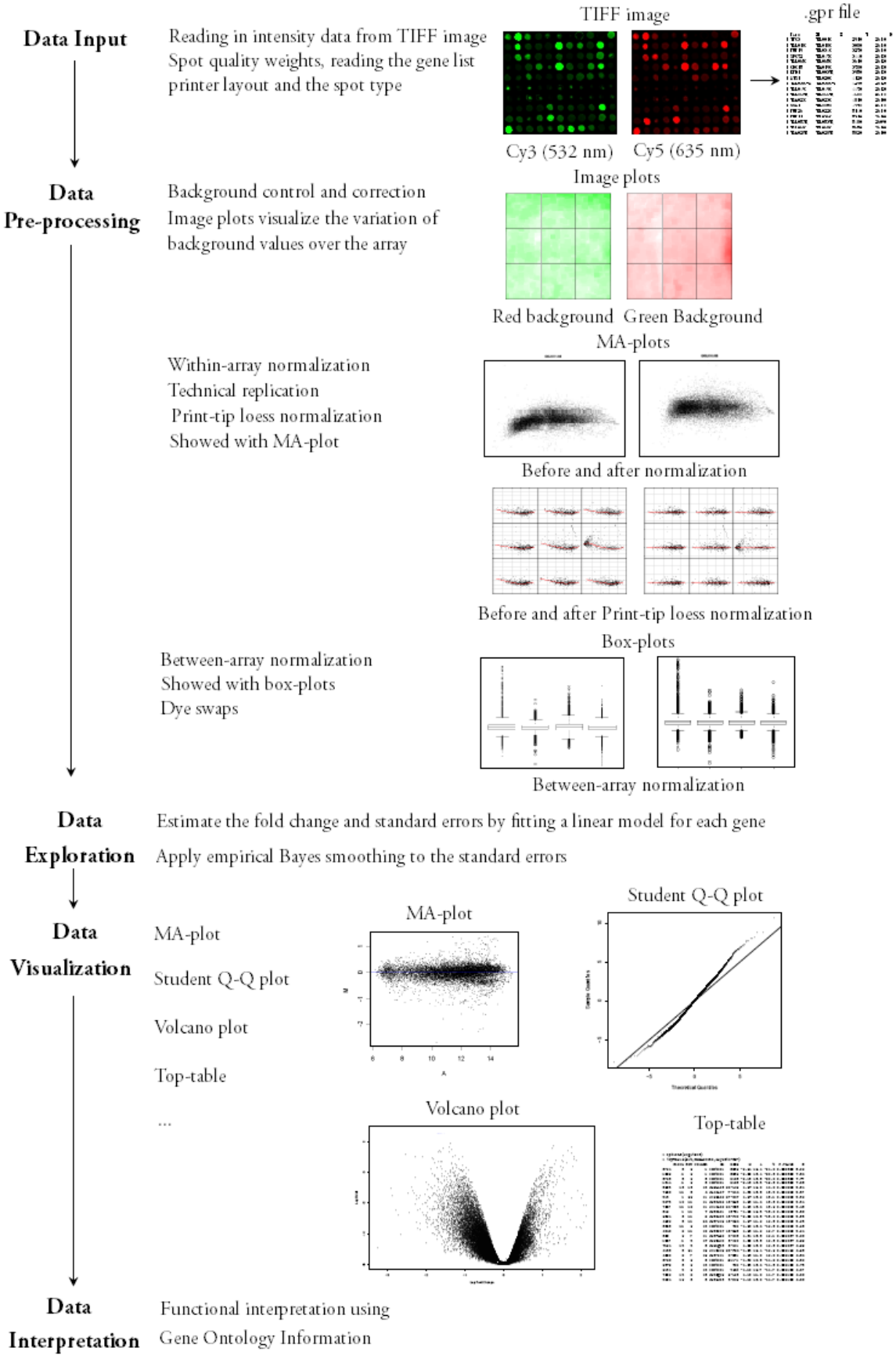


Fig. 2.7 Process of microarray data analysis by using LIMMA- and KTH- package in R platform.

2.2.7 Zeaxanthin and lutein measurement

Fresh tubers harvested from the field trial (Oberviehhausen, 2006) were cut into slides of ca. $30 \times 30 \times 10$ mm (length × width × height) and freeze-dried using Christ ALPHA 1-4 (Martin Christ Gefriertrocknungsanlagen GmbH, Osterode am Harz, Germany). Freeze-dried samples were subsequently ground to powder at RT and kept in 2 ml reaction tubes. An aliquot of 0.1 g freeze-dried powder was transferred into a 2 ml reaction tubes. The sample was mixed with 0.01 g K_2CO_3, 0.18 ml internal standard (500 µM ß-apo-8´-carotenal, which was prepared by dissolving 0.208 mg of ß-apo-8´-carotenal in 1 ml of MeOH/ THF 1:1) and 0.72 ml MeOH/THF (1:1), homogenized by vortexing for 1 min, and carotenoids were extracted using ultrasonic bath (4°C) for 3 min. As a next step, 0.5 ml BHT (0.1 % in hexane) and 0.25 ml NaCl solution (10 %) was added, probes mixed thoroughly for 15 seconds and carotenoids extracted in the ultrasonic bath for 3 min. Consequently, samples were centrifuged at 4000 U/min for 3 min and supernatant was collected into a new 15 ml tube. Then, 0.6 ml fresh hexane (0.1 %) was added to the mixture and the extraction procedure repeated four times. The supernatant collected from five hexane-extracts was evaporated in a concentrator (Eppendorf Concentrator 5301, Eppendorf, Wesseling-Berzdorf, Germany) at RT. For HPLC analysis, the dried residue was re-dissolved in 1 ml HPLC mobile phase. The whole carotenoids extraction procedure was carried out under dim light protection.

Carotenoid extracts from potato tuber samples were analyzed using Merck Hitachi-HPLC system L 6200 (Hitachi, Tokyo, Japan), coupled with Phenomenex Develosil RP aqueous C30-column (250 x 46 mm, 5 µm) (Phenomenex, Korea) and an UV-Vis Detector (450nm) (Hitachi, Tokyo, Japan). Obtained peaks were hereafter quantified with HSM7000 software delivered with Merck Hitachi-HPLC system L 6200 (Hitachi, Tokyo, Japan). The gradient mobile phase consisted of three different solvents: (1) 0.05 % of triethylamine in methanol; (2) acetone; and (3) 0.05 % of ammonium acetate in distilled water. The flow rate of the solvent was fixed at 1 ml/min. Sample injection volume was 20 µl and the temperature of the column oven was set to 25°C.

Compounds were identified according to the retention time and quantification was done according to internal standard (ß-apo-8´-carotenal).

3 Results

3.1 Morphology and agronomic performance of GE potato clones

3.1.1 Under greenhouse conditions

GE zeaxanthin-rich potato clones SR47 and SR48, and their conventional counterpart cultivar Baltica were grown under standard greenhouse conditions during natural vegetation period of 2005 and 2006. The general morphology including haulm length of mature plants, number of main stems per plant, flowering status and number of tubers per plant was recorded (Tables 3.1 and 3.2).

Table 3.1 Agronomic characteristics of Baltica, SR47 and SR48 under greenhouse conditions[a]

Clone	Emergence and senescence time (days)	Haulm length (cm)	Number of stems	Number of tubers per plant
Baltica	5-7 and 83-87	84.3± 3.3	2.3± 0.8	25.6± 2.1
SR47	5-7 and 83-87	85.4± 4.6	2.6± 0.8	25.4± 2.2
SR48	5-7 and 83-87	84.5± 2.9	2.8± 1.5	25.1± 2.0

[a]Data presented: means of 30 plants harvested in 2005 and 2006 (15 plants/clone/year); Student's t-test was applied to determine levels of significance. P-value ≤ 0.05 was regarded as significant.

Table 3.2 Characteristics of the inflorescence of Baltica, SR47 and SR48 under greenhouse conditions[a]

Clone	Begin and end of inflorescence[b]	Number of flowering branches per plant	Number of flowers per flowering branch	Number of berries per plant
Baltica	28 -31; 73 -76	12.3±2.9	16.7±2.9	13.6±8.6
SR47	28 -31; 73 -76	13.4±2.1	14.1±2.8	9.0±4.7*
SR48	27 -31; 73 -76	13.1±3.4	12.6±3.2	12.3±9.5

[a] Data presented: means of 30 plants harvested in 2005 and 2006 (15 plants/clone/year); Student's t-test was applied to determine levels of significance. P-value ≤ 0.05 was regarded as significant.

[b] Time calculated: days after planting (days).

No significant differences were observed between Baltica and the two GE potato clones regarding haulm length, number of main stems and number of tubers per plant under greenhouse conditions. The time from planting to emergence, planting to maturity

and planting to senescence was neither identified significantly different compared to Baltica. No further difference was found in general morphology of the GE plants. All stems very equally coloured in a green / light red-violet shade. The leaves were middle to dark green and the flowers white.

The flowering times of SR47 and SR48 under greenhouse conditions were synchronous with that of Baltica. Minor differences (6.5 - 8.9% higher) were found in number of flowering branches per plant of SR47 and SR48 as compared to Baltica, however they were not statistically significant (Student's t-test with cut-off p-value 0.05). Differences of 15.6 - 24.6% were also found in number of flowers per branch. The colour and size of berries displayed no obvious difference (data not shown). The number of berries per plant of SR47 was statistically different (33.8% less) from that of Baltica, but SR48 had 9.6% less berries.

The tubers of Baltica and GE clones were all short-oval to round-oval with shallow evenly distributed eyes and smooth yellow skin. The colour of flesh of SR47 and SR48 was visibly different from Baltica, ranging between dark-yellow and orange. In most cases, the colour of flesh of SR47 was darker than that of SR48 (data not shown).

3.1.2 Under open-field conditions

3.1.2.1. Morphology

Field trials were conducted in Roggenstein (2005 and 2007), and in Oberviehhausen (2006 and 2007) during the natural vegetation period (from April to September or October). Conventional cultivars Baltica, Ditta, Sibu, Selma and Désirée, as well as SR47, SR48 were grown as shown in the planting plan (Appendix D). General morphology, including the number of main stems per plant, flowering status and yield was assessed, and results are demonstrated in Table 3.1. Fig. 3.1 shows an example of general morphology of Baltica, SR47 and SR48 from Roggenstein in 2005.

Baltica SR47 SR48

Fig. 3.1 Morphology of Baltica, SR47 and SR48 in Roggenstein (2005) at PGS 30

Flowering times of GE vs. Baltica plants displayed no difference between Roggenstein (2005 and 2007) and Oberviehhausen (2006 and 2007) (data not shown). The number of stems, flowering branches per plant, and flowers per branch of SR47 and SR48 were not statistically different from that of Baltica (Table 3.3). As observed also under greenhouse conditions, the number of berries of the two GE potato clones was significantly lower than that of Baltica according to Student's t-test (P-value ≤ 0.05).

Table 3.3 Morphology of Baltica, SR47, SR48, Désirée, Ditta, Selma and Sibu under open-field conditions[a]

Variety	Number of stems	Number of flowering branches per plant[b]	Number of flowers per flowering branch[b]	Number of berries per plant[b]
Baltica	3.9±2.0	8.1±3.2	8.5±1.8	21.2±9.3
SR47	2.9±1.0	3.7±2.0	4.9±1.8	0.8±1.4 * [c]
SR48	3.2±2.2	4.6±2.5	4.8±2.2	1.8±1.7 * [c]
Désirée	4.0±1.6	6.5±2.2	12.8±2.0*	8.0±3.6
Ditta	4.2±1.5	3.9±1.3	11.7±3.5	0
Selma	3.6±1.6	7.7±2.3	14.6±1.8*	0
Sibu	3.4±0.9	17.0±3.9*	13.3±1.2*	3.4±2.1*

[a] Data presented: means of 30 plants per potato clone (2005, Roggenstein);

[b] Significant differences between GE vs. Baltica clones are labelled with asterisk according to Student's *t*-test (P-value ≤ 0.05);

[c] Data assessed from GE plants grown under netting conditions (for details see section 2.2.1.2).

As hypothesized, no significant differences in number of stems was found for the other four conventional cultivars in comparison to Baltica. However, Sibu had significant higher number of flowering branches (52.4%) than Baltica. Furthermore, Sibu, Selma and

Désirée had 50.6 - 71.8% more flowers per flowering branch than Baltica (Table 3.3). No berries at all were developed by Sibu and Selma.

Neither obvious, nor easily visible differences among berries of GE vs. Baltica plants were identified in field experiments (Fig. 3.2). The shapes of berries of Baltica, SR47 and SR48 were similar to cherry-tomatoes with light green colour and their diameter varied from 21 – 29 mm. No statistical differences were found between berry diameters of GE potato clones and Baltica. The berries from Désirée and Dita were similar to that of Baltica in shape, colour and size. The only difference was the violet radius in the upper platen of the berry displayed by Ditta (Fig. 3.2).

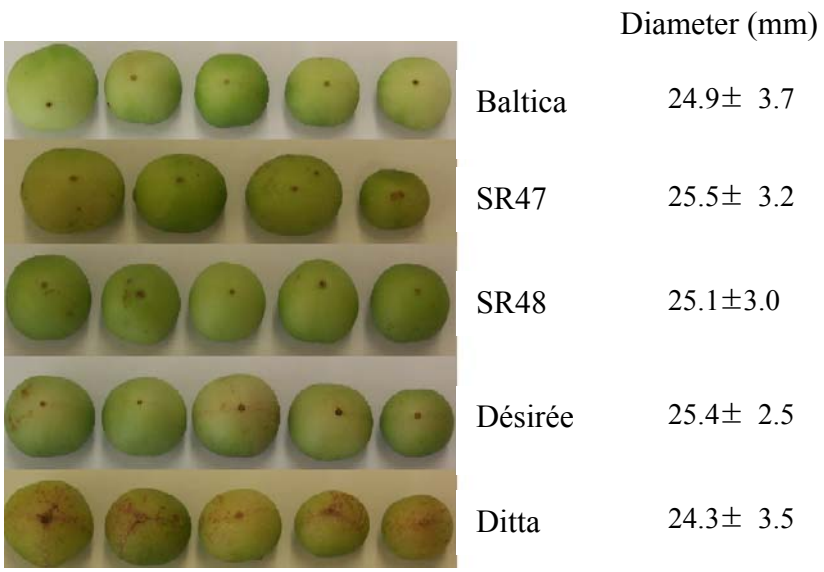


Fig. 3.2 Berries of Baltica, SR47, SR48, Désirée and Ditta (Roggenstein, 2005). Data shown here are means of 30 berries per potato clone collected from Roggenstein in 2005. No berries were produced by Sibu and Selma.

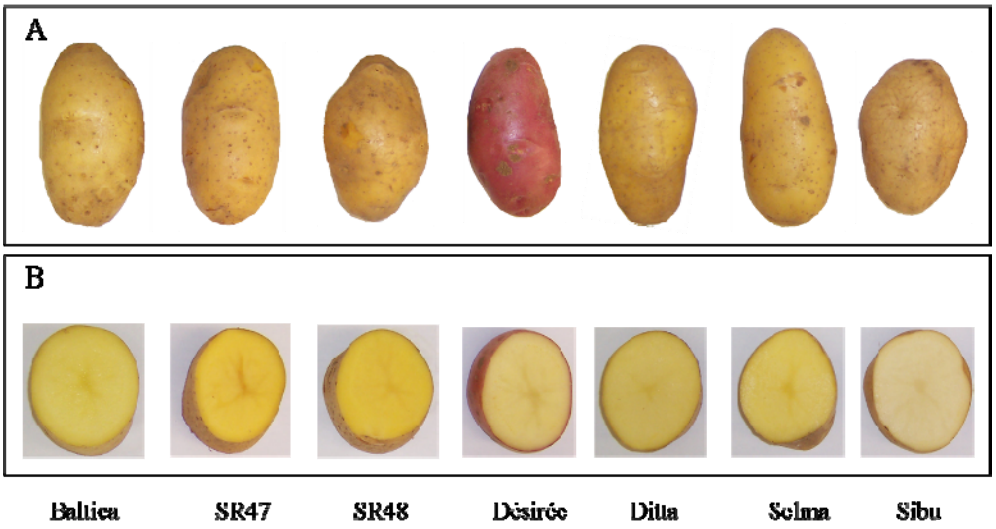


Fig. 3.3 Shape and pigmentation of tubers of Baltica, SR47, SR48, and the conventional cultivars Désirée, Ditta, Selma and Sibu in field trials. Tubers were photographed immediately after harvesting and cutting. A: general shape and skin colour of the tubers; B: colour of the tuber flesh.

3.1.2.2. Yield

Tubers from field trials (Roggenstein in 2005 and 2007; Oberviehhausen in 2006) were weighed in order to obtain yield estimates [dt/ha]. Fig. 3.4 shows average yield of the seven potato clones harvested from Oberviehhausen 2006 and Roggenstein 2007.

