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Expression Analysis of Zeaxanthin Epoxidase of Genetically Engineered Zeaxanthin-rich Potatoes in Comparison to Conventional Cultivars under Field Conditions

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ABSTRACT

Two genetically engineered (GE) zeaxanthin-rich potato (*Solanum tuberosum* L.) clones, derived from potato cultivar 'Baltica' were evaluated under open-field conditions with respect to agronomic performance, stability and tuber-specific expression of the inserted zeaxanthin epoxidase (*zep*) gene. Data collected from two field sites totalling four environments in Germany demonstrated that general morphology and tuber yield of GE potato clones were not impaired by the metabolic changes in tuber tissue. Quantitative real-time PCR analysis of *zep* gene expression in leaves, roots and tubers collected at three different developmental stages from the two GE potato clones and the conventional counterpart clone 'Baltica' showed that the transgene maintained its ability to induce the accumulation of zeaxanthin in tubers, while no significant *zep* expression changes were found in leaves and roots. The results clearly demonstrated that the tuber-specific promoter led to a strict tissue-specific expression of the inserted gene in the two GE potato clones in each of the four environments. Additionally, HPLC measurement showed that the tubers from two GE clones contained 19.5 to 58.7 µg/g dw of zeaxanthin, while the zeaxanthin content in the tubers of 'Baltica' was under detection level. HPLC results together with qRT-PCR results confirmed the inverse relationship between *zep* expression level and the accumulation of zeaxanthin in GE tubers. Furthermore, *zep* expression analysis of four other conventional cultivars showed that gene expression differed in a similar or even greater range among the four conventional cultivars investigated than the variation between GE clones and 'Baltica'.

Keywords: transgenic potato, field trial, quantitative real-time PCR analysis

INTRODUCTION

Zeaxanthin and its isomer lutein are important macular pigments present in human retina (Carpentier *et al.* 2009). These yellow pigments are postulated to participate in photo protection, and the diminished macular pigment might be related to retinal damage (Khachik *et al.* 2006; Whitehead *et al.* 2006). They occur in nature in many vegetables, fruits and flowers, while the content of lutein are 17-63 times higher than zeaxanthin in common consumed vegetables such as kale, parsley and spinach (Humphries and Khachik 2003). Although no dietary intake reference has yet been set for lutein and zeaxanthin, some epidemiological studies have indicated that intake of approximately 6 mg/day seems desirable for healthy people (Seddon *et al.* 1994). However, the amount of lutein/zeaxanthin, especially zeaxanthin, from average daily fruit/vegetable consumption could not meet the recommended level to prevent against AMD. Therefore, attempts were made to increase amounts of zeaxanthin/lutein in a staple crop like for example potato (Römer *et al.* 2002). The potato cultivar 'Baltica' was genetically engineered for increased zeaxanthin content by applying two strategies that both rely on tuber-specific down-regulation of the *zep* gene, either through co-suppression or antisense suppression. cDNA fragments encoding the potato zeaxanthin epoxidase (ZEP) were cloned into the pBin19-related transformation vector pPGB121S in the sense or antisense orientation, respectively. 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. SR47/00#18 (transformed with sense-oriented *zep* gene) and SR48/00#17 (transformed with antisense-oriented *zep* gene) were selected due to their unaltered phenotype, relative high contents of zeaxanthin and total carotenoids. On average, the contents of zeaxanthin and total carotenoids in SR47/00#18 were 40.1 and 60.8 µg/g DW, and 16.5 and 44.2 µg/g DW in SR48/00#17 (Römer *et al.* 2002). Bioavailability studies in human of zeaxanthin from the two zeaxanthin-rich GE potatoes showed that the consumption of the GE potatoes significantly increases chylomicron zeaxanthin concentrations. This result suggests that zeaxanthin-rich GE potatoes potentially could be used as an important dietary source of zeaxanthin (Bub *et al.* 2008).