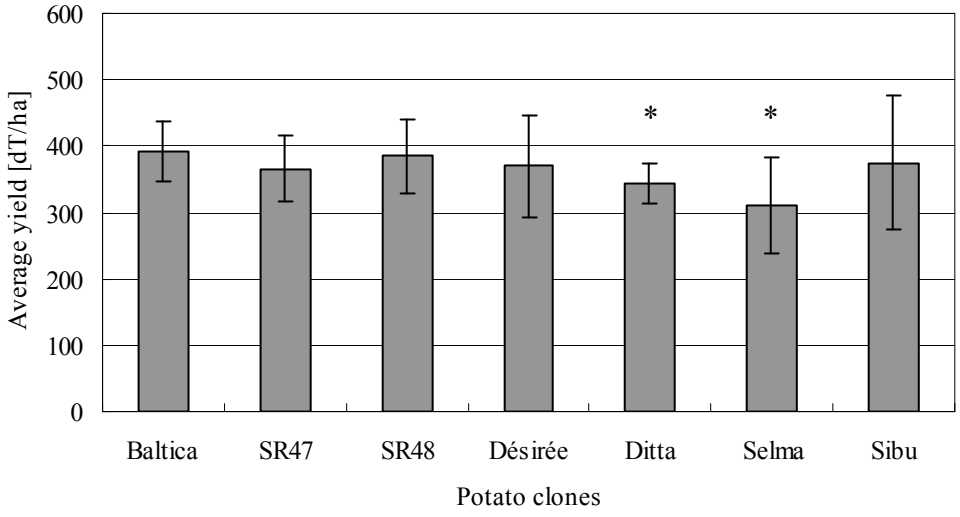


Fig. 3.4 Average yield of field trials in Oberviehhausen (2006) and Roggenstein (2007). Student´s t-test (P-value ≤ 0.05) was used to analyze the yield difference between Baltica and SR47, SR48, Désirée, Ditta, Selma and Sibu respectively. Potato clones with significant yield difference compared to Baltica are marked with asterisk above the correspondence column.

Baltica had the highest average tuber yield of 392 dt/ha among all seven potato clones, the GE potato clone SR48 averaged tuber yield of 385 dt/ha. Second GE clone SR47 ranked fifth among all (366 dt/ha). Sibu with average yield of 375 dt/ha and Désirée 370 dt/ha followed SR48. Although the average tuber yields of SR47 and SR48 were 6.6% and 1.8% lower than that of Baltica, respectively, no statistically significant differences were found in tuber yield among Baltica, SR47 and SR48. Average tuber yields of Ditta (343 dt/ha) and Selma (311 dt/ha) were significantly lower as compared to Baltica, with 12.5% and 20.7%, respectively.

3.1.2.3. Out-crossing rate

Gene transfer through pollen is usually one of most concerning issues in GE plant safety evaluation procedure. In this study, in order to measure the out-crossing rate of the conventional potato clones with GE potato clones through pollen transfer, berries from conventional potato clones, planted with different distance to GE potato plants were collected at PGS 90. Due to lack of berries from Sibu and Selma, only true seeds from Baltica, Ditta and Désirée were analyzed.

Transgene-specific PCR was employed to genomic DNA extracted from seedlings germinated from potato true seeds. Positive PCR results were treated as proof of out-crossing with GE potato clones. Details of the analysis can be found in Material and Methods. Table 3.4 lists natural out-crossing rate of Baltica, Ditta and Désirée with GE potato pollen.

Table 3.4 Estimation of the out-crossing rate of available true seeds from Baltica, Ditta and Désirée

Compared items	Baltica	Ditta	Désirée
Number of seeds checked	6000	2280	6000
Germination rate	88.8%	85.4%	96.4%
Out-crossing probability	0.17‰	0	0

The out-crossing rate of Baltica, Ditta and Désirée with GE potato clones was found to be extremely low. Only 1 from 6000 seeds from Baltica was confirmed to carry an

insertion of the *zep* gene. Furthermore, the berry containing transgenic seed was harvested from the plots neighbouring GE plants, where the distance was less than 6 meters. In none of the cases pollen transfer from GE plants to Ditta or Désirée cultivars was detected.

3.1.2.4. Zeaxanthin and lutein contents in tubers

Zeaxanthin and lutein contents of mature tubers of all seven varieties were estimated by employing HPLC analysis (Table 3.5). Among all peaks identified, only Zeaxanthin and lutein were quantified.

Table 3.5 Lutein and zeaxanthin contents in tubers of seven potato cultivars (µg/g dw) [a]

Sample	Lutein content	Zeaxanthin content
Baltica	5.5 ± 2.4	ND[b]
SR47	2.4 ± 0.6	47.6 ± 11.1
SR48	3.4 ± 1.6	28.1 ± 8.6
Ditta	4.7 ± 1.4	ND
Désirée	2.9 ± 0.9	ND
Sibu	1.9 ± 0.5	ND
Selma	5.5 ± 2.2	ND

[a] Values expressed as mean ± standard derivation (n=9).

[b] Not detectable.

On the basis of dry weight, SR47 was identified to contain 47.6 ± 11.1 µg/g dw of zeaxanthin, while SR48 28.1 ± 8.6 µg/g dw. No zeaxanthin was detected in the five conventional potato cultivars. It was evidenced that GE potato clones of SR47 and SR48 maintained the expected high level of zeaxanthin content under open-field conditions.

3.2 Expression analysis of *zep*

In order to investigate the expression level of transformed *zep* gene in different tissues and growth stages, absolute and relative expression levels of *zep* were determined

from plant materials harvested from greenhouse and open-field trials by using quantitative real-time RT-PCR method. The results presented here demonstrate: (1) *zep* expression patterns of GE potato clones SR47 and SR48 as compared to their conventional counterpart cultivar Baltica; (2) *zep* expression patterns of further four conventional potato cultivars as compared to Baltica.

3.2.1 Comparison between GE potato clones with Baltica

3.2.1.1. *zep* expression levels in different tissues of Baltica

Before comparing the expression level of *zep* between GE potato clones and Baltica in different tissues, it was necessary to get an insight of the absolute *zep* mRNA abundance in different tissues of Baltica. Absolute *zep* expression levels in different potato tissues (leaf, berry, root and tuber) were examined by qRT-PCR in order to determine the range of tissue-specific *zep* expression levels (Fig. 3.5).

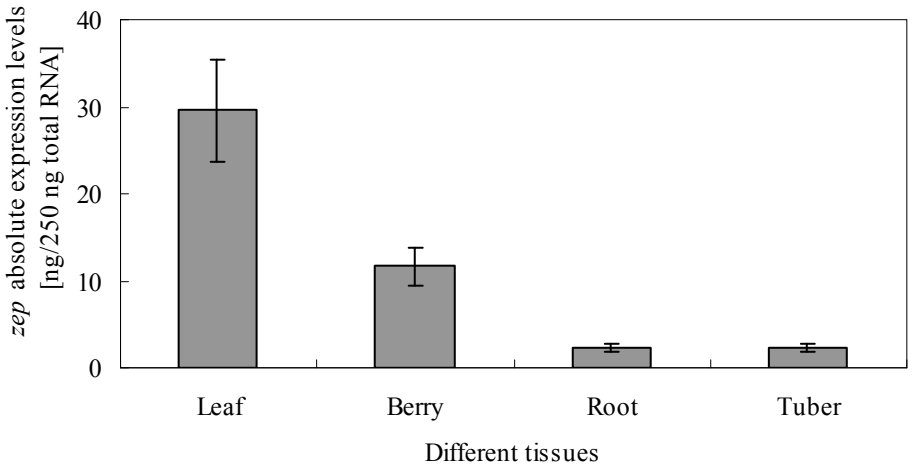


Fig. 3.5 Expression analysis of *zep* in different tissues of Baltica. Total RNA was extracted from leaves, berries, roots and tubers at PGS 90 in field trials (Roggenstein 2005). qRT-PCR was applied to detect absolute mRNA abundances of *zep*. Absolute expression levels of *zep* were presented as the quantity of *zep* mRNA in 250 ng total RNA.

The expression of *zep* was detected in all Baltica tissues examined, with transcripts being considerably more abundant in leaves and berries than in roots and tubers (Fig. 3.5). Approximately 24-40 ng of *zep* mRNA per 250 ng of total RNA was detected in leaves, while only 5-13 ng in berries and 1-4 ng in roots and tubers. The corresponding copy number of *zep* mRNA ranged from 10^{10} per 250 ng of total RNA in leaves and berries, to 10^{8} in roots and tubers. *Zep* expression levels were around 3-12 times higher in aerial parts of the plant than in underground parts.

3.2.1.2. Expression analysis of *zep* in leaves

To examine whether the inserted *zep* gene could affect the expression of endogenously expressed *zep* in leaves of GE potato clones of SR47 and SR48, qRT-PCR was performed with total RNA isolated from leaf samples harvested in greenhouse (2005 and 2006) and open-field (Roggenstein in 2005 and 2007 and Oberviehhausen in 2006 and 2007). Leaf samples were harvested at principle growth stages (Meier, 2001) PGS 30, PGS 60 and PGS 90. All *zep* expression levels were presented in absolute and relative (to Baltica) scale (Fig. 3.6).

Absolute *zep* expression data (Fig. 3.6 A) exhibited small overall changes in *zep* expression levels at different PGS in leaves of both GE clones and Baltica. Under greenhouse conditions, the abundance of *zep* mRNA in leaves increased slightly during the growth of the plants in all three potato clones. In both field trials (Roggenstein 2005 in Fig.3.6 B and Oberviehhausen 2006 in Fig. 3.6 C), the highest expression level of *zep* was found at PGS 60, decreasing slightly at PGS 90 of SR47, SR48 and Baltica. No significant expression differences were found in leaves of both GE plants as compared to Baltica. Relative expression levels of *zep* (Fig. 3.6 D, E and F) clearly demonstrated no significant differences of *zep* expression in leaves at different growth stages of both GE plants as compared to Baltica.

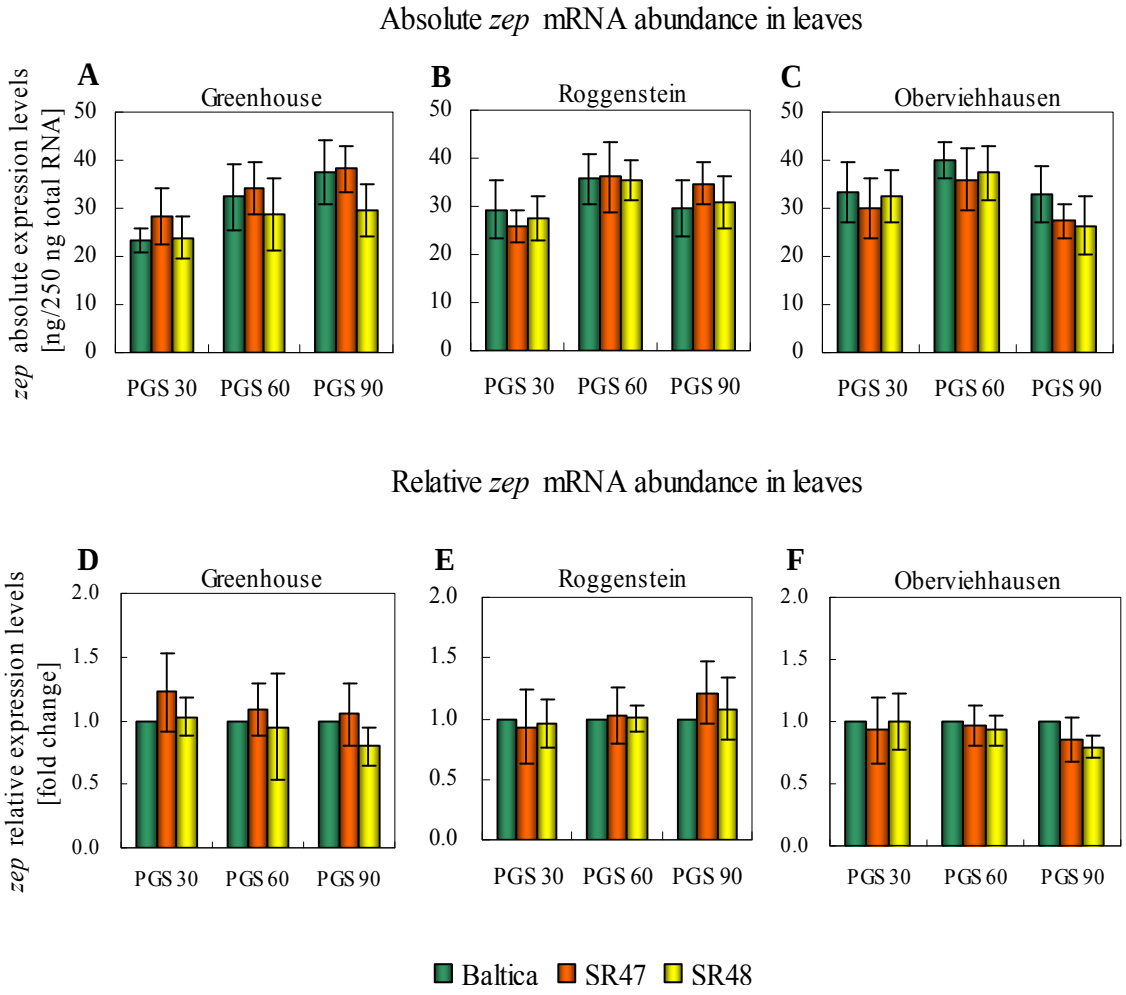


Fig. 3.6 Absolute and relative expression of *zep* in leaves of Baltica, SR47, SR48 at different PGS under greenhouse and open-field conditions. Leaf samples were taken from greenhouse (2005 and 2006) and open-field (Roggenstein 2005 and 2007; Oberviehhausen 2006 and 2007). qRT-PCR was performed with total RNA isolated from leaves. A, B, C: Absolute expression levels of *zep* were calculated according to a standard curve (Bustin 2000; Pfaffl 2001). D, E, F: Relative expression levels of *zep* were calculated first relative to a housekeeping gene (elongation factor, EF), then relative to the *zep* expression in Baltica. Data are presented as means ± SD from three biological repetitions consisting of three technical replicates and were averaged over the two corresponding environments.

3.2.1.3. Expression analysis of *zep* in roots

Expression levels of *zep* were determined in roots of GE clones and Baltica similarly to the method used for leaves. Root samples were taken at same PGS stages as for the aforementioned experiment.

Absolute expression data exhibited only slight differences of expression level of *zep* at different PGS in roots of both GE clones and Baltica under both greenhouse and open-field conditions (Fig. 3.7 A, B and C). *Zep* expression patterns were highly similar for plants harvested from greenhouse and field trials, and the highest amount of *zep* mRNA was identified for PGS 60. Furthermore, absolute expression data demonstrated higher expression level of *zep* in Oberviehhausen than in Roggenstein at all growth stages. The relative expression level of *zep* (Fig. 3.7 D, E and F) clearly indicated no significant differences of expression level of *zep* in roots at different growth stages for both GE plants as compared to Baltica.

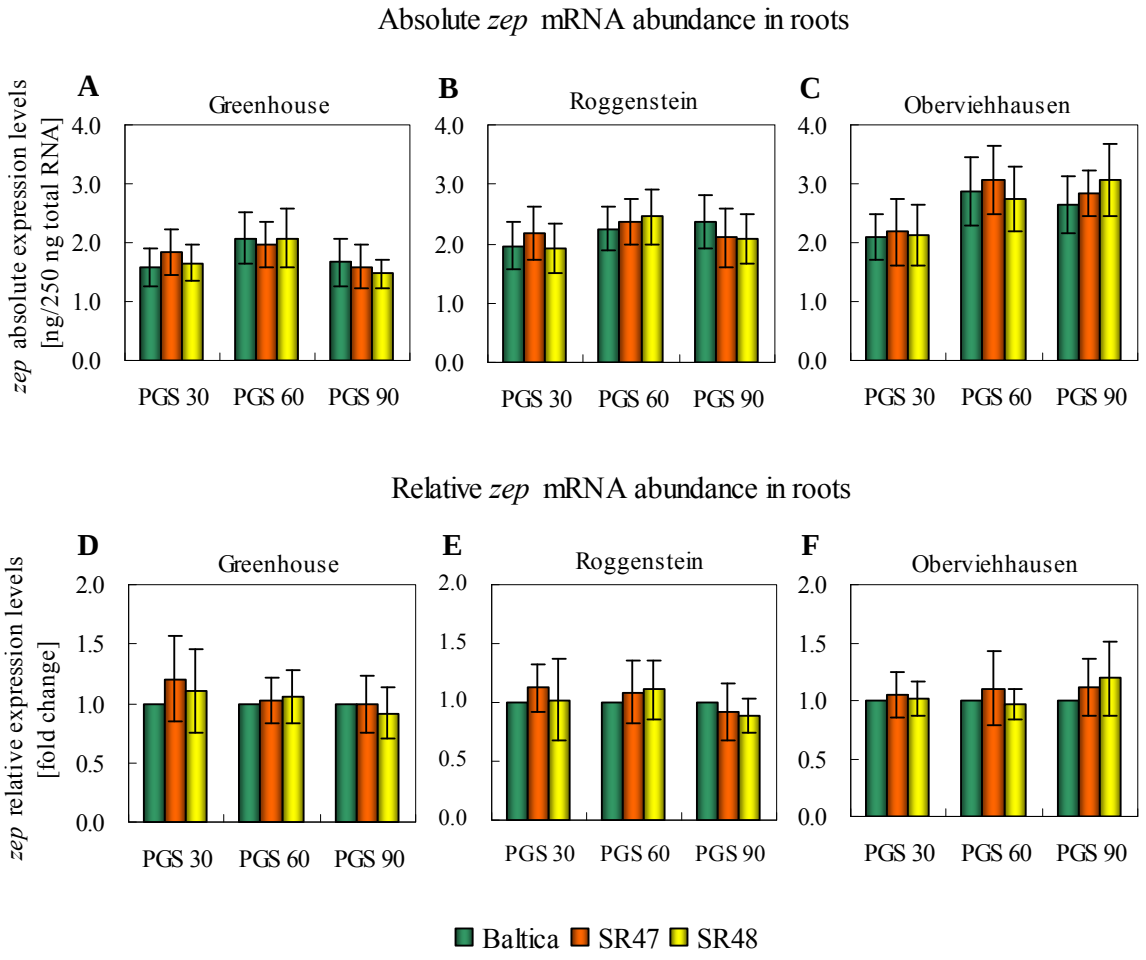


Fig. 3.7 Absolute and relative expression of *zep* in roots of Baltica, SR47 and SR48 at different PGS under greenhouse and field conditions. Root tissue sampling, *zep* expression measurements and data analysis were same as for leaf material (Fig. 3.6). A, B, C: Absolute expression levels of *zep* in roots. D, E, F: Relative expression levels of *zep* in roots.