Although the results are promising for filling up the dietary gap of zeaxanthin by consuming zeaxanthin-rich GE potatoes, further experiment needs to be carried out to confirm the tuber-specific expression of inserted gene under field conditions besides the tests made under greenhouse conditions (Römer *et al.* 2002). ZEP catalyzes the intercomversions between the carotenoids violaxanthin, antheraxanthin, and zeaxanthin in higher plants under stress conditions to form zeaxanthin that protects the photosynthetic apparatus against the effect of excessive light (Havaux and Kloppstech 2001). It also functions in the first step of the biosynthesis of the abiotic stress hormone abscisic acid (ABA) in

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higher plants (Xiong and Zhu 2003). Therefore, it is of great interest to assess the performance of the GE plants with *zep* gene down-regulation under field conditions.

Additionally, it is possible that the GBSS promoter has a slight non-tuber-specific expression which could lead to a reduction in *zep* expression in tissues other than tuber in GE potato plants (Visser *et al.* 1991). Thus, it is also important to evaluate whether the GBSS promoter leads to a strict tuber-specific expression in the two GE potato plants under field conditions or not.

Parallel projects were carried out under the same field conditions with emphasis on rhizosphere microbial communities (Weinert *et al.* 2009) and abundance and diversity of rhizobacteria with *in vitro* antagonistic activity (Weinert *et al.* 2010). Both studies drew the conclusions that environment and cultivar were more important factors than genetic modification affecting the microbial community of soil. Thus, differences observed between GE clones and the conventional counterpart clone 'Baltica' did not exceed natural cultivar-dependent variability.

The main aims of this work were to (1) evaluate the general morphology and performance of the two transgenic zeaxanthin-rich potato clones in comparison to their non-transgenic counterpart cultivar 'Baltica' and four other conventional cultivars under field conditions; (2) verify the stable and tuber-specific expression of the inserted *zep* gene under field conditions by using qRT-PCR; and (3) compare the differences in *zep* expression between GE and non-GE plants with the natural variation in conventional cultivars under the same agricultural field conditions. The latter objective was to provide also a more biologically meaningful evaluation method of GE plants as suggested by Cheng *et al.* (2008).

MATERIALS AND METHODS

Chemicals and reagents

All chemicals used in this study were purchased from Sigma-Aldrich Chemicals (St. Louis, MO, USA), unless specified otherwise. Chemicals and reagents used in RNA isolation were analytical grade. Reference samples (zeaxanthin and lutein), internal standard (β-apo-8'-carotenal) and solvents (methanol and acetone) used in HPLC detection were HPLC grade.

Plant materials

Two transgenic potato clones SR47/00#18 (SR47) and SR48/00#17 (SR48) (SaKa-Ragis Pflanzenzucht GbR, Windeby, Germany) were used in this study and will be referred throughout this work as SR47 and SR48, respectively. Both transgenic potato clones were derived from the potato cultivar 'Baltica', which was genetically engineered for *zep* by co-suppression (SR47) and antisense (SR48) technologies (Römer *et al.* 2002; Sandman *et al.* 2002; Lübeck *et al.* 2006). Conventional cultivars '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 investigating natural trait variation among different cultivars.

Field experimental design

Field trials were conducted at two locations in Germany over three years: Roggenstein (RGS) in 2005, 2007 and Oberviehhausen (OVH) in 2006, 2007. Both field sites were located in Bavaria (Germany) and differed considerably in their soil characteristics. The Roggenstein soil was characterized by 26.1% sand, 44.0% silt, 28.1% clay, organic carbon (C_{org}): 1.1%, total nitrogen (N_t): 0.1% and pH 6.6. The Oberviehhausen soil contained 54.6% sand, 31.3% silt, 14.1% clay, C_{org}: 1.9%, N_t: 0.2%, and had a pH of 6.5.

Growing season was from April to September of each year. 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 m × 3 m and consisted of four rows with thirty plants each. The distance between two rows was 0.75 m and between plants in the same row 0.3 m. All experiments were conducted following standard agronomical procedures. Samples were taken from leaves, roots and tubers at principal growth stage (PGS) 30, 60 and 90 in 2005 and 2007 (RGS), and 2006 and 2007 (OVH) according to BBCH-scale of the phenological development stages identification system (Meier 2001). Five plants chosen randomly from each plot were harvested and subsequently pooled for gene expression study. Potato tubers were harvested with a mechanical lifter. Tubers were handpicked and weighed for yield determination.

RNA isolation

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 pine tree RNA isolation method, originally described by Chang *et al.* (1993). The extraction buffer (20 g/l CTAB, 20 g/l PVP 40, 100 mM Tris-HCl pH 8.0, 25 mM 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 β-mercaptoethanol (AppliChem GmbH, Darmstadt, Germany) and 7.2 µl spermidine, 2-3 g ground 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, NY, 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 saline sodium dodecyl sulfate Tris-EDTA buffer (1 M NaCl, 0.5% SDS, 10 mM Tris pH 8.0, 1 mM 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 diethylpyrocarbonate treated water. Total RNA was quantified by measuring absorbance at 260 nm using spectrophotometer (Genesys 10 Bio, Thermo Electron Corporation, Waltham, MA, USA) and stored at -80°C for later use.

PCR analysis

When the two GE potato clones were propagated in the greenhouse for field experiments, PCR analysis was performed with primers to amplify the plant selectable *npt* II marker gene to confirm transgenesis of the plants. Total genomic DNA was isolated from potato tubers using CTAB method (Saghai-Maroof *et al*. 1984). Fifty ng DNA were used in the PCR reaction with *npt* II specific primers (forward: 5′ CGC TAT CAG GAC ATA GCG TTG 3′; reverse: 5′ GAC CGG CAA CAG GAT TCA ATC 3′; annealing temperature 45°C), which were designed using Primer Premier software 5.0 (Premier Biosoft International, Palo Alto, CA, USA). The PCR samples were run through 30 cycles by using the following temperature regimes: 94°C denaturation for 30 sec, 45°C annealing for 30 sec, 72°C elongation for 1 min and cycles were followed by a final extension of 2 min at 72°C.

Northern blot analysis

Expression studies of *zep* were performed using total RNA isolated from tubers at PGS 60 of the two GE potatoes and 'Baltica'. 30 µg total RNA from tubers were separated by loading on denaturing formaldehyde gel. The separated RNA samples were then blotted on positively charged nylon membrane (Pall Biodyne Plus, Pall Corp., Port Washington, NY, USA) and hybridized with a digoxigenin-labeled *zep* gene probe at 42°C according to Römer *et al*. (2002).

Quantitative real-time PCR analysis

Absolute and relative quantification of *zep* were determined by qRT-PCR analysis, utilizing the method described by Ros *et al.* (2004). A *zep* complementary RNA (cRNA) fragment used as a standard in *zep* quantification was prepared. In brief, a 652-bp DNA fragment encoding part of ZEP was amplified by PCR using zep1 primers (forward: 5′ CCA AGT CCG ACG CCA AGA TAA 3′; reverse: 5′ TTG GTG CTG ATG GCA TAA GGT CT 3′; annealing temperature: 62.5°C; designed using Primer Premier 5). After cloning and transforming the 652-bp DNA fragment, linear plasmid DNA which contains target DNA fragment was transcribed by using zep2 primers (forward: 5′ AAG TGC CGA GTC AGG AAG CC 3′; reverse: 5′ AGT CCG ACG CCA AGA TAA GC 3′; annealing temperature: 55°C; designed using Primer Premier 5.0). 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 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) in 96-well optical reaction plates with fitted optical caps (Applied Biosystems, Foster City, CA, 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) 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 the sequences 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. Abundance of *zep* mRNA is shown as ng *zep* cRNA/250 ng total RNA. 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: forward: 5′ ATT GGA AAC GGA TAT GCT CCA 3′; reverse: 5′ TCC TTA CCT GAA CGC CTG TCA 3′. Relative expression levels of *zep* were calculated first relative to 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.

Zeaxanthin and lutein measurements

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 × 46 mm, 5 µm) (Phenomenex, Torrance, CA, USA) and an UV-Vis Detector (450 nm) (Hitachi). Obtained peaks were quantified with HSM7000 software delivered with Merck Hitachi-HPLC system L 6200 (Hitachi). 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 (β-apo-8'-carotenal).