3.2.1.4. Expression of *zep* in tubers

In order to verify the tuber-specific change of *zep* expression and its transcription kinetics, tubers from different developmental stages were analyzed by qRT-PCR. Swelling stolons, developing and mature tubers from both greenhouse (2005 and 2006) and field trials (Roggenstein in 2005 and 2007 and Oberviehhausen in 2006 and 2007) were used to determine absolute and relative *zep* expression levels (Fig. 3.8).

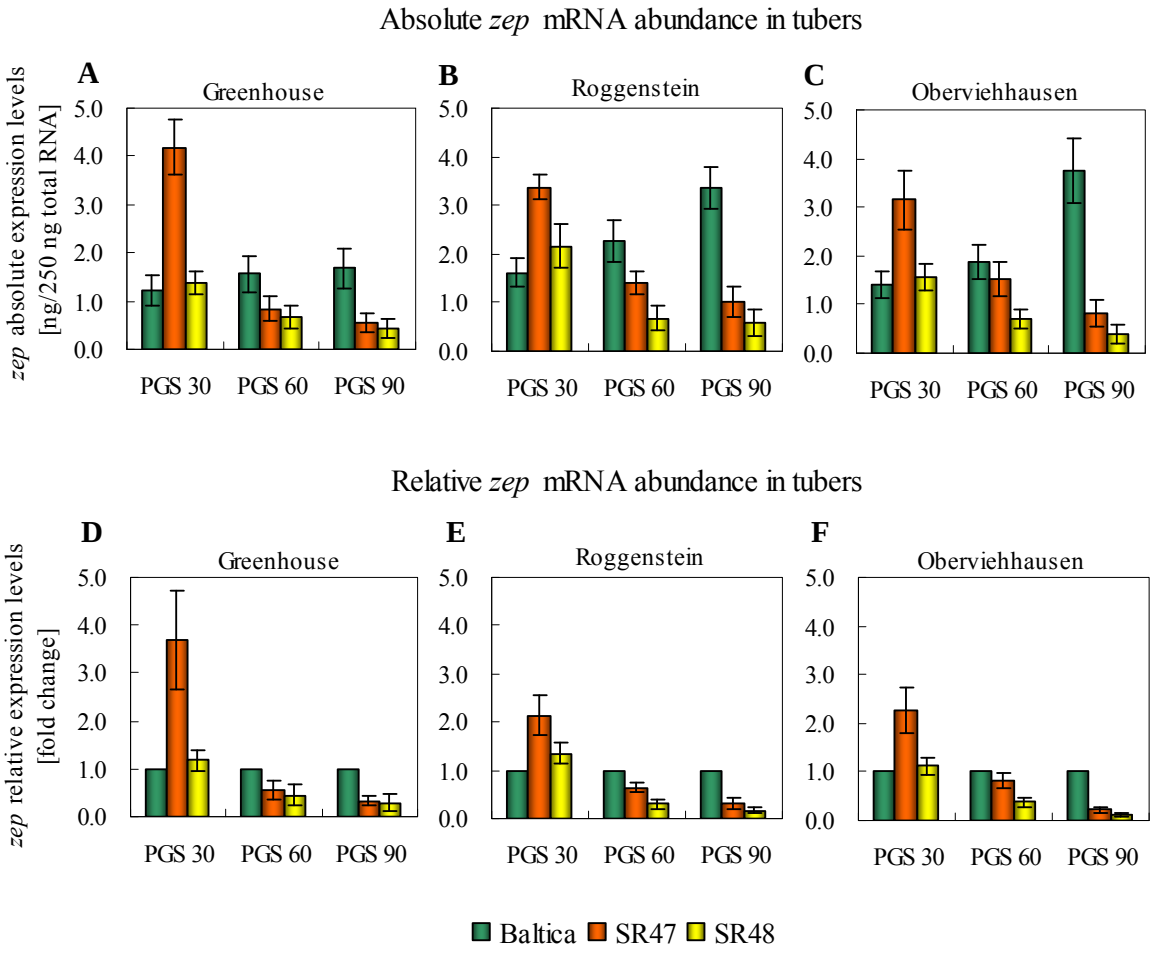


Fig. 3.8 Absolute and relative expression of *zep* in tubers of Baltica, SR47 and SR48 at different PGS under greenhouse and open-field conditions. Swelling stolons (10-15 mm diameter, corresponding to PGS 30), developing tubers (25-35 mm, PGS 60) and mature tuber (40-60 mm, PGS 90) were sampled according to BBCH Monograph (Meier, 2001). *zep* expression measurements and data analysis were same as for leaf material (Fig. 3.6). A, B and C: Absolute expression levels of *zep* in tubers. D, E and F: Relative expression levels of *zep* in tubers.

The absolute and relative expression data of *zep* in tubers exhibited considerable changes of *zep* expression patterns in both GE clones compared to Baltica (Fig. 3.8). In Baltica tubers, the abundance of *zep* mRNA increased during potato growth under both field conditions (Fig. 3.8 A). No significant difference of *zep* mRNA abundance was found between Roggenstein and Oberviehhausen at all development stages. The abundance of *zep* mRNA in developing tubers (PGS 60) of Baltica in Roggenstein was approximately 1.3 times higher, and in mature tubers (PGS 90) 2.1 times higher than in swelling stolons (PGS 30). A similar pattern was also found in Oberviehhausen where the abundance of *zep* mRNA was 1.2 times higher in developing tubers (PGS 60) and 2.3 times higher than that in swelling stolons (PGS 30).

In tubers of SR47 under both field conditions, the absolute *zep* expression level decreased along the growth stages. SR47 showed a reverse pattern of *zep* expression to Baltica. In swelling stolons of SR47, the abundance of *zep* mRNA was 3.5-4.8 times higher than in Baltica and dropped down dramatically from developing to mature stage tubers. Noticeably, the mRNA amount of *zep* was 20-50% and 60-80% lower in SR47 than that of Baltica at the developing and mature tuber stage, respectively. Similar expression patterns of *zep* were also observed in tubers of SR48 throughout all developmental stages compared to Baltica. However, *zep* mRNA abundance of SR48 was significantly different from that of SR47 at different developmental stages. In swelling stolons, SR48 and Baltica had similar abundance of *zep* mRNA, while in developing tubers *zep* mRNA abundance dropped down more significantly in SR48 than in SR47. In mature tubers, expression level of *zep* in SR48 was further decreased but to a noticeably lower extent.

The highest relative expression level of *zep* in both GE clones under both field conditions was found in swelling stolons (Fig. 3.8 B); while the *zep* expression level of SR48 was similar to that of Baltica, a more than 2-fold up-regulation of *zep* expression was observed in swelling stolons of SR47 (co-suppression). Dramatic decrease of *zep* expression of both GE clones started from developing tuber stage and reached their lowest level in mature tuber stage with only 1/3 to 1/5 of the *zep* expression level in Baltica.

3.2.2 Comparison among conventional cultivars

In order to obtain a meaningful concept of natural variation of *zep* expression levels in different conventional potato cultivars, qRT-PCR was applied to different tissues (leaves, roots and tubers) collected at different growth stages from Ditta, Sibu, Selma and Désirée grown under the same field conditions as GE potato clones and Baltica (Fig. 3.9).

Two different *zep* expression patterns were found in leaves of Baltica, Ditta, Sibu, Selma and Désirée (Fig. 3.9 A and B). In Baltica, Ditta and Sibu, *zep* mRNA abundance increased from PGS 30 to PGS 60 by 11.1-17.4%, but decreased from PGS 60 to PGS 90 (15.6-17.6%). The highest *zep* transcript levels were found at PGS 60 ranging from 34.7 to 48.4 ng/250 ng total RNA. However, in Selma and Désirée, the highest *zep* expression level appeared at PGS 30 (36.1 and 49.5 ng/250 ng total RNA, respectively). From PGS 30 to PGS 60, there was a *zep* expression reduction of 9.4% in Selma and 2.2% in Désirée, and a further reduction of 21.9% and 13.0% was found in Selma and Désirée, respectively, from PGS 60 to PGS 90.

There were three different *zep* transcription patterns in roots of these five conventional potato cultivars. Baltica, Ditta and Sibu showed the same pattern, in which *zep* mRNA amount increased from PGS 30 to PGS 60 by 6.9- 49.4% while it decreased from PGS 60 to PGS 90 by 2.2- 27.6% (Fig. 3.9 C and D). The second pattern was found in Selma where *zep* mRNA transcripts increased along plant development by 8.8% (from PGS 30 to PGS 60) and 48.0% (from PGS 60 to PGS 90). The third pattern was found in Désirée where *zep* mRNA transcripts decreased slightly (0.7%) from PGS 30 to PGS 60 and then increased considerably by 32.7% from PGS 60 to PGS 90.

Zep transcript data of tubers showed larger variation than for leaves and roots among these five conventional potato cultivars (Fig. 3.9 E and F). The increase of *zep* mRNA amount differed between 26.9 and 134.4% among the five cultivars when considering PGS 30 to PGS 60, and 30.2- 80.8% for PGS 60 to PGS 90.

In summary, the fluctuation of *zep* expression in leaves and roots of different conventional potato clones was higher than the difference between two GE potato clones

and their conventional counterpart cultivar Baltica. In tubers, considerable fluctuations were also found at different stages of different conventional potato cultivars.

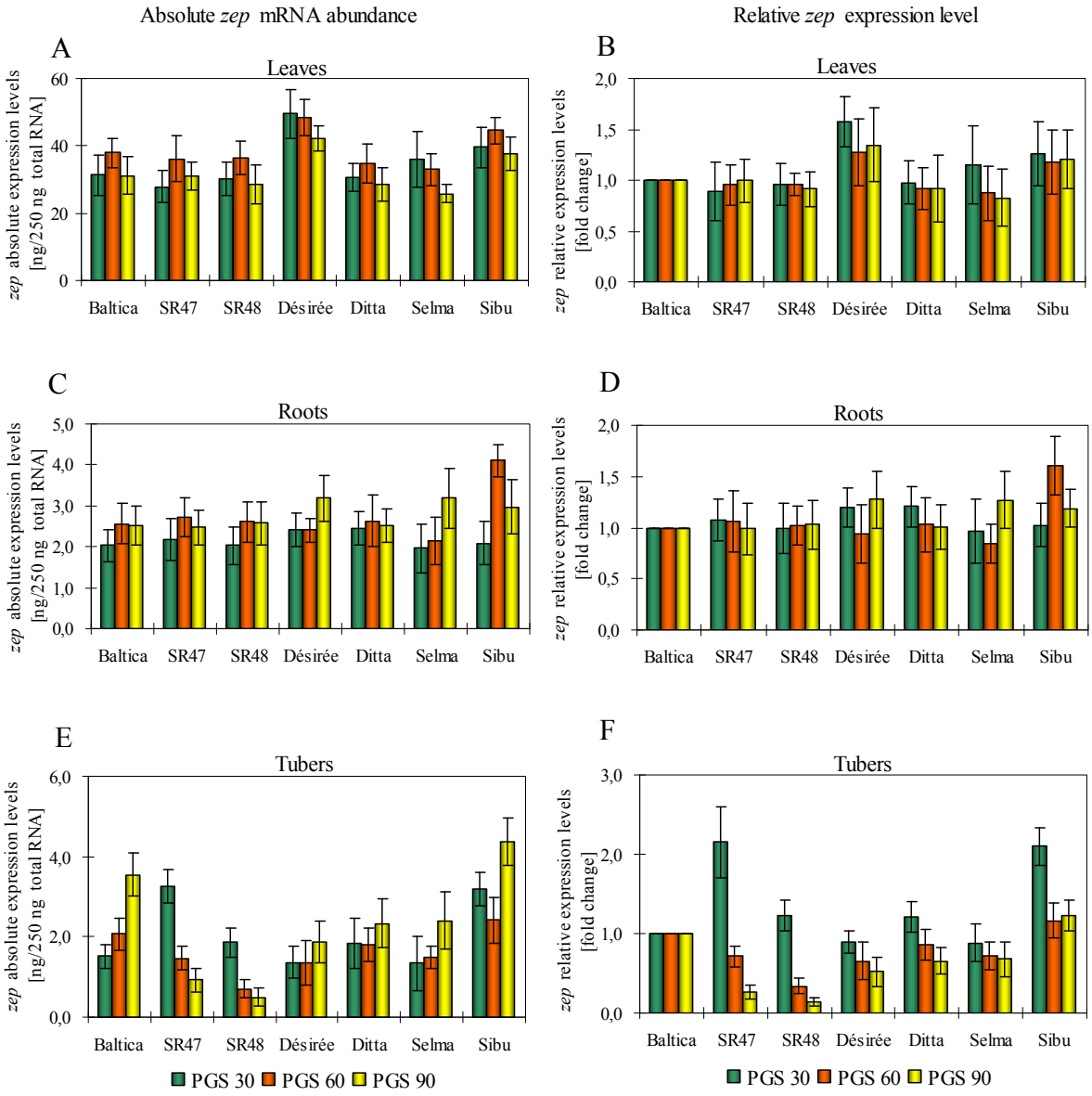


Fig. 3.9 Absolute and relative expression of *zep* in leaves, roots and tubers of Baltica, SR47, SR48, Ditta, Sibu, Selma and Désirée under open-field conditions. Samples were taken from research field Roggenstein (2005 and 2007) and Oberviehhausen (2006 and 2007). qRT-PCR was performed with total RNA isolated from leaves, roots and tubers. A, C, E: Absolute expression levels of *zep* were calculated according to a standard curve (Bustin, 2000; Pfaffl, 2001). B, D, F: Relative expression levels of *zep* were calculated first relative to a housekeeping gene (elongation factor, EF), then relative to the *zep* expression in Baltica. Data are presented as means ± SD from three biological repetitions consisting of three technical replicates and were averaged over the two environments.

3.3 Expression profiling

In order to investigate any unintended perturbations in potato transcriptome after transgenesis, global gene expression analysis was implemented by using cDNA array technology. This part of results presents comparative expression profiling of two GE potato clones and their conventional counterpart cultivar Baltica by applying (1) cDNA macroarray: expression profiling of DNA fragments derived from SH cDNA libraries of leaves and tubers of GE potatoes; (2) cDNA microarray: expression profiling of 11,412 verified and usable DNA fragments in current public database, which derived from potato stolons, roots, microtubers, dormant tubers, germinating eyes, and healthy and *Phytophthora infestans*-challenged plant materials and (3) comparison of the results from the two array technologies.

3.3.1 Analysis through cDNA macroarray

3.3.1.1. Construction and contents of cDNA libraries

Four differentially expressed cDNA libraries were constructed by using SH method (Vertis Biotechnologie AG, Freising, Germany) as described in material and methods. Two libraries were derived from leaf materials at PGS 60 and two from tuber materials at the same growth stage. Table 3.6 shows the number of clones randomly picked from each library. The unigenes were classified into nine functional categories according to partial sequence identities to known enzymes and proteins (Table 3.7).

Table 3.6 Contents of cDNA libraries

Name of the library	Number of clones sequenced	Number of unigenes
SR47-leaf-library	96 clones	94 unigenes
SR48-leaf-library	96 clones	93 unigenes
SR47-tuber-library	144 clones	138 unigenes
SR48-tuber-library	144 clones	137 unigenes

Table 3.7 Functional classifications of genes in cDNA libraries

Classifications	Leaf-libraries		Tuber-libraries	
	SR47	SR48	SR47	SR48
Cellular metabolism	24 (25.5%)	21 (22.6%)	33 (23.9%)	52 (37.9%)
Signalling	5(5.3%)	2 (2.2%)	3 (2.2%)	1 (0.7%)
Transporters	3 (3.2%)	1 (1.1%)	5 (3.6%)	4 (2.9%)
Binding protein	9 (9.6%)	4 (4.3%)	9 (6.5%)	8 (5.8%)
Transcription	5 (5.3%)	8 (8.6%)	7 (5.1%)	7 (5.1%)
Stress/defence	2 (2.1%)	8 (8.6%)	2 (1.4%)	2 (1.5%)
Unknown protein	33 (35.1%)	22 (23.7%)	61 (44.2%)	48 (35.0%)
No similarities found	13 (13.8%)	23 (24.7%)	17 (12.3%)	15 (10.9%)
Total	94 (100%)	93 (100%)	138 (100%)	137 (100%)

In SR47-leaf-library, 94 of the 96 sequenced clones encoded individual genes. Of the 94 unigenes identified by BLAST analysis, 24 (25.5%) were involved in general cellular metabolism, while almost half (48.9%) of the encoded genes represented unclassified proteins or clones for which no similarity to any database sequence could be found. The other unigenes showed homology to genes functioning in signalling, transcription, stress/defence, transport and binding activities.

In the SR48-leaf-library, similar distribution of unigenes to SR47-leaf-library was found. 93 (96.9%) out of 96 sequenced clones encoded individual genes, with 21 (22.6%) clones indicating homology to general cellular metabolism and almost half (47.8%) of the clones belonging to molecular function unknown group. Among the clones with homology to genes involved in signalling, transport, binding, transcription, growth/development and stress/defence activities, most interesting ones presented connection to carotenoids biosynthesis pathway (*Nicotiana tabacum* violaxanthin de-epoxidase precursor (*TVDE1*) mRNA) and photosynthesis (*Solanum tuberosum* chlorophyll a/b binding protein (*Lhcb*1-4) gene, nuclear gene encoding chloroplast protein).

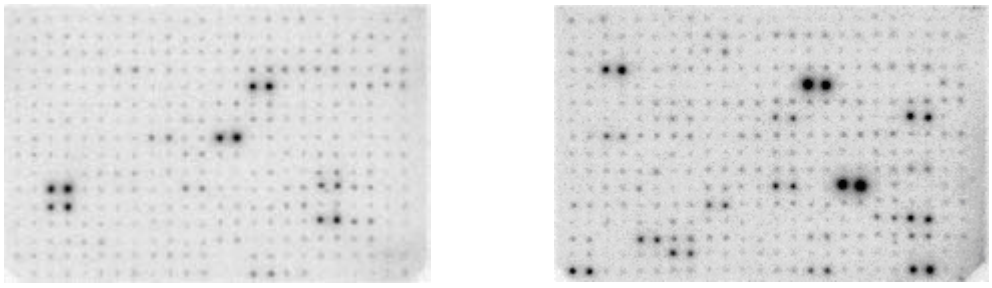
In SR47-tuber-library, 138 (95.8%) out of 144 sequenced clones encode unigenes. Of the 138 unigenes, 33 (23.9%) exhibited homology to general cellular metabolism genes and 78 (56.5%) were classified as unknown function/no similarities found. Among 9 genes with binding function, one gene was related to cellular retinaldehyde

binding/alpha-tocopherol transport or cellular retinaldehyde-binding/triple function proteins from *Medicago truncatula*. The other interesting clone was a putative light-inducible tissue-specific ST-LS1 gene from *Solanum tuberosum*.

A total of 137 (95.1%) unigenes were identified from 144 sequenced clones in SR48-tuber-library. None of the 137 unigenes displayed homology to genes involved in photosynthesis/chloroplast or carotenoids/xanthophyll synthesis pathways, but 52 (37.9%) clones indicated homology to general cellular metabolism, and 63 (45.9%) were attributed to the category of sequences of unknown molecular function.

3.3.1.2. Expression analysis in leaves and tubers of GE clones

For further analyses, unigenes from the four cDNA libraries were spotted onto nylon filters and expression analysis for each gene was carried out. For that, spotted cDNA macroarrays were hybridized with ^{32}P-labelled cDNA samples prepared from total RNA of leaves (PGS 30, PGS 60 and PGS 90) and tubers (PGS 60 and PGS 90) from Baltica, SR47 and SR48. Fig. 3.10 shows images of two macroarray filters after hybridization as example. Signals from each image were normalized according to hybridization design and relative expression levels (expression fold-change) were calculated (see section 2.2.5.3).



A: leaf cDNA library **B:tuber cDNA library**

Fig. 3.10 Images of scanned filters after hybridization with ^{32}P-labelled cDNA. A: leaf cDNA macroarray hybridized with labelled cDNA from GE leaf material; B: tuber cDNA macroarray hybridized with labelled cDNA from GE tuber material.

3.3.1.2.1. Expression analysis in leaves

Table 3.8 shows the number of genes with differences in gene expression levels in leaves of GE potato clones compared to Baltica. Most of the genes up- or down-regulated showed a fold-change between 1.1 and 2.0. Only few genes exhibited a more than two-fold change of gene expression levels in all the samples compared.

Table 3.8 Distribution of genes with different expression fold changes in leaves of GE potato clones

		Up-regulated		Unchanged	Down-regulated	
		1.1-2.0 fold	2.1-2.6 fold	1 fold	1.1-2.0 fold	2.1-2.5 fold
SR47	PGS 30	112 (60.9%)	13 (7.1%)	10 (5.4%)	48 (26.1%)	1 (0.5%)
	PGS 60	55 (30.0%)	1 (0.5%)	17 (9.2%)	105 (57.1%)	6 (3.3%)
	PGS 90	42 (22.8%)	2 (1.1%)	18 (9.8%)	115 (62.5%)	7 (3.8%)
SR48	PGS 30	48 (26.1%)	1 (0.5%)	26 (14.2%)	109 (59.2%)	0
	PGS 60	52 (28.3%)	0	26 (14.2%)	104 (56.5%)	2 (1.1%)
	PGS 90	42 (22.8%)	0	22 (12.0)	115 (62.5%)	5 (2.7%)

Student t-test was applied to the normalized data and only the genes with p-value smaller than 0.01 were considered as genes with significantly different expression levels. Table 3.9 lists the details of moderately differentially expressed genes in leaves of GE and conventional counterpart potato clones.

Scatter plots of normalized signal intensities gave an overview of changes in the expression of each gene in leaves and tubers from different growth stages of the two GE potato clones compared to Baltica (Fig.3.11). In each subfigure, logarithmic gene expression levels in SR47 or SR48 (y-axis) were plotted against logarithmic gene expression levels in Baltica (x-axis). Diagonal lines were drawn in each subfigure to facilitate visualisation which genes were up-regulated or repressed.

In leaves at PGS 30, two out of 187 (~1.1%) genes in SR47 were found to be significantly up-regulated more than two-fold compared to Baltica; no genes displaying significant differential expression patterns were found in SR48 at the same growth stage (Fig. 3.11 A and B). At PGS 60, expression levels of two (~1.1%) genes in SR47 were identified to be significantly up-regulated (both 2.1-fold), while in SR48 one (0.5%) gene showed a 2.2-fold down-regulation (Fig. 3.11 C and D). At PGS 90, two (1.1%) genes in

SR47 were significantly differentially expressed, of which a gene with similarity to an ATP-dependent protease (CD4B) from tomato was up-regulated (2.2-fold) and the other (thioredoxin h-like protein from *Panax ginseng*) down-regulated 2.3-fold. In SR48 at PGS 90, only one gene was found to be significantly up-regulated two-fold (Fig. 3.11 E and F).

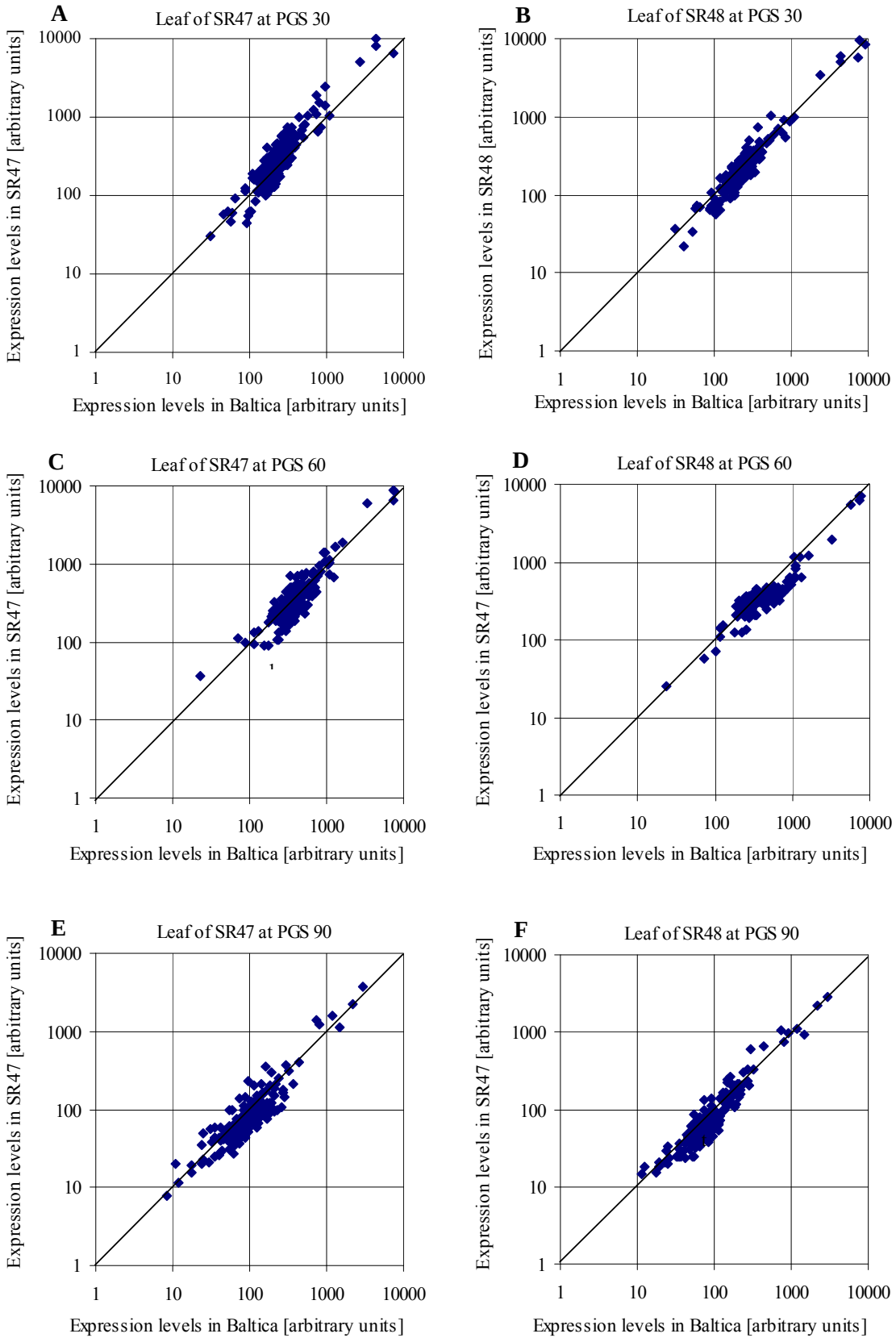


Fig. 3.11 Scatter plots of gene expression in leaves of SR47 and SR48 compared to Baltica. Marks above the diagonal represent up-regulated genes, marks below the diagonal down-regulated genes.

Table 3.9 Moderately differentially expressed genes in leaves of GE potato clones[a]

PGS	Accession No[b]	Homology	E-value	Fold change[c]	p-value	Gene Ontology[d]		
						Biological process	Cellular component	Molecular function
In leaves of SR47								
PGS 30	NM_114372	40S ribosomal protein S20 (*Arabidopsis thaliana*)	2.00E-43	+2.3	0.0083	protein biosynthesis (GO: 0006412)	structural constituent of ribosome (GO: 0003735)	rRNA binding (GO: 0019843)
	AJ277206	S-adenosylmethinonine synthetase (*Camellia sinensis*)	1.00E-16	+2.2	0.0093	ethylene biosynthetic process (GO:0009693)	cell wall (GO:0005618)	ATP binding (GO:0005524)
PGS 60	XM_478918	NADH-ubiquinone oxidoreductase 18 kDa subunit, mitochondrial precursor-like protein, (*Oryza sativa* japonica cultivar-group)	2.00E-13	+2.1	0.0001	-	-	NADH dehydrogenase (ubiquinone) activity (GO:0008137)
	AK248045	HTC in fruit (*Solanum lycopersicum*)	1.00E-179	+2.1	0.0015	-	-	-
PGS 90	M32604	Tomato ATP-dependent protease (CD4B) gene (*Lycopersicon esculentum*)	0	+2.2	0.0008	nucleotide-excision repair (GO:0006289)	chloroplast (GO:0009507)	ATP binding (GO:0005524)
	EU136387	mRNA, thioredoxin h-like protein (*Panax ginseng*)	1.00E-55	-2.3	0.0081	-	-	-
In leaves of SR48								
PGS30	No significantly differentially expressed genes were found							
PGS 60	AAM23004	Orcinol O-methyltransferase [Rosa hybrid cultivar]	3.00E-53	-2.2	0.0093	-	-	-
PGS 90	BT013952	*Lycopersicon esculentum* clone 132975F, mRNA	0	+2.0	0.0054	-	-	-

[a] Only genes with at least two-fold change and p-value <0.01 were considered differentially expressed;

[b] Accession no. from NCBI database refers to the best hit of the homology search;

[c] Genes with positive fold change were up-regulated in leaves of corresponding GE potato clones and negative fold changes were down-regulated;

[d] Gene ontologies are listed only when the information was available.

3.3.1.2.2. Expression analysis in tubers

Expression of 275 unigenes from tuber libraries was determined in developing (PGS 60) and mature (PGS 90) tubers. Scatter plots of expression levels of the 275 unigenes in tubers of SR47, SR48 and Baltica are shown in Fig. 3.12. Table 3.10 shows the number of genes with different expression fold changes in tuber of GE potato clones and details of the genes found to be significantly differentially expressed in tubers are listed in Table 3.11.

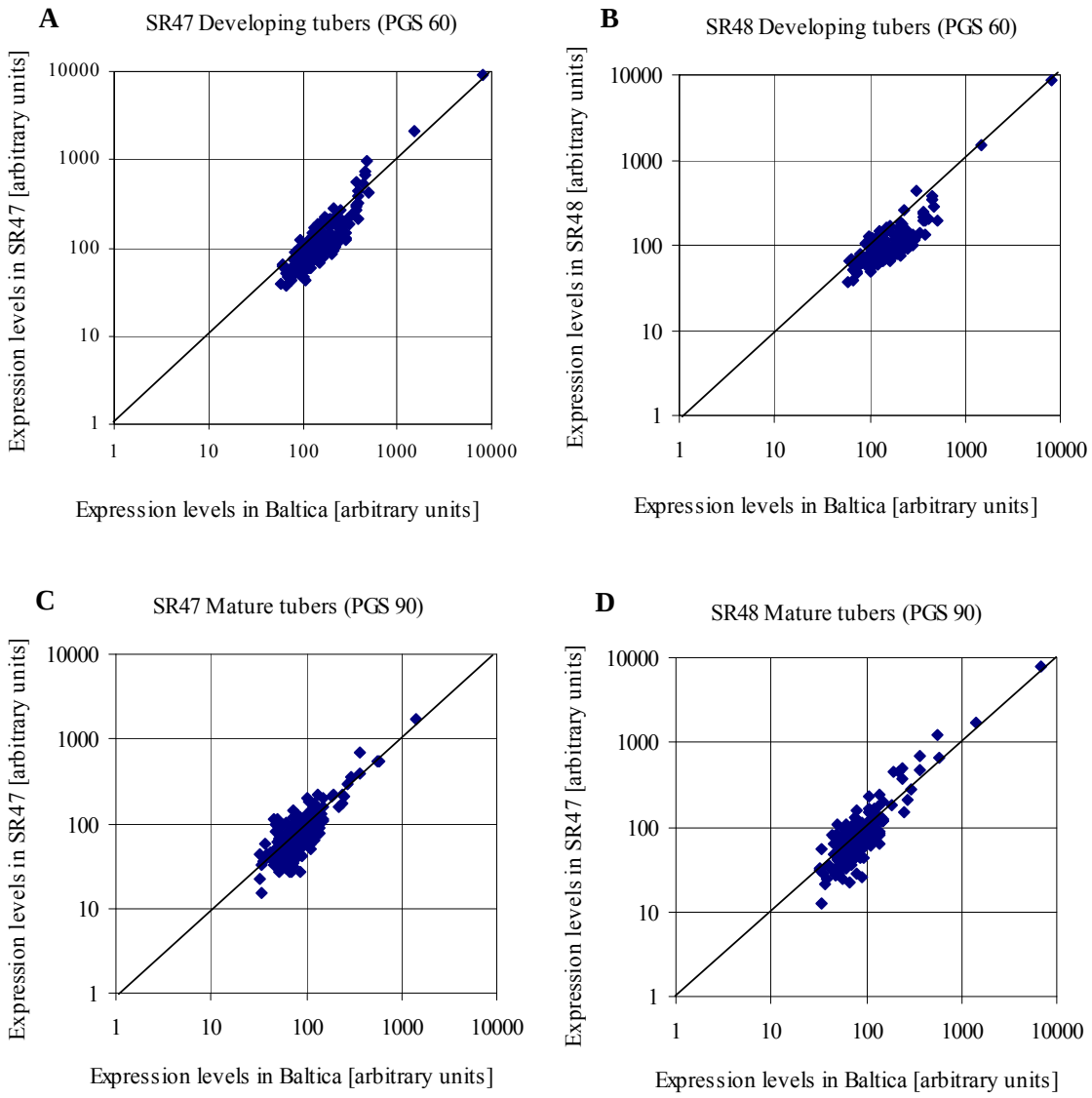


Fig. 3.12 Scatter plots of gene expression in tubers of SR47 and SR48 compared to Baltica. Marks above the diagonal represent genes up-regulated in SR47 or SR48. Marks below the diagonal represent genes down-regulated in SR47 or SR48.