RESULTS

Field trials were conducted in RGS (2005 and 2007) and OVH (2006 and 2007) with the two GE zeaxanthin-rich potatoes and their conventional counterpart cultivar 'Baltica' to evaluate the agronomical performance, stability and tissue-specific expression of *zep* in GE potato plants. Four other conventional cultivars ('Désirée', 'Ditta', 'Selma' and 'Sibu') were also included in all field trials to evaluate natural varietal variation and compare possible trait differences between GE and non-GE plants.

Morphology, yield, zeaxanthin and lutein contents of GE potato clones

The shape and skin characteristics of SR47 and SR48 were not visibly different from that of 'Baltica'. However, flesh colour of SR47 and SR48 tubers was more yellow to orange compared to 'Baltica' and the other conventional potato clones. **Fig. 1** presents shapes and flesh colours of tubers of all seven potato clones.

Tubers were weighed to obtain yield estimates. No statistically significant differences were found in tuber yield among 'Baltica', SR47 and SR48 considering the means across four environments. The yields of 'Ditta' and 'Selma' were significantly lower when compared to 'Baltica' because of cultivar-specific characteristics (**Fig. 2**).

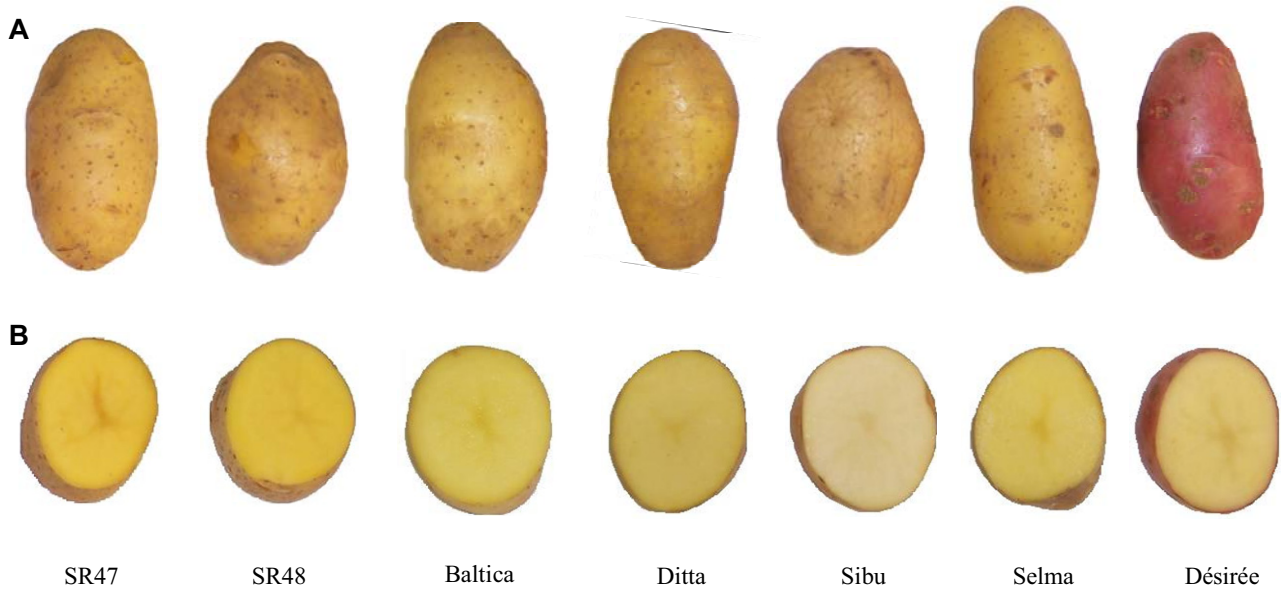

Fig. 1 Phenotype of potato tubers of SR47, SR48, 'Baltica', 'Désirée', 'Ditta', 'Selma' and 'Sibu' used in field trials. Tubers were photographed immediately after harvesting and cutting. (**A**) General shape and skin colour of the tubers. (**B**) Colour of the tuber flesh.