Table 3.10 Distribution of genes with different expression fold changes in tubers of GE potato clones

		Up-regulated		Unchanged	Down-regulated		
		1.1-2.0 fold	2.1-2.6 fold	1 fold	1.1-2.0 fold	2.1-3.0 fold	3.1-4.0 fold
SR47	PGS 60 (developing tuber)	35 (12.7%)	2 (0.7%)	24 (8.7%)	198 (72.0%)	16 (5.8%)	0
	PGS 90 (mature tuber)	25 (9.1%)	1 (0.4%)	17 (6.2%)	182 (66.2%)	46 (16.7%)	0
SR48	PGS 60 (developing tuber)	91 (33.1%)	4 (1.5%)	31 (11.3%)	136 (49.5%)	13 (4.7%)	0
	PGS 90 (mature tuber)	89 (32.4%)	9 (3.3%)	29 (10.5%)	138 (50.2%)	8 (2.9%)	2 (0.7%)

Table 3.11 Moderately differentially expressed genes in tubers of GE potato clones[a]

Developing stage	Accession No[b]	Homology	E-value	Fold change[c]	p-value	Gene Ontology[d]		
						Biological process	Cellular component	Molecular function
				SR47				
Developing tubers (PGS 60)	DQ191631	Annexin p34-like protein mRNA (*Solanum tuberosum*)	0	-2.2	0.0033	response to osmotic (GO: 0006970) oxidative stress (GO: 0006979)	peroxidase activity (GO: 0004601) calcium ion binding (GO: 0005509)	cytosol (GO: 0005829) membrane (GO: 0016020)
	NM_118791	AtCBL3 (Calcineurin B-Like 3) mRNA (*Arabidopsis thaliana*)	1.00E-79	-2.3	0.0038	-	-	-
	AF271258	Peptide deformylase-like protein mRNA (*Lycopersicon esculentum*)	0	-2.3	0.0065	Translation (GO: 0006412)	Chloroplast (GO: 0009507)	peptide deformylase activity (GO: 0042586)
	AJ489324	2-on-2 hemoglobin (glb3 gene) mRNA (*Datisca glomerata*)	3.00E-25	-2.0	0.0084	oxygen transport (GO: 0015671)	-	heme binding (GO: 0020037) iron ion binding (GO: 0005506) oxygen binding (GO: 0019825)
	AK224787	cDNA clone: FC15DH04, HTC in fruit (*Solanum lycopersicum*)	9E-133	-2.1	0.0094	-	-	-
Mature tubers PGS 90	DQ191652	RNA binding protein-like protein mRNA (*Solanum tuberosum*)	4.00E-30	-2.3	0.0090	-	mitochondrion (GO: 0005739)	double-stranded DNA binding (GO: 0003690) RNA binding (GO: 0003723)

(To be continued on next page)

Table 3.11 (Continued)

Developing stage	Accession No[b]	Homology	E-value	Fold change[c]	p-value	Gene Ontology[d]		
						Biological process	Cellular component	Molecular function
				SR48				
Developing tubers (PGS 60)	DQ294266	Mitochondrial carrier-like protein mRNA (*Solanum tuberosum*)	3E-43	-2.4	0.0015	transport (GO: 0006810) mitochondrial transport (GO: 0006839)	mitochondrial inner membrane (GO: 0005743)	binding (GO: 0005488)
	NP_914888	Hypothetical protein OSJNBa0052O12.6 [*Oryza sativa* (japonica cultivar-group)]	4E-45	-2.4	0.0038	-	-	-
	AK246435	cDNA, clone: FC11AC02, HTC in fruit (*Solanum lycopersicum*)	0	-2.4	0.0050	-	-	-
	AK224787	cDNA clone: FC15DH04, HTC in fruit (*Solanum lycopersicum*)	9E-133	-2.0	0.0061	-	-	-
	AK246673	cDNA clone: FC18DD02, HTC in fruit (*Solanum lycopersicum*)	0	-2.2	0.0066	-	-	-
	DQ191631	Annexin p34-like protein mRNA (*Solanum tuberosum*)	0	-2.5	0.0087	response to osmotic (GO: 0006970) oxidative stress (GO: 0006979)	-	peroxidase activity (GO: 0004601) calcium ion binding (GO:0005509)
	ABI34375	Hypothetical protein SDM1_53t00013 (*Solanum demissum*)	9E-26	-2.4	0.0091	-	-	-
	NM_101390	Oxidoreductase (ATCCMH) mRNA (*Arabidopsis thaliana*)	2E-34	-2.3	0.0098	-	-	-
	BT012772	mRNA sequence from clone 113750F (*Lycopersicon esculentum*)	0	-2.8	0.0098	-	-	-
Mature tubers´ (PGS 90)	No significant differentially expressed genes were found							

[a] Only genes with at least two-fold difference and p-value <0.01 were considered differently expressed.
[b] Accession no. from NCBI database refers to the best hit of the homology search.
[c] Genes with negative fold change were down-regulated in leaves of corresponding GE potato clone.
[d] Gene ontologies are listed only when the information was available.

Fig. 3.12 shows the relative expression of genes in GE potato tubers at PGS 60 (developing tuber) and PGS 90 (mature tuber). More genes were moderately down-regulated in tubers of GE potatoes at PGS 60 (Fig. 3.12 A and B) than at PGS 90 (Fig. 3.12 C and D). Table 3.10 lists the distribution of the genes up- or down-regulated at different PGS in GE tubers. A total of 77.8% (214 out of 275) genes in SR47 at PGS 60 were down-regulated with a fold change of 1.1 to 3.0; 54.2% (149 out of 275) genes were down-regulated with a fold change of 1.1 to 3.0 in SR48 at the same growth stage. After statistical analysis of the expression data, altogether six genes (five at PGS 60 and one at PGS 90) were found to show a significant change of expression in tubers of GE potato clone SR47 (Table 3.11). Noticeably, all the significantly differentially expressed genes in SR47 were down-regulated, although in a relatively limited degree (2.1-2.3 fold change). For the GE clone SR48 nine genes were found to be significantly differentially expressed. None of the genes was identified in mature tubers (PGS 90) of SR48.

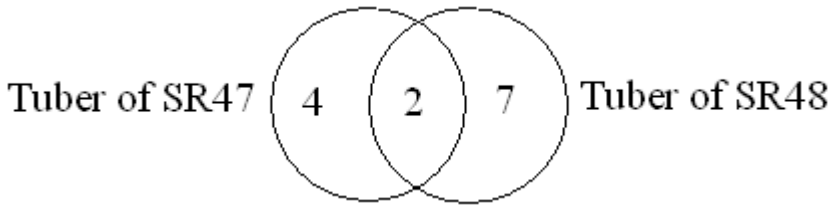


Fig. 3.13 Venn diagram of the genes found in tubers of SR47 and SR48.

Two genes, common to both GE potato clones, were down-regulated in tubers with a fold value of around 2 (Fig. 3.13). The fold change information can be found in table 3.11). One of the genes showed similarity to an annexin p34-like protein mRNA from *S. tuberosum* (Genebank accession no. DQ191631). The other one was related to an unknown cDNA fragment derived from fruit of *S. lycopersicum* (Genebank accession no. AK224787).

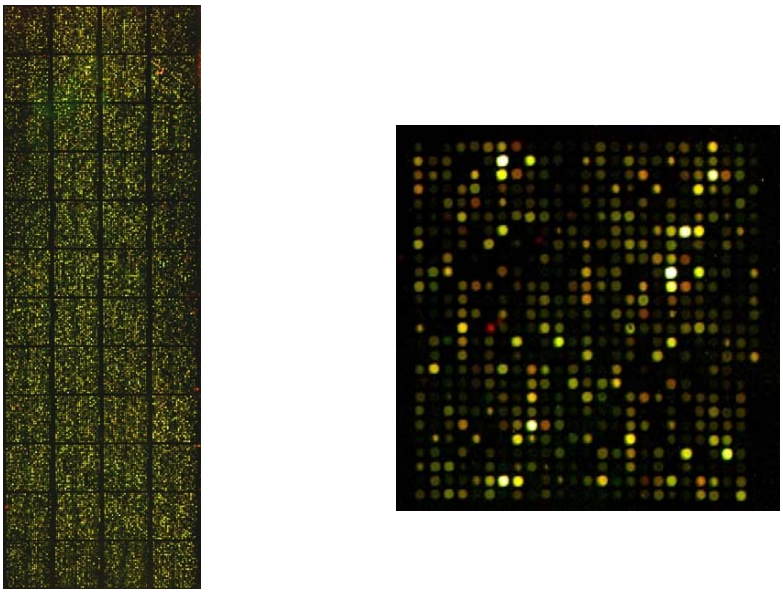
3.3.2 Analysis through cDNA microarray

In this part of the study, *10K TIGR potato microarray* was used to validate the trustworthiness of the 15 (according to the Venn diagram there would be only13, because

two are in common) significantly (greater than two-fold) differentially expressed genes in tubers of GE clones found by cDNA macroarrays and to explore any other possible difference in a more comprehensive way.

3.3.2.1. Hybridization and quality assessment of the data

Developing tubers harvested from open-field trial (Roggenstein 2005) at PGS 60 of GE potato clones SR47, SR48 and conventional counterpart cultivar Baltica were utilized in microarray analysis. RNA from each sample was extracted directly by using DynaBeads oligo (dT) 25 (Dynal Biotech, Oslo, Norway) (see section 2.2.3.3), labelled and hybridized pair-wise to the spotted microarray slides as indicated in the experimental design (see section 2.2.7). Fig 3.14 shows an example of a scanned 10K TIGR potato array after hybridization.



A **B**

Fig. 3.14 Hybridization images of TIGR potato microarray (Slide 1: SR47 vs. Baltica). A: overview; B: one subarray.

MA-plots of the unnormalized data of each array and imagine plots visualizing the variation of background intensities showed the good quality of the hybridization data (data not shown). After background correction, within-array-, between-array-, print-tip loess-

and dye-swap normalization, and averaging the technical replicates, data was ready for estimating differential gene expression (fold change analysis) in GE potato tubers by applying a linear model and an empirical Bayes approach for moderated t-statistics.

3.3.2.2. Comparative analysis of gene expression

The main advantage of microarray analysis is that it can detect thousands of genes simultaneously and compare the expression of the same gene in two samples. For such huge amount of data, a proper analysis and visualization method is very important. Among many visualization methods for comparative gene expression analysis, volcano plots give a clear overview of the differentially expressed genes in target samples. In this study, volcano plots were chosen to visualize comparative gene expression analysis in tubers of the two GE clones compared to their conventional counterpart cultivar Baltica (Fig. 3.15 and 3.16). Table 3.12 lists potential differentially expressed genes in GE potato tubers.

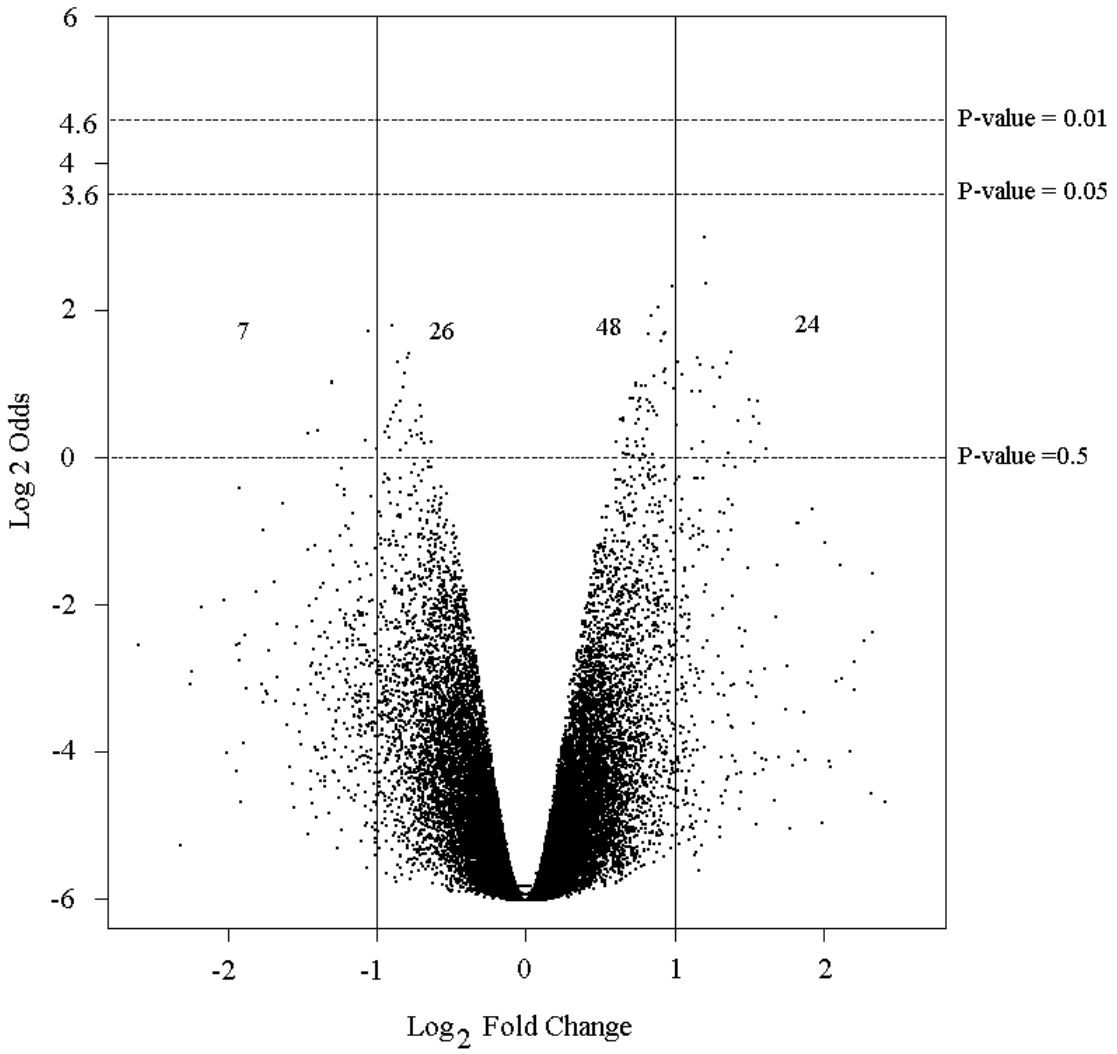


Fig. 3.15 Volcano plot for visualizing gene expression changes in tubers of SR47 (PGS 60, Roggenstein 2005). Each point represents one gene in the 10K TIGR cDNA microarray. The x-axis corresponds to the log2 of the expression fold change between SR47 and Baltica, the y-axis corresponds to the Log Odds[2] for differential expression of all genes. Significantly differentially expressed genes (with log odds above 4.6, 99% of chance of being statistically significant) should appear above the first line. Genes up-regulated > two-fold are on the right of the right vertical line, and the ones repressed ≥ two-fold are on the left of the left vertical line. The four numbers showed in this Fig. stand for the number of genes with p-value ≤ 0.5 and with expression fold changes ≤ -2, between -2 and 1, between 1 and 2 and ≥ 2, respectively.

[2] Log Odds (or B-value) stands for the probability that a certain gene is differentially expressed. A Log Odds value of 0 corresponds to a 50-50 chance that the gene is differentially expressed. The higher the Log Odds for each gene is, the higher the probability that the gene is differentially expressed and not a false positive (Smyth, 2004). An adjusted p-value of 0.01 corresponds to a Log Odds (B-value) of 4.6 and an adjusted p-value of 0.05 to 3.6.

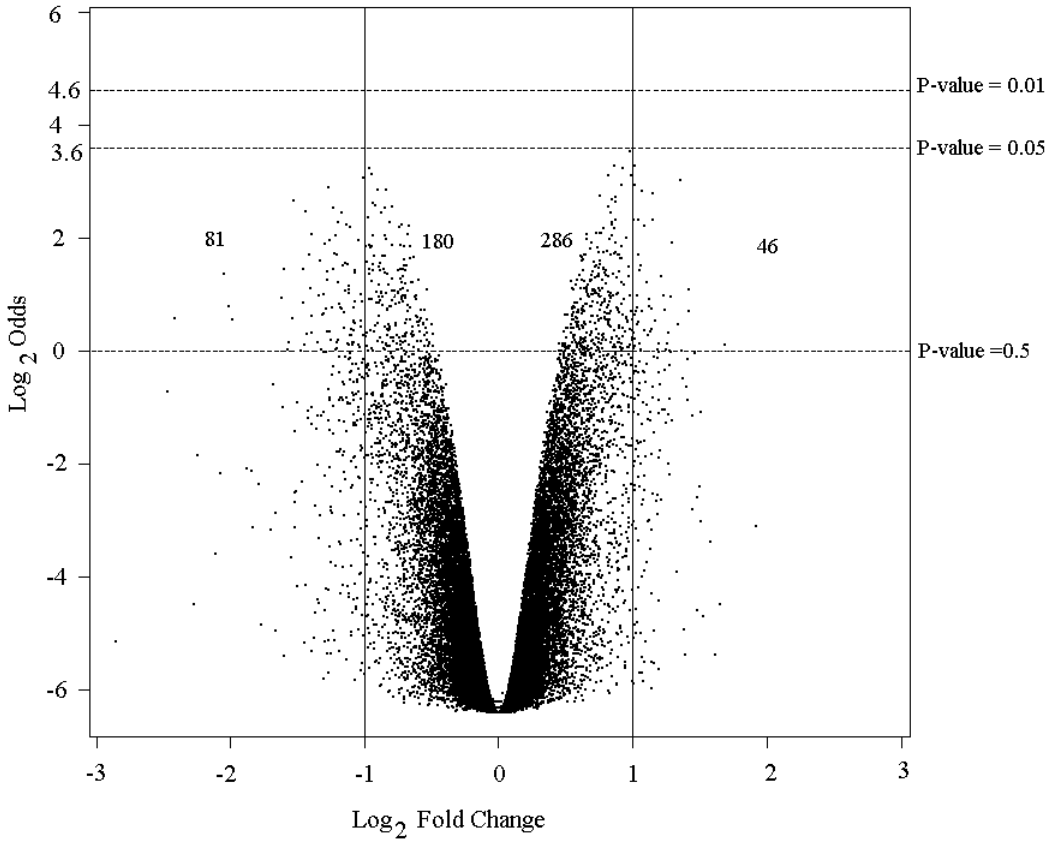


Fig. 3.16 Volcano plot for visualizing gene expression changes in tubers of SR48 (PGS 60, Roggenstein 2005). More details were described in Fig. 3.15.