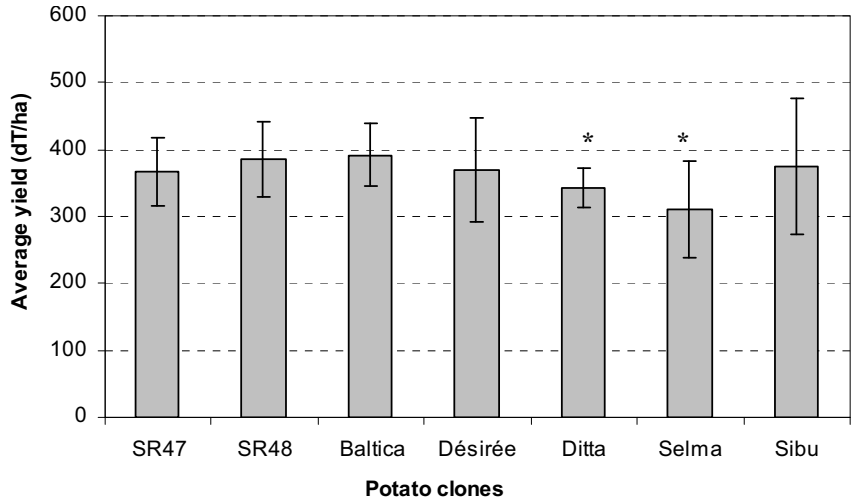

Fig. 2 Average tuber yields in OVH (2006) and RGS (2007). * Significant differences between means of the clones obtained from field trials, based on Student's *t*-test (α= 0.05).

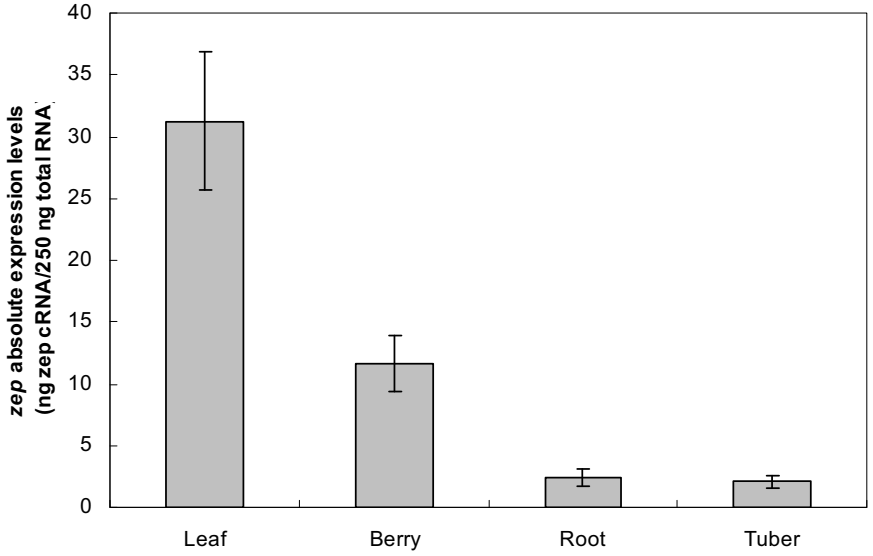

Fig. 3 Expression analysis of *zep* in different tissues of 'Baltica'. Total RNA was extracted from leaves, berries, roots and tubers at PGS 90 over the four environments. Absolute expression levels of *zep* were presented as the quantity of *zep* cRNA in 250 ng total RNA. Berries were available only in RGS 2005 because of local biosafey consideration of gene transfer..

Zeaxanthin and lutein contents of mature tubers of the 7 varieties were estimated through HPLC analysis. **Table 1** summarizes the contents of zeaxanthin and lutein found in different potato cultivars. On the basis of DW, SR47 had 47.6 ± 11.1 µg/g zeaxanthin and SR48 had 28.1 ± 8.6 µg/g. Zeaxanthin could not be detected in the five conventional potato cultivars.

PCR and Northern blot analyses

Amplification of an expected 591-bp product from the *npt* II gene in the two GE potato clones confirmed transgenesis of the plants (data not shown). Furthermore, Northern blot analysis was employed to total RNA from tubers at PGS 60 of the two GE potato plants and 'Baltica' using a *zep* gene probe. It showed weak to negative hybridization signals for samples from the two GE plants compared to 'Baltica' (data not shown), supporting down-regulation through co-suppression (SR47) and antisense (SR48) mechanisms.