As visible in volcano plots (Fig. 3.15 and 3.16), no genes were significantly differentially expressed in tubers at PGS 60 of both GE potato clones with a cut-off B-value= 4.6 (p-value= 0.01) and 3.6 (p-value= 0.05). However, when cut-off p-value was increased to 0.5, seven and 24 genes were found to be down- and up-regulated, respectively, in tuber tissue of SR47 with a moderate range of fold changes (greater than two-fold). In tubers of SR48, the numbers were 81 for the fraction of up-regulated genes and 40 for the one that showed down-regulation (Fig. 3.16).

Although no genes were found to be significantly differentially expressed in developing tubers of GE potato clones when compared to their conventional counterpart cultivar Baltica at cut-off B-value at 4.6 or 3.6, analysis command of "Toptable" exports lists of genes ranked with p-value (Wirta, 2005). Top 30 genes of the two GE clones were exported and Table 3.12 lists only the genes with expression fold changes more than ±2 in developing GE potato tubers among the top 30 genes.

Table 3.12 Top ranked genes found through microarray analysis in GE potato tubers[a]

Homology	GB. No.	Gene Ontology	Fold Changes[b]	AveExpr[c]	t[d]	P-value[e]	adj.P-Val[f]	B[g]
		In tubers of SR47						
Chitin-binding lectin 1 precursor [*Solanum tuberosum* (Potato)]	BQ113833	Protein amino acid binding (GO:0005515)	+2.3	10.45	10.62	8.32e-06	0.187	3.17
LSD-One-Like 1 [*Brassica campestris* (Field mustard)]	BQ114140	Not available	+2.2	8.13	7.16	1.27e-04	0.187	1.35
Xyloglucan endotransglucosylase-hydrolase XTH7 [*Lycopersicon esculentum* (Tomato)]	BQ114680	Glycosidase activity (GO:0016798)	+2.3	7.29	7.03	1.44e-04	0.187	1.25
CAPIP2 [*Capsicum annuum* (Bell pepper)]	BQ506071	No available information	+2.1	9.53	6.85	1.71e-04	0.187	1.12
		In tubers of SR48						
No homology available	BQ512918	Not available	-2.3	9.95	-9.55	1.71e-05	0.0341	3.42
Putative acid phosphatase [*Hordeum vulgare* (Barley)]	BQ513581	acid phosphatase activity (GO:0003993)	+2.6	9.56	8.94	2.72e-05	0.0341	3.03
DnaJ-like protein [*Solanum tuberosum* (Potato)]	BQ512749	inositol/phosphatidylinositol phosphatase activity (GO:0004437)	-2.3	10.25	-8.73	3.21e-05	0.0341	2.89
TK1-like deoxyribonucleoside kinase [*Lycopersicon esculentum* (Tomato)]	BQ507320	thymidine kinase activity (GO:0004797)	+2.1	9.86	8.64	3.44e-05	0.0341	2.83
No homology available	BQ514051	molecular function (GO:0005554)	-2.5	9.46	-8.42	4.13e-05	0.0341	2.67

[a] Criteria of the gene selection: genes were ranked according to their B-value, which calculated from a linear model fit after adjusting for multiple testing;
[b] Genes with positive or negative fold change were up- or down-regulated in tubers of corresponding GE potato clone compared with the expression in Baltica;
[c] AveExpr: average log2-expression for the probe over all arrays and channels;
[d] t: moderated t-statistics;
[e] P-Value: raw p-value;
[f] adj. P-Value: adjusted p-value according to Benjamini and Hochberg (1995);
[g] B: log odds that the gene is differentially expressed.

Four genes with expression fold changes more than two were found in SR47 developing tubers. These four genes were all up-regulated with limited fold changes around two.

Five genes were found in GE developing tubers of SR48. Three of the five genes were down-regulated with expression fold changes in a range of 2.3-2.5. However, two of the three down-regulated genes have no available homology. Two genes were up-regulated with 2.6 and 2.1 fold in GE tubers of SR48.

No common differentially expressed genes were found both in SR47 and SR48. And no genes have direct correlation with carotenoid synthesis pathway, which the inserted *zep* gene may cause unexpected changes.

3.3.3 Comparison between macro- and microarray

In macroarray hybridization experiments, fifteen genes in the two GE tubers were found to be significantly differentially expressed with a moderate range of expression fold changes compared to Baltica. Since tubers are the consume part of potato plants and more differentially expressed genes were found in developing tubers (PGS 60) through macroarray analysis, microarray analysis was applied to tubers at PGS 60.

Table 3.13 shows the expression fold changes and corresponding p-values in microarray analysis of differentially expressed genes found through macroarray analysis. Five from six differentially expressed genes in tubers of SR47 have corresponding gene spots on microarray and all these five genes were proved unchanged with expression fold changes around one. Four from nine differentially expressed genes in tubers of SR48 were also showed no significant fold changes through microarray analysis.

Besides, no common genes were found when compare differentially expressed genes found through macroarray analysis and top ranked genes with expression fold changes more than two achieved through microarray analysis.

Table 3.13 Comparison between cDNA macro- and microarray

Developing stage	Accession No	Homology	Macroarray		Microarray	
			Fold change[a]	p-value	Fold change	p-value
		SR47				
Developing tubers (PGS 60)	DQ191631	Annexin p34-like protein mRNA (*Solanum tuberosum*)	-2.2	0.0033	-1.1	0.69
	NM_118791	AtCBL3 (Calcineurin B-Like 3) mRNA (*Arabidopsis thaliana*)	-2.3	0.0038	-1.1	0.74
	AF271258	Peptide deformylase-like protein mRNA (*Lycopersicon esculentum*)	-2.3	0.0065	-1.1	0.57
	AJ489324	2-on-2 hemoglobin (glb3 gene) mRNA (*Datisca glomerata*)	-2.0	0.0084	1.1	0.58
	AK224787	cDNA clone: FC15DH04, HTC in fruit (*Solanum lycopersicum*)	-2.1	0.0094	-	-
Mature tubers PGS 90	DQ191652	RNA binding protein-like protein mRNA (*Solanum tuberosum*)	-2.3	0.0090	1.1	0.39
		SR48				
Developing tubers (PGS 60)	DQ294266	Mitochondrial carrier-like protein mRNA (*Solanum tuberosum*)	-2.4	0.0015	-1.1	0.67
	NP_914888	Hypothetical protein OSJNBa0052O12.6 [*Oryza sativa* (japonica cultivar-group)]	-2.4	0.0038	-1.2	0.08
	AK246435	cDNA, clone: FC11AC02, HTC in fruit (*Solanum lycopersicum*)	-2.4	0.0050	-	-
	AK224787	cDNA clone: FC15DH04, HTC in fruit (*Solanum lycopersicum*)	-2.0	0.0061	-	-
	AK246673	cDNA clone: FC18DD02, HTC in fruit (*Solanum lycopersicum*)	-2.2	0.0066	-	-
	DQ191631	Annexin p34-like protein mRNA (*Solanum tuberosum*)	-2.5	0.0087	-1.2	0.11
	ABI34375	Hypothetical protein SDM1_53t00013 (*Solanum demissum*)	-2.4	0.0091	-	-
	NM_101390	Oxidoreductase (ATCCMH) mRNA (*Arabidopsis thaliana*)	-2.3	0.0098	-1.2	0.23
	BT012772	mRNA sequence from clone 113750F (*Lycopersicon esculentum*)	-2.8	0.0098	-	-
Mature tubers (PGS 90)		No significantly differentially expressed genes were found				

[a] Genes with positive or negative fold change were up- or down-regulated in tubers of corresponding GE potato clone compared with the expression in Baltica.

4 Discussion

4.1 Morphology and agronomic performance of GE plants

The general morphology and agronomic performance of GE potatoes did not show obvious differences compared to their conventional counterpart cultivar Baltica under greenhouse and open-field conditions. Plant growth, development and tuber yield of the two GE plants were highly consistent with Baltica except decreased number of florets per inflorescence and berries. This unexpected character of GE potatoes could be considered as an advantage in view of gene transfer and biosafety of GE plants for future agricultural use.

4.1.1 Field testing of GE plants

Potatoes were one of the first crop plants in which GE plants were successfully regenerated (An *et al.*, 1986). Potato transformation offers new opportunities to transfer genes for cultivar improvement and has several advantages over other breeding methods, such as the effective transfer of new genes directly into existing elite cultivars without many generations of additional crosses (Conner, 2003). However, plant transformation is highly unpredictable with respect to the nature of transgene integration, the magnitude, specificity and stability of transgene expression and the frequency of off-types observed within populations of independently derived transformed plants (Conner and Christey, 1994). Therefore, it is important to select a large population of independently transformed individuals in the laboratory and screen the population to identify the clones with desired expression of transgene, while maintaining the phenotype of the parental cultivar in greenhouse. Furthermore, confirmation of the chosen GE plants in field trials is critically important for future release and agricultural employment and it is the most appropriate environment to gather valuable data for assessing biosafety, by which the issues of public

concern over the GE potatoes can be best addressed (McPartlan and Dale, 1994). Many reports on field tests of GE potatoes confirmed that the phenotypic performance conferred by the transgene was maintained under field conditions (Davidson *et al.*, 2002). However, there are many examples reporting the opposite (Kaniewski *et al.*, 1990; Heeres *et al.*, 2002). These research results illustrate that it is critically important to verify that transgenes confer the desired phenotype under field conditions in a case by case manner.

Two GE potato clones used in this work were selected from 127 individual GE clones with desired high-zeaxanthin content in tubers, which were obtained from a large number of transformants from six transformation series (Römer *et al.*, 2002). In Römer´s report, no phenotypic divergence except the expected high-zeaxanthin content in tubers was evident under greenhouse conditions. In this work, general morphology and agronomic performance of the GE potato clones (SR47 and SR48) were compared with the corresponding control clone (Baltica) under both greenhouse and open-field conditions. Results collected in this work were in agreement with those reported by Römer *et al.*, (2002). In this three-year field trial (2005 in Roggenstein, 2006 in Oberviehhausen, 2007 in both sites of Roggenstein and Oberviehhausen), the two GE potato clones were similar to Baltica in their general phenotypic appearance. These phenotypic traits were also uniform between the two GE clones and were consistent over the three-year field trial. The data further confirmed that the GE clones maintained the agronomic characters of Baltica and showed high potential as successful GE potatoes with higher zeaxanthin and total carotenoid contents.

4.1.2 Transgene dispersal

One of the public concerns about GE plants is transgene transfer by pollen to neighbouring crops of the same or closely related species (Dale, 1993). The regulatory authorities generally specify isolation distances from other plants of the same species when granting permits for field testing. From many years of experience of the production of seeds from traditional plant cultivars, isolation distances have become well established

for most crops. However, it is not that important for asexually propagated clonal crops, such as potato. Therefore, accurate information on effective pollination distances is comparably little (Conner and Dale, 1996).

Several published reports that quantify the frequency and distance of transgene dispersal during potato field trials are available (Dale *et al.*, 1992; Conner, 1993; McPartlan and Dale, 1994; de Vries *et al.*, 2003). These studies were performed independently in several countries, using different cultivars and different transgenic phenotypes. The majority of these independently performed studies reported very similar results: a very limited dispersal of pollen over very short distances near the edge of a trial. For adjacent row (<1 m), the out-crossing rate ranged from 0.046 – 23.64 %; for up to 3 m the rate ranged from 0 – 2.07 %; for 3 – 9 m the rate ranged from 0 – 0.02 % and for a distance over more than 10 m, the transgene dispersal by pollen to other potato plants is very limited and unlikely to happen (Tynan *et al.,* 1990; Dale *et al.,* 1992; Conner, 1993; McPartlan and Dale, 1994; de Vries *et al.*, 2003).

In this work, the conventional potato cultivars Baltica, Ditta, Sibu, Selma, Désirée, used as potential recipients of transgene-containing pollen, were planted in sub-plots with distances of 0.6 – 15 m from the GE potato plants SR47 or SR48. Large number of seedlings from Baltica, Ditta and Désirée were tested for transgene insertion, only one seedling from Baltica was found as transgene positive and its location was identified in the row where GE and non-GE potato plants were in alternate plots. No positive seedling was found for Ditta and Désirée despite the same distance. The out-crossing rate in this case was extremely low (0.17 ‰). The variety Baltica frequently produces heavy intensity of flowers and subsequently berries (average number of flowers per inflorescence was 8 – 10; number of berries was 10 – 30 per plant in the field trials). Although the number of flowers of GE plants was not significantly lower than for Baltica, the number of berries from GE plants, especially of SR47, were significantly lower.

Although the portion of aborting flowers varied with environmental condition, another possible reason could be the occurrence of somaclonal variation. Mori *et al.* (2007) reported transgenic *Agapanthus* plants that exhibited variable somaclonal variation, i.e. decreased number of florets per inflorescence, smaller florets, and reduced pollen fertility.

More somaclonal variation in GE plants as well as non-GE, tissue culture-derived plants of different species has been frequently reported (Larkin and Scowcroft, 1981; Karp, 1995; Lynch *et al.*, 1995; Singh *et al.*, 1998; Arencibia *et al.* 1999; Cervera *et al.*, 2000).

The correlation of genomic changes in GE plants and occurrence of somaclonal variation was investigated by Labra *et al.* (2004). They concluded that somaclonal variation observed in the GE *Agapanthus* plants may also due to tissue culture processes, including induction and subculture of embryogenic calluses used as a target material for transformation and/or selection and regeneration of transgenic tissues after *Agrobacterium* co-cultivation.

Conversely, the un-expected weaker fertility of GE potatoes observed in this work could be considered as a great advantage for further agricultural use because of a decreased risk of gene-transfer through true seeds into the environment.

4.1.3 Zeaxanthin content under open-field conditions

In contrast to the unchanged morphological characters, flesh colour of the GE tubers was obvious dark-yellow to orange, which coincides with the enriched zeaxanthin content in GE tubers. Results showed that the GE tubers harvested in Oberviehhausen 2006 contained 47.6 ± 11.1 µg/g dw (SR47) and 28.1 ± 8.6 µg/g dw (SR48). The contents were in agreement with the results from Römer *et al.* (2002) and Gerjets and Sandmann (2006) and showed that the desired trait of high-zeaxanthin content was stably inherited.

In recent years many successful examples of other higher plants engineered for higher carotenoid contents were reported: canola seed with β- and α-carotene accumulation, tomato fruits with enhanced lycopene and carrot plants with ketocarotenoid accumulation in leaves and roots (Guiliano *et al.*, 2008). However, among all the genetically modulated high-carotenoid plants there is only a very limited number with high-zeaxanthin accumulation. “Golden Rice” was engineered for higher beta-carotene (pro-vitamin A) content, but for lutein and zeaxanthin accumulation that accounts,

however, for less than 1% of total carotenoids (Ye *et al.*, 2000; Paine *et al.*, 2005). Another example with zeaxanthin accumulation is tomato fruit, with up to 24 μg/g fresh weight of zeaxanthin (Dharmapuri *et al*., 2002). Compared to this genetically modulated tomato fruits, the GE potatoes used in this work have less amount of zeaxanthin, but when taking into account the important role of potato in world's staple food supply market, the enhancement of certain nutrients in potato will certainly make an important improvement on the daily nutrition intake.

4.2 Expression analysis of *zep*

The study of transgene expression is of vital importance for GE plants. Transgene expression levels are influenced by many factors, in particular the site of integration of the transgene within the plant genome, gene silencing, and the promoter attached. Although some of these factors can be excluded out to some degree in the experimental design, it is still necessary to correlate phenotypic differences between transgenic and control plants with transgene expression (Page and Minocha, 2004).

Expression data of *zep* collected through this study showed that the endogenous *zep* in leaves and roots of GE plants was unaffected by the transferred *zep* which is under control of a tuber-specific promoter. The inserted *zep* gene, in sense and anti-sense orientation, respectively, only triggered the down-regulation of *zep* transcription in tuber tissue and was stably inherited under both greenhouse and open-field conditions.