Expression of *zep* 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 insight on absolute *zep* mRNA abundance in different tissues of 'Baltica'. The expression of *zep* was detected in all 'Baltica' tissues examined over the four environments (except the berries that were only collected in RGS 2005 because of biosafety considerations), with transcripts being considerably more abundant in leaves and berries than in roots and tubers (**Fig. 3**). Approximately 30 ng of *zep* mRNA per 250 ng of total RNA were detected in leaves, while there were 11.0 ± 3.0 ng in berries and only 2.4 ± 0.7 and 2.1 ± 0.5 ng in roots and tubers, respectively.

Table 1 Lutein and zeaxanthin contents in seven potato cultivars (µg/g dw)[a]

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

[a]Values expressed as means ± standard deviation (n=9).
[b]Not detectable.

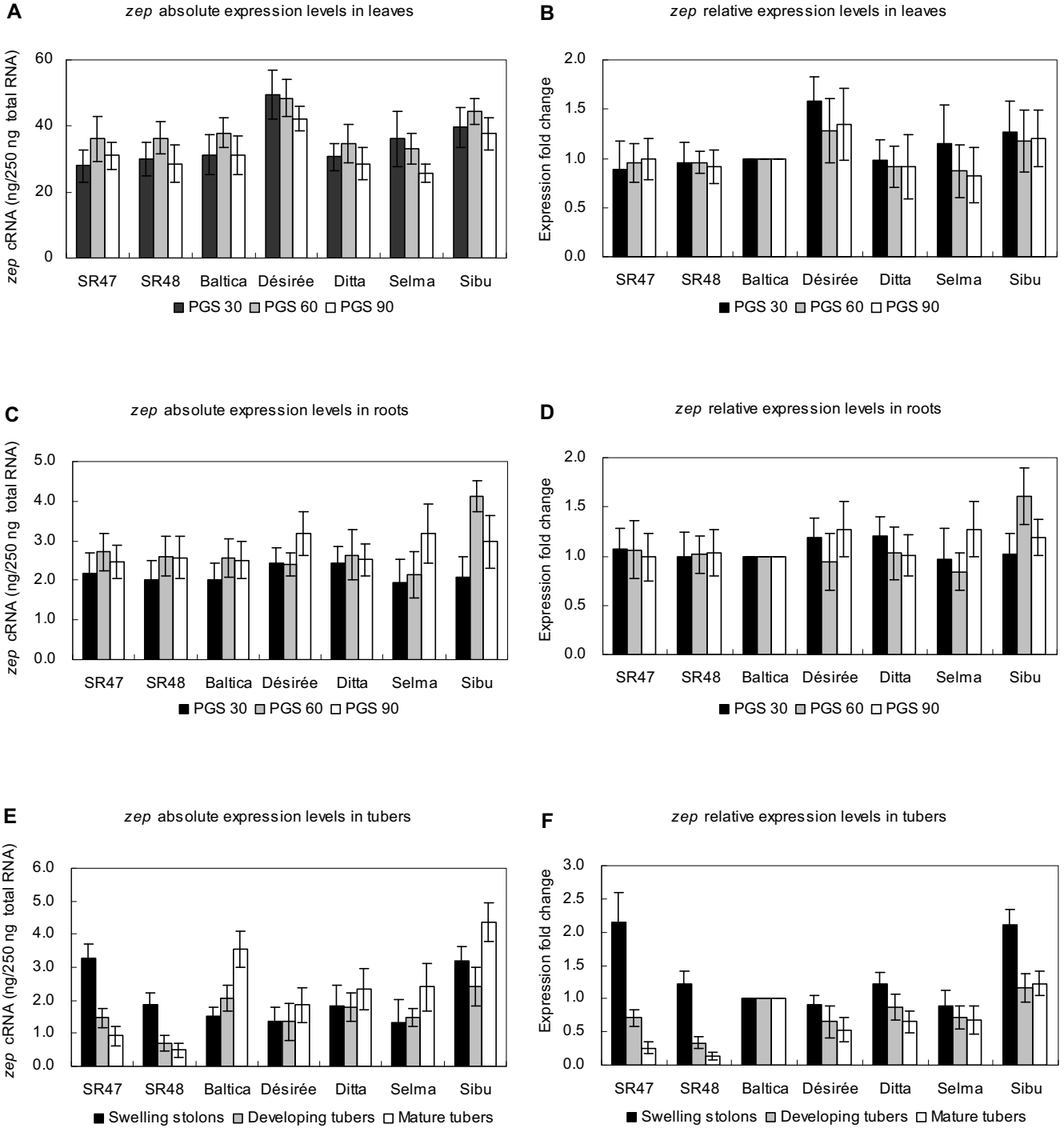

Fig. 4 Absolute and relative expression of *zep* in leaves, roots and tubers of SR47, SR48, 'Baltica', 'Désirée', 'Ditta', 'Selma' and 'Sibu' under field conditions. Samples were taken from RGS (2005 and 2007) and OVH (2006 and 2007). QRT-PCR was performed with total RNA isolated from leaves, roots and tubers. (**A, C, E)** Absolute expression levels of *zep* were calculated according to a standard curve. Abundance of *zep* mRNA is showed as ng *zep* cRNA/250 ng total RNA. (**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 each consisting of three technical replicates and were averaged over the four environments.

Expression of *zep* in leaves and roots

To examine whether the inserted *zep* gene could affect the expression of endogenous *zep* in leaves of transgenic SR47 and SR48 clones, qRT-PCR was performed with total RNA isolated from leaf samples harvested from field trials (RGS in 2005 and 2007 and OVH in 2006 and 2007). Leaf samples were collected at PGS 30, 60 and 90.

Absolute *zep* expression data (**Fig. 4A**) exhibited limited overall changes in leaves of both GE clones and 'Baltica' at different PGS. At both field sites, the highest expression level of *zep* was found at PGS 60, decreasing slightly at PGS 90 for SR47, SR48 and 'Baltica'. No significant expression differences were found in leaves of both transgenic plants as compared to 'Baltica'. Relative expression levels of *zep* (**Fig. 4B**) clearly demonstrated no significant differences of *zep* expression in leaves at different growth stages of both transgenic plants as compared to 'Baltica'.

Absolute expression data exhibited slight differences in *zep* expression level in roots of both transgenic clones and 'Baltica' at different PGS (**Fig. 4C**). Highest amount of *zep* mRNA was identified for PGS 60. Likewise for leaves, relative expression level of *zep* (**Fig. 4D**) clearly indicated no significant differences in roots at different growth stages for both transgenic plants compared to 'Baltica'.

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 (PGS 30), developing (PGS 60) and mature tubers (PGS 90) from all field trials were used to determine absolute and relative *zep* expression levels (**Figs. 4E, 4F**). The absolute and relative expression data of *zep* in tubers exhibited considerable changes of *zep* expression patterns in both transgenic clones compared to 'Baltica'.

In 'Baltica' tubers, the abundance of *zep* mRNA increased during potato growth at both field locations. No significant difference of *zep* mRNA abundance was found between RGS and OVH at all developmental stages (data not shown). The average abundance of *zep* mRNA in developing tubers of 'Baltica' in RGS was 1.3 times higher, and in mature tubers 2.1 times higher than in swelling stolons (**Fig. 4E**).

In tubers of SR47 under 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 average abundance of *zep* mRNA was 1.3 times higher than in 'Baltica' and dropped down dramatically from developing to mature tubers. Noticeably, the mRNA amount of *zep* was around 30% and 75% 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 transgenic clones under field conditions was found in swelling stolons (**Fig. 4F**); 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. Dramatic decrease of *zep* expression of both transgenic clones started from developing tuber stage and reached their lowest level in mature tuber stage: SR47 and SR48 showed only 30% and 20%, respectively, of the *zep* expression level in 'Baltica'.

Comparison of *zep* expression of conventional potato cultivars

In order to obtain a meaningful concept of natural varietal variation of *zep* expression levels, qRT-PCR was applied to different conventional potato cultivars.