4.2.1 *zep* expression in a tissue-specific manner

Tissue-specific expression here has two implications: (1) the distribution of endogenous *zep* mRNA abundance is tissue-specific and (2) the expression of inserted *zep* gene in sense and anti-sense orientation resulted only in the down-regulation of endogenous *zep* gene expression in tuber tissue.

The endogenous *zep* mRNA abundance is tissue-specifically expressed, higher in aerial parts (leaves, stem and berries) but lower in roots and tubers, as the expression analysis showed in this work. Similar results were found in *Nicotiana plumbaginifolia* (Audran *et al.*, 1998) and *Lycopersicon esculentum* Mill. (Wang *et al.*, 2008b). This is because in leaves zeaxanthin functions as an excess energy quencher. When light energy input exceeds the capacity for energy utilization through photosynthetic electron transport, the xanthophyll cycle is activated and zeaxanthin is accumulated in the thylakoids. The zeaxanthin accumulation constitutes the major protective process of the photosynthetic apparatus (Demmig-Adams and Adams, 1992). However, *zep* also plays an important role in non-chlorophyllous tissues. Although the abundance of *zep* mRNA in roots and tubers is 20 – 30 times lower than in leaves (Fig. 3.5), research results indicated that zeaxanthin epoxidation is a regulatory step in abscisic acid (ABA) biosynthesis (Frey *et al.,* 1999). The phytohormone ABA plays an important role in the regulation of various physiological processes including seed development, dormancy and response to environmental stresses, such as drought, salinity or cold (Giraudat *et al.*, 1994; Thompson *et al.*, 2000) and is synthesized from xanthophylls (oxygenated carotenoids), e.g. zeaxanthin, violaxanthin and neoxanthin (Taylor *et al.*, 2000). Therefore, endogenous *zep* expression and regulation in different plant tissues play an important role in maintaining the normal and healthy status of plants.

As the application of genetically engineered plants has widened, the need to develop methods to fine-tune control of transgene expression is mandatory. This aim can be achieved by using regulatory DNA sequence known as tissue-specific promoters, to drive the expression of transgene in specific plant tissue or at certain developmental stages (Song *et al.*, 2000). The availability of a broad spectrum of promoters that differ in their ability to regulate the temporal and spatial expression patterns of the transgene can dramatically increase the successful application of transgenic technology. A variety of promoters is necessary at all levels of genetic engineering in plants: from basic research discoveries, development of economically viable crops and plant commodities, to addressing legitimate concerns raised about the safety and containment of GE plants in the environment (Potenza *et al.*, 2004). A broad range of tissue-specific promoters from fruits

and grains, tubers, flowers, pistils, anthers, pollen, roots and root nodules, leaves and green tissue are available (Potenza *et al.*, 2004).

In the two GE potatoes used in this work, the *zep* tissue-specific expression was realized through granule-bound starch synthase (GBSS) promoter. Starch in potato tubers consists of up to 25% amylose, and GBSS is the key enzyme in amylose biosynthesis. A 5′ flanking potato GBSS promoter fragment of 800 bp was sufficient to cause high levels of reporter gene expression in both stolons and tubers, with little to no activity in leaves (Visser *et al.*, 1991). However, like patatin promoters, sugars can induce the GBSS promoter in leaves, but not to levels as high as those of the patatin promoters (Shekhawat *et al.*, 2007).

The introduced *zep* gene in sense and anti-sense orientation in the two GE potato clones was shown only to be expressed in tuber tissue through the results collected in this study. Consistently with the tuber-specific nature of the GBSS promoter used for the silencing constructs, no significant variations of *zep* expression, with respect to the conventional counterpart plant, were observed in leaves and roots. In the two GE clones, consistent with the hypothesized silencing of *zep* gene in tuber, the expression levels of *zep* showed significant down-regulation (up to 1/5) in mature tubers. The dark-yellow to orange colour of the tuber flesh and the results from HPLC confirmed that more zeaxanthin and total carotenoids were accumulated in GE potato tubers due to the silenced *zep* gene.

4.2.2 *zep* expression kinetics in different tissues

It is not only important to investigate the transient expression of *zep* in different tissues of potato plants at a certain growth stage, but also to know how it changes over time. Although the expression levels in mature tubers of GE plants answered the question of tuber specific expression of target gene, the analysis of *zep* expression kinetics could provide more information about the dynamics of the abundance of *zep*.

In this work, *zep* expression kinetics was analyzed in leaves, roots and tubers of GE plants and their conventional counterpart plant. *zep* was found to be expressed stably and constitutively in leaves and roots of GE and non-GE plants in the course of the growth under both greenhouse and open-field conditions. *zep* expression levels in these two tissues varied a limited extent at different stages. And only minor differences were found between greenhouse and open-field conditions. Audran and his colleagues (1998) reported that mRNA level of *zep* in leaves of *Nicotiana plumbaginifolia* displayed a day/night cycle, however the protein levels remained constant. Similar expression pattern was also found in *Arabidopsis thaliana* (North *et al.*, 2005). However, the expression of *zep* in leaves and roots increased under the condition of drought stress. In roots and seeds, no diurnal fluctuation in mRNA levels was observed (Audran *et al.*, 1998).

In this work*, zep* expression kinetics in tubers showed a reverse pattern in GE plants compared to their conventional counterpart plant. In non-GE plant, *zep* expression showed the lowest level at swelling stolon stage, then increased along tuber development and reached the highest level in mature tuber stage. In two GE potato plants, *zep* reached the highest point at swelling stolon stage, but decreased in developing and mature tuber stages. However, further research on *zep* and other close connected enzymes is necessary. Although many of the genes encoding the enzymes of the carotenoid biosynthetic pathway have been cloned from both plant and microbial sources over the past decade (reviewed in Cunningham and Gantt, 1998; Hirschberg, 2001; Fraser and Bramley, 2004), its regulation is not yet well understood. The rules that govern which carotenoids accumulate and to what extent, remain to be clarified in many cases. And this is of particular importance in plant storage organs that form major parts of our diet.

4.2.3 Post-transcriptional gene silencing

Two GE potatoes used in this work were established by using co-suppression (SR47) and antisense (SR48) gene silencing methods. Although the methods used are different, both GE potato have higher zeaxanthin and total carotenoids accumulation. The expression analysis in this work indicated that pos-transcriptional regulation of *zep* leads to the accumulation of zeaxanthin and total carotenoids.

The discovery of antisense phenomena of plant gene silencing and co-suppression (van der Krol *et al.*, 1988; Smith *et al.*, 1988; Napoli *et al.*, 1990; van der Krol *et al.*, 1990) provided the most powerful and widely-used approaches for investigating the roles of specific enzyme in metabolism, crop improvement and gene therapy (Wang *et al.*, 2008a; Eamens *et al.*, 2008). The underlying mechanisms responsible for gene silencing in plants remained unknown for many years. Nuclear run-on assays indicated that co-suppression occurred after the transcription of the transgene, and it was then called post-transcriptional gene silencing (PTGS) (Van Blokland *et al.*, 1994). Some hypotheses were proposed for the mechanisms of co-suppression, such as the threshold and RNA-dependent RNA polymerase (RdRp) hypothesis (Stam *et al.*, 1997; Cogoni and Macino, 1997; Vaucheret at al., 1998). The revelation of the double-strand-RNA (dsRNA)-induced mechanism (Ngo *et al.*, 1998) led to a vast expansion of interest in researching the molecular details and biological functions of RNA silencing in all eukaryotes. According to a threshold model, when RNA transcripts of a gene accumulate beyond a critical, threshold level, they are selectively degraded by RNases. An accumulation of high levels of RNA transcripts of a gene may lead to the production of aberrant sense RNA transcripts of the transgene. An accumulation of aberrant RNA transcripts is proposed to activate RNA-dependent RNA polymerase of plant origin, which transcribes the RNA transcripts to produce antisense RNA. The antisense RNA transcripts would associate with the accumulated normal and aberrant RNA transcripts of the transgene as well as the endogenous gene. This will produce RNA duplexes, which present targets for double-stranded RNA-specific RNases like RNase H. Degradation of the RNA transcripts of a gene is postulated to somehow

lead to a hyper-methylation of the DNA sequences homologous to the degraded RNA sequences. This often leads to a drastic reduction in the level of expression of the transgene in question and also of the homologous endogenous gene (Stam *et al.*, 1997). Other research results showed that the copy number, DNA methylation and structure of the integrated T-DNA of the transgene may play a role in the process of co-suppression (Stam *et al.*, 1997; Vaucheret at al., 1998). Subsequent discoveries of the various related small RNA (sRNA) pathways revolutionized the way we study gene regulation and developmental control in plants and animals (Eamens *et al.*, 2008).

Some other hypotheses proposed for the mechanisms of antisense were also available. For example, antisense RNAs act on their complementary target RNAs to block translation, induce mRNA decay, inhibit primer maturation, or induce premature termination of target RNA transcription (Brantl *et al.*, 2002).

4.2.4 Natural and GE-derived variation in gene expression

It is a matter of fact that natural variation among different cultivars always exists. The variation could be in physical, biochemical or physiological, and in small scale, also transcriptional (gene expression). By thousands of years´ agricultural practice, people accept the natural variation and made (are making) full utilization of it to breed varieties with desired characters.

Many other factors can also make effects on gene expression. The physical and chemical characteristics of soil, periodicity and quantity of rainfall, distribution of temperature, healthy status of the crops and husbandry method and schedule play very important roles in effecting certain gene or group of gene expression.

It cannot be denied that the insertion of a gene, especially randomly, has a relative higher possibility to cause differential gene expression. In this study, expression of the target gene *zep* showed no significant changes in leaves and roots compared to their conventional counterpart plant at three different growth stages. The changes of *zep* expression in GE tubers were moderately bigger in range than the other four conventional

potato cultivars. This is the reason why the global expression comparison was applied and tried to explore any other potentially differentially expressed gene or group of genes. However, it is not enough to set the range of natural variation through only four cultivars. A Database including the natural variation for each crop cultivar is needed to be established in order to assess the differences between GE and non-GE crops in a more reasonable manner (Cheng *et al.*, 2008).

A recent study on carotenoid and tocopherol compounds among 16 native Andean potato cultivars revealed a wide range difference in amount of those natural antioxidants (Andre *et al.*, 2007). The amount of α-tocopherol in tubers ranged from 2.73 to 20.80 µg/g dw. The upper level was clearly above the quantities generally reported for commercial cultivars. One thing is clear that it is impossible to predict the potential natural variation of any trait of plants. Detection of expression changes only provides information on transcript level. Thus, it should and must be integrated with the studies on the other levels such as metabolism level as a whole system. Only under that circumstances, a more accurate and safe assessment of GE crops can be reached.

4.3 Global expression comparison

The global expression analysis data shown in this work illustrate that no un-intended significant changes were found at the transcriptional level of the two GE potatoes compared to their conventional counterpart plant through two independent array technologies. Macroarray technology was first applied to search for differentially expressed genes. Eight (six in SR47 and two in SR48) and fifteen (six in SR47 and nine in SR48) genes were found to be moderately (no genes were found with more than 3-fold change) differentially expressed in leaves and tubers, respectively. Considering the accuracy and capacity limitation of the macroarray approach, we further applied microarray expression analysis as independent validation method. Microarray results showed that the moderate differentially expressed genes found through macroarray could

not be validated and no new differentially expressed genes were found through microarray experiment.

4.3.1 Comparison through cDNA macroarray

4.3.1.1 Contents and classification of Subtractive Hybridization (SH) cDNA libraries

Among the 462 gene sequences (187 in leaf libraries and 275 in tuber libraries), almost 50% of the genes represented unclassified proteins or clones for which no similarity to any database sequence could be found. The second biggest class was cellular metabolism. There were 24.1% (average of two leaf libraries with 25.5% and 22.6%, respectively) and 30.9 (average of two tuber libraries with 23.9% and 37.9%, respectively. There are many reasons that can lead to variation in expression of genes involved in basic cellular metabolism other than the insertion of transgene, including temporal and spatial factors like different growth stages and different parts of the plant. Furthermore, no homologies were found direct or indirect with genes related to carotenoids pathway. The missing of *zep* gene in cDNA libraries could due to the randomly picking up method. However, composition of these four cDNA libraries indicates to some degree that the inserted gene in this case did not cause significant changes in global expression level in GE plants.

4.3.1.2 Moderately differentially expressed genes found in leaves of GE plants

In leaves of SR47, altogether six genes were found to be differentially expressed at different growth stages (two at each stage of PGS 30, PGS 60 and PGS 90), while one gene was down-regulated with 2.3 fold, other genes were up-regulated with fold changes of 2.1-2.3. Only two differentially expressed genes were found in leaves of SR48, one at PGS60, the other at PGS90 with expression fold changes of -2.2 and 2.0, respectively. The expression fold changes were relatively low throughout all growth stage repetitions.

As the whole plant is an elastic and dynamic system, the effect of such changes with very limited number of genes may not lead to any essential harmful effects in leaves. In the previous study on the two GE potato clones also used in this work, Gerjets and Sandmann's reported (2006) that there was no significant change of chlorophyll and carotenoids in leaves of these two GE potato clones were found and their photosynthetic efficiency was unchanged compared with their conventional counterpart cultivar Baltica.

Gene Ontology information showed that three of the six differentially expressed genes have functions in RNA-, ATP-binding and redox reactions. These genes could also be classified as stress-related genes and may regulate the activity of stress signaling components (Xiong and Zhu, 2001; Xiong *et al.*, 2002). Thus, environmental factors can also cause expression changes of certain genes.

Furthermore, the limited moderately differentially expressed genes found in leaves of GE potato plants could also due to somaclonal variation. Tissue culture was used to regeneration of GE plants and short-term testing of insert construct. Somaclonal variation can potentially occur when plants are being regenerated from selected, successfully genetically transformed cells (Mori *et al.*, 2007).

4.3.1.3 Moderately differentially expressed genes found in tubers of GE plants

Six differentially expressed genes were found in tubers of SR47, five occurred in developing tuber stage. Nine differentially expressed genes were found in tubers of SR48 solely at developing stage. Compared to leaves, more differentially expressed genes were found in tubers and especially in developing tubers and all differentially expressed genes were down-regulated with fold changes between 2.0-2.3.

A noticeable phenomenon is that two same genes were found to be down-regulated in both GE tubers. One differentially expressed gene is homologous to annexin p34-like protein (-2.2 fold in SR47 and -2.5 in SR48) and the other is of unknown function (-2.1 fold in SR47 and -2.0 in SR48). Annexins belong to a ubiquitous multigenic family of multifunctional, Ca^{2+}- and membrane-binding proteins. They are present in both animal and plant cells where over 200 species- and tissue-specific isoforms have been identified

(Calvert *et al.*, 1996; Morgan and Fernandez, 1997). Annexins are thought to participate in the maintenance of Ca^{2+} homeostasis, in signal transduction, in cell proliferation and growth regulation; function as both substrates and modulators of various protein kinases and regulators of synthesis of lipid second messengers (Bandorowicz-Pikula *et al.*, 2001). Whether the appearance of these two differentially expressed genes in both GE plants due to coincidence of randomly clone picking when constructing cDNA libraries, or to a certain common regulating mechanism triggered by inserted gene still need further investigation.

4.3.1.4 Limitations of macroarray technology

The major limitation of macroarray technology is low probe density, and to some extent lower accuracy because of manual-printed filters used for hybridization. Macroarrays consist of DNA probes which are immobilized onto nylon membranes. In this work, DNA probes were genes taken from SH-based cDNA libraries. The size of the membranes is normally between 8 × 12 cm and 22 × 22 cm with spots of approximately 1 mm diameter, thus the density is quite limited. Generally, macroarrays are low in costs than microarrays. However, genes spotted onto macroarrays need to be selected carefully because of the density limitation. Thus the costs of expression study by using macroarray will be highly increased when combined with other methods for selecting target genes, such as subtractive hybridization.

Microarrays allow for monitoring the expression of thousands of genes simultaneously since they have a much higher probe density, typically 20-30,000 probes per slide. The Institute for Genomic Research (TIGR, USA) provides several versions of potato cDNA array with approximately 10,000 elements (11,412 verified cDNAs). Furthermore, the hybridization protocol has been optimized for achieving very reproducible images with low background and high signal to noise ratio. Thus, TIGR potato microarray was applied in this work to validate the results from macroarray.

4.3.2 Comparison through cDNA microarray

4.3.2.1 Reliability of microarray analysis

The microarray experiment, including experimental design, execution and analysis accomplished in this work was in agreement with "Minimum information about a microarray experiment (MIAME)" (Brazma *et al.*, 2001). We were highly aware of the importance of data handling. In this study, we have implemented tools from the Bioconductor package (www.bioconductor.org), which is a freely available package with a bundle of up-to-date tools for analysis of DNA microarray data. Currently Bioconductor toolkit is considered as one of the most sophisticated software for microarray data analysis, which is based on the R statistical programming language. The statistical analysis methods (B-test) we used allow for a comprehensive analysis of the data instead of only using simple fold changes in gene expression (Quackenbush, 2002; Smyth *et al.*, 2004; Draghici *et al.*, 2006). So the results obtained in this study that there were no obvious un-intended transcriptional perturbations in GE plants are based on solid analysis.

Generally, differentially expressed genes of interest selected from microarray studies supposed to be verified by using a higher sensitive technique, mostly a qRT-PCR (Yang and Speed, 2002). Although qRT-PCR is considered as a "golden standard" or a necessary validation, it has also been stated that qRT-PCR results are not always better or validation is not always necessary (Gaj *et al.*, 2008). On practical level, qRT-PCR can be labor intensive, time consuming, and expensive, with typically only a small fraction of the results being validated. Furthermore, there are no generally accepted guidelines or recommendations concerning the definition of qRT-PCR validation for microarray analysis (Smith, 2007). Besides, it's not feasible to validate all results of a microarray experiment, selection of which genes should be validated raises bias. Since genes selection is normally non-randomly, qRT-PCR results from certain gene or groups of genes can not stand for the un-validated remaining genes (Miron *et al.*, 2006).

The reasons that no qRT-PCR validation was applied to microarray analysis in this work are (1) the whole procedure, from experiment design till data analysis, was applied in a very accurate manner; (2) macroarray analysis showed similar results of only very limited differentially expressed genes found and (3) no differentially expressed genes or any interest genes related with carotenoid synthesis pathway were found.

4.3.2.2 Results from microarray analysis

No significantly differentially expressed genes were found in the two GE potato tubers compared to their conventional counterpart plant through microarray expression analysis. However, the biological meaning of the majority genes in microarray analysis with minor changes stays unknown. In certain state, small changes in expression level might be critical (Smith, 2007). Comparison of transcriptional and physiological data can provide a better understanding of the functional implication of these genes as well as associated mechanisms of regulation (Auman *et al.*, 2007). More studies focus on linking changes of gene expression and biological functions are needed in order to make further biological interpretation of these results. Based on the results from this study, we suggest that the microarray approach has the potential to become a useful tool for screening of unintended effects caused by inserted gene in transcriptional level of GE plants.

4.3.2.3 Limitations and perspectives of microarray technology

However, there are several limitations of this powerful technology. For the technology itself, one limitation is a large proportion of the transcriptome is beyond the reach of current technology. For example, microarrays are adequate for detecting the direction of change in expression of genes expressed above a certain threshold (Draghici *et al.*, 2006), but how about the genes flagged as unreliable data and filtered because of their weak signal? Although multiple statistical tests were applied in order to take the higher variability of gene expression measurements of low abundant genes into account, it is still a lot of work to do to avoid missing important information.

Another limitation of microarray technology is that it can only provide the indirectly information with regard to the cellular complement of primary and secondary metabolites. Therefore, it is necessary to adapt proteome and metabolome analyses as complements (Fiehn *et al.*, 2002). Gene expression profiling combined with protein and metabolite profiling will provide comprehensive information and could be very useful instruments in safety assessment of GE plants (Cellini *et al.*, 2004).

For microarray data analysis, the center of the problem is how to identify differentially expressed genes. In the beginning of the microarray era arbitrarily fold-change cut-offs were used to identify differentially expressed genes. For example, a two-fold up- or down-regulation might have been considered as differentially expressed genes. Nowadays, however, it has to be identified using statistical tools, such as the t-test, SAM or B-test and the results (Wirta, 2005). However, the cut-off of these values is still artificial. Taking the cut-off value of fold change as an example, most researchers chose fold change cut-value greater than two, mainly because it is widely used in microarray literatures. Others may set different fold change cut off (more or less than two fold) according to their own biological understanding of gene expressions. Some technologies could help in setting sensible criteria to identify differentially expressed genes, such as (1) choosing a threshold for differential expression if there were known differentially expressed or non-differentially expressed genes; (2) printing artificial genes on microarray, then spike corresponding RNA into target RNA before labeling and hybridization; (3) careful and thorough graphical exploration and (4) the choice of ranking statistic methods (Thorne, 2007).

However, microarray technology and other related technologies are constantly improving with high speed. Microarrays with full coverage of transcriptome of crop plants emerge continuously with sophisticated statistical tools, thus it can be adapted to the analysis of the very complex data arising from the profiling techniques.

4.3.3 Comparison between cDNA macro- and microarray

Macroarray analysis applied in this work contains 275 cDNA fragments derived from SH cDNA libraries of tuber materials, while TIGR potato microarray used in this work contains 11,412 verified fragments derived from potato stolons, roots, microtubers, dormant tubers, germinating eyes, and healthy and *Phytophthora infestans*-challenged libraries. Microarray has a better coverage of information. Although few genes (eight in leaves and fifteen in tubers of GE plants) were found to be significantly differentially expressed, the expression fold changes of those genes were with very limited scale around 2.3. Since cut-off value in selecting differentially expressed genes varied in different research background (Gaj *et al.*, 2008), it is very difficult to set a clear and biological meaningful cut-off value especially when most of the genes were with unchanged or minor changes of expression levels.

Based on the results from microarray analysis, no significantly differentially expressed genes were found in tubers of GE plants. This conclusion might seem to be inconsistent with the results from macroarray analysis, however, determination of a cut-off value is generally empirical (Kim *et al.*, 2002). The significantly differentially expressed genes through macroarray analysis with expression fold changes of two might not be chosen as differentially expressed genes when fold change cut-off value was set to three. As stated above, a meaningful cut-off value and biological interpretation can only be set to transcriptional analysis when more metabolic and physiological data are available (Auman *et al.*, 2007). According to the current available information and understanding, the results from macro- and microarray analysis in this work showed no significantly differentially expressed genes of interest were found in GE plants.

4.4 Conclusion and outlook

Compared to the long history of conventional breeding, genetic engineering technology has a short history of no more than 40 years. However, the undeniable fact is, despite the short history of this new technology and the growing concern of its safety issue, it provides immense opportunities to improve existing crops regarding resistance to pests and diseases and specific quality characteristics (Conner, 2006). GE crops have also the potential to be an inexpensive source of specialized products, and to be a part of the solution for feeding the increasing world population (Wenzel, 2006).

Although GE methods are indeed more reliable and efficient (Batista *et al.*, 2008), it is necessary to evaluate GE crops in a very careful and comprehensive manner under agricultural field conditions. The main part of this work was to investigate agronomic performance and gene expression profiles of GE potato plants compared to their conventional counterpart plant under field conditions for three successive years. The agronomic data collected from field conditions provides fundamental information for future possible application of these two GE potatoes. Gene expression analysis confirmed the tissue-specific expression of inserted gene in tuber tissue under field conditions. Application of macro- and microarray technologies in this work in revealing any unintended changes in transcriptome provided a comprehensive survey of tens of thousands of genes. Although this work did not cover all aspects of biosafey assessment of GE crops and thus no comprehensive conclusion could be drawn, the available results from this work provide a base for further analysis and evaluation of the two GE zeaxanthin-rich potatoes, i.e. combining metabolites profiling data with transcript profiling.

More and more scientific studies convincingly address and clarify the issue of safety of GE crops and foods. Based on scientific data, no unequivocal evidence demonstrates adverse effects of any of the currently commercialized GE food products (Batista and Oliveira, 2009). Microarray technology, together with proteomics and metabolomics, are providing solid confirmation that GE rice (Batista *et al.*, 2008), wheat

(Baudo *et al.*, 2006), soybean (Cheng *et al.*, 2008) and thale cress (El Ouakfaoui and Miki, 2005) are substantially equivalent to their non-GE counterparts. Radiation and chemical treatment used to create mutations in many existing crops also lead to many more gene activity perturbation than transgene insertion (Zhang *et al.*, 2006; Dubouzet *et al.*, 2007; Batista *et al.*, 2008).

As a matter of fact, the advantage of genetic engineering does not suppress the use of conventional breeding approaches. Conventional breeding is better suited for improving many traits simultaneously, or improving traits controlled by many genes, or traits for which the controlling gene has not been identified. It is also relatively inexpensive, technically simple, and free of government regulation (Manshardt, 2004). So, conventional breeding and genetic engineering are different but complementary ways of improving crops. Although in the current situation, genetically engineered products need more careful and comprehensive assessment than conventionally derived products, the aim is to exclude any potential unintended side effects. We have the reason to believe that along the fast development of detection technologies and genetic knowledge, genetic engineering will continue providing us more economic, nutritional and environmental friendly crops.

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Appendix A: Chemicals

Name of Chemical	Grade	Supplier
Alpha[^{32}P]-dCTP, 3000 Ci/mmol		Hartmann Analytic, Braunschweig
Agarose		Serva, Heidelberg
Chloroform	p.a.	Sigma-Aldrich, Taufkirchen
Dimethyl sulfoxide (DMSO)	p.a.	Sigma-Aldrich, Taufkirchen
DTT(Dithiothreitol)		Roth, Karlsruhe
DEPC(Diethyl Pyrocarbonate)	p.a.	Roth, Karlsruhe
EDTA (Ethylendiamintetraacetic acid)		Roth, Karlsruhe
Ethanol	p.a.	Merck, Darmstadt
Ethidium bromide	reinst	Sigma-Aldrich, Taufkirchen
Formaldehyde	p.a.	Merck, Darmstadt
Formamide	p.a.	Fluka, Taufkirchen
Glycerol	99.5%	Roth, Karlsruhe
HCl concentrated.	p.a.	Roth, Karlsruhe
IPTG		Fluka, Taufkirchen
Isopropanol (2-Propanol)	p.a.	Sigma-Aldrich, Taufkirchen
LiCl	p.a.	Roth, Karlsruhe
$MgCl_2$		Roth, Karlsruhe
$MgSO_4$		Sigma-Aldrich, Taufkirchen
3-(N-morpholino)-2-propansulfon acid (MOPS)		Roth, Karlsruhe
NaCl	p.a.	Roth, Karlsruhe
NaOH		Roth, Karlsruhe
Sodium acetate		Merck, Darmstadt
Sodium citrate		Roth, Karlsruhe
Peptone		Difco, Detroit, USA
PVP (Polyvinylpyrrolidon)		Sigma-Aldrich, Taufkirchen
SDS (Sodium lauryl sulphate)	crist.	Roth, Karlsruhe
Sephadex G-50 Superfine		Amersham Pharmacia, Wien
Tris (Tris-(hydroxymethyl)-aminomethane)		Roth, Karlsruhe
TRIzol		
ß-Mercaptoethanol	p.a.	Roth, Karlsruhe

Appendix B: Kits, Enzymes and Oligonucleotides

Kits	
Name	Supplier
Big Dye Terminators Version 1.1	Applied Biosystems, Lincoln
M-MLV Reverse Transcriptase (H-)	Invitrogen, Karlsruhe
QIAquick® Gel Extraction Kit	Qiagen, Hilden
QIAquick® PCR Purification Kit	Qiagen, Hilden
RNeasy mini prep	Qiagen, Hilden
SYBR green	Qiagen, Hilden
SuperScript First-Strand Synthesis System	Invitrogen (Karlsruhe)
Pronto!^{TM} Microarray Validation Kit	Corning, NY, USA

Enzymes		
Enzyme	units/µl	Supplier
KpnI	15	Amersham Pharmacia Biotech, Vienna
PstI	15	Amersham Pharmacia Biotech, Vienna
XbaI	15	Amersham Pharmacia Biotech, Vienna
Taq DNA Polymerase	5	Qiagen, Hilden

Oligonucleotides			
Oligonucleotides for standard-PCR			
Name	Sequence		
5' pBL2SK	5'GAC TGG AAA GCG GGC AGT GAG3'		
3' pBL2SK	5'TGC TGC AAG GCG ATT AAG TTG3'		
5' pBSKoL	5'ATG CTT CCG GCT CGT ATG TT3'		
5' pBSKoR	5'CAG GGT TTT CCC AGT CAC GA3'		
Oligonucleotides for sequencing			
M13 reverse	5'CAG GAA ACA GCT ATG AC3'		
M13(-20)forward	5'GTA AAA CGA CGG CCG A3'		
T7	5'GTA ATA CGA CTC ACT ATA GGG C3'		
Oligonucleotides for reverse transcription			
Oligo(dT)$_{12-18}$	TTTTTTTTTTTT(TTTTTT)		
Oligonucleotides for RT-PCR		Tm	Products
ZEP	5'AAG TGC CGA GTC AGG AAG CC3'	55°C	150bp
ZEP	5'AGT CCG ACG CCA AGA TAA GC3'		
VDE	5'ATT TCC CCT TCA CTC AAC TCT3'	58°C	145bp
VDE	5'ATG ACC ACC AAC TCC AAC CTC3'		
EF	5'ATT GGA AAC GGA TAT GCT CCA3'	55°C	101bp
EF	5'TCC TTA CCT GAA CGC CTG TCA3'		

Appendix C: Media and Buffers

Name	Components and concentration
LB_{Amp}-medium	10 g/l peptone, 5 g/l yeast extract, 10 g/l NaCl, 100 mg/l Ampicillin, pH7.0
SOC-Medium	20 g/l peptone, 5 g/l yeast extract, 0.6 g/l NaCl, 0.2 g/l KCl, 1.0 g/l $MgCl_2$, 3.5 g/l D-glucose
50x TAE	2 M Tris base, 2M glacial acetic acid, 50mM EDTA, pH7.0
50x TBE	2 M Tris, 2M Boric Acid, 50mM EDTA, pH7.0
5x electrophoresis buffer	0.2 M MOPS, 50 mM NaAc, 5 mM EDTA, add DEPC-treated water
TE	10mM Tris-HCl(pH 7.5), 1mM EDTA (pH 7.5)
SSTE	1M NaCl, 5 g/l SDS, 10mM Tris-HCl (pH 8.0), 1mM EDTA (pH 8.0)
RNA Extraction Buffer	20 g/l CTAB, 20 g/l PVP 40, 100mM Tris-HCl(pH 8.0), 25mM EDTA, 2M NaCl
20x MOPS	400mM MOPS, 100mM sodium acetate, 20mM EDTA, pH7.0
Strip-Buffer*	200mM NaOH, 2 g/l SDS
Church Buffer	250mM sodium phosphate(pH 7.2), 70 µg/ml SDS, 1 mM EDTA, 10 µg/ml tRNA, 1 µg/ml oligo d(A)40-60
20x SSC	3M NaCl, 300mM sodium citrate
Wash-buffer 1*	2x SCC, 1 g/l SDS
Wash-buffer 2*	40mM $NaPO_4$, 1 g/l SDS

* Buffer not requiring autoclaving.

Appendix D: Planting Plan

Row No.	Block 1	Plot. No.	Block 2	Plot. No.	Block 3	Plot. No.
1	Baltica		Ditta		Sibu	
2	Baltica	1	Ditta	15	Sibu	29
3	Baltica		Ditta		Sibu	
4	Baltica		Ditta		Sibu	
5	SR 47/00 #18		Desirée		Baltica	
6	SR 47/00 #18	2	Desirée	16	Baltica	30
7	SR 47/00 #18		Desirée		Baltica	
8	SR 47/00 #18		Desirée		Baltica	
9	Ditta		Sibu		Selma	
10	Ditta	3	Sibu	17	Selma	31
11	Ditta		Sibu		Selma	
12	Ditta		Sibu		Selma	
13	SR 48/00 #17		Baltica		Ditta	
14	SR 48/00 #17	4	Baltica	18	Ditta	32
15	SR 48/00 #17		Baltica		Ditta	
16	SR 48/00 #17		Baltica		Ditta	
17	Sibu		SR 47/00 #18		SR 48/00 #17	
18	Sibu	5	SR 47/00 #18	19	SR 48/00 #17	33
19	Sibu		SR 47/00 #18		SR 48/00 #17	
20	Sibu		SR 47/00 #18		SR 48/00 #17	
21	Selma		SR 48/00 #17		Desirée	
22	Selma	6	SR 48/00 #17	20	Desirée	34
23	Selma		SR 48/00 #17		Desirée	
24	Selma		SR 48/00 #17		Desirée	
25	Desirée		Selma		SR 47/00 #18	
26	Desirée	7	Selma	21	SR 47/00 #18	35
27	Desirée		Selma		SR 47/00 #18	
28	Desirée		Selma		SR 47/00 #18	
29	Sibu		SR 48/00 #17		Desirée	
30	Sibu	8	SR 48/00 #17	22	Desirée	36
31	Sibu		SR 48/00 #17		Desirée	
32	Sibu		SR 48/00 #17		Desirée	
33	Selma		SR 47/00 #18		Sibu	
34	Selma	9	SR 47/00 #18	23	Sibu	37
35	Selma		SR 47/00 #18		Sibu	
36	Selma		SR 47/00 #18		Sibu	
37	SR 48/00 #17		Selma		SR 47/00 #18	
38	SR 48/00 #17	10	Selma	24	SR 47/00 #18	38
39	SR 48/00 #17		Selma		SR 47/00 #18	
40	SR 48/00 #17		Selma		SR 47/00 #18	
41	Desirée		Baltica		Baltica	
42	Desirée	11	Baltica	25	Baltica	39
43	Desirée		Baltica		Baltica	
44	Desirée		Baltica		Baltica	
45	Baltica		Sibu		Ditta	
46	Baltica	12	Sibu	26	Ditta	40
47	Baltica		Sibu		Ditta	
48	Baltica		Sibu		Ditta	
49	SR 47/00 #18		Ditta		SR 48/00 #17	
50	SR 47/00 #18	13	Ditta	27	SR 48/00 #17	41
51	SR 47/00 #18		Ditta		SR 48/00 #17	
52	SR 47/00 #18		Ditta		SR 48/00 #17	
53	Ditta		Desirée		Selma	
54	Ditta	14	Desirée	28	Selma	42
55	Ditta		Desirée		Selma	
56	Ditta		Desirée		Selma	
	9 m	3 m	9 m	3 m	9 m	3 m