Two different *zep* expression patterns were found in leaves (**Figs. 4A, 4B**): 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 *zep* cRNA/250 ng total RNA. However, in 'Selma' and 'Désirée', the highest *zep* expression level appeared at PGS 30 (36.1 and 49.5 ng *zep* cRNA /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' from PGS 60 to PGS 90, respectively.

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% (**Figs. 4C, 4D**). 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. 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 (**Figs. 4E, 4F**).

DISCUSSION

Potato was one of the first crops to be transformed by *Agrobacterium tumefaciens* (Stiekema *et al*. 1988; De Block 1988) and, since then, transgenic potatoes with different characteristics such as potato virus Y (PVY) resistance, high-starch content and vaccine production have been generated in several potato cultivars (Mullins 2006). For transgenic potatoes with novel traits that eventually benefit agriculture and society, an assessment from molecular level to field testing is of critical importance. In this study, general morphology and agronomic performance of the two GE potato clones did not show obvious differences compared to 'Baltica' under field conditions: plant growth, development and tuber yield of the two GE plants were highly consistent with 'Baltica'. In contrast to the unchanged morphological parameters, the flesh colour of the transgenic tubers was dark-yellow to orange, which coincides with the enriched zeaxanthin content in transgenic tubers. Zeaxanthin contents of the field-grown GE clones were in agreement with those reported by Römer *et al.* (2002) and Gerjets and Sandmann (2006). This also showed that the desired trait of high-zeaxanthin content was stably inherited under field conditions.

Most common procedure to confirm transformed plants is based on PCR amplification of the selectable marker gene. To be sure about genetic transmission of the transformed *zep* gene, PCR amplification of *npt* II was performed when tubers were multiplied for the field trials. Northern blot hybridization further supported the gene-down-regulation strategies. However, to get more accurate expression data of *zep* gene in different tissues at different growth stages, qRT-PCR was employed in this study.

The endogenous *zep* mRNA abundance was found to be higher in leaves compared to roots. Similar results were found in *Nicotiana plumbaginifolia* (Audran *et al.* 1998) and *Lycopersicon esculentum* Mill. (Wang *et al.* 2008). This is because zeaxanthin functions in leaves 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. Zeaxanthin accumulation constitutes the major protective process of the photosynthetic apparatus (Demmig-Adams and Adams 1992). Because of its important biological functions, *zep* mRNA abundance was measured at three different growth stages in leaves and roots of GE potato clones to investigate whether the inserted *zep* gene affects endogenous *zep* expression in non-target tissues. Expression data of *zep* collected through this study showed that the endogenous *zep* level in leaves and roots of transgenic plants was unaffected by the inserted *zep* gene under control of the tuber-specific GBSS promoter. Therefore, the unchanged expression level of *zep* in leaves of transgenic potato plants may be an explanation of the similar tuber yield between transgenic potato plants and their conventional counterpart cultivar.

Expression analysis of *zep* in different tissues proved that the introduced *zep* gene with GBSS promoter in sense and antisense orientation in the two GE potato clones only triggered the down-regulation of *zep* transcription in tuber tissue and was stably inherited through the three-year field trial. In 'Baltica', *zep* expression levels were lowest at swelling stolon stage, then increased during tuber development

and reached the highest peak in mature tubers. In contrast, *zep* gene was maximally expressed at swelling stolon stage in the two GE potato clones, and decreased in developing and mature tuber stages up to 80%. The dark-yellow to orange colour of the tuber flesh and the results from HPLC (**Table 1**) confirmed that more zeaxanthin and lutein were accumulated in GE potato tubers due to the down-regulated *zep* gene. The result is in agreement with previous studies on these GE potato clones under greenhouse conditions (Römer *et al.* 2002). Down-regulation of endogenous gene/ gene expression through RNA-mediated gene silencing, such as the strategies (co-suppression and antisense) used to construct the two GE potato clones in this study was first observed in *Petunia* (Napoli *et al*. 1990). It is also referred to as RNA interference (RNAi) in animals, post transcriptional gene silencing (PTGS) in plants and quelling in fungi (Patel *et al*. 2008). It has been a challenge to understand the phenomenon, while current research seems to reach an agreement that the core components of RNA-mediated silencing mechanism are composed of small RNA working in conjunction with Argonaute proteins to target specific nucleic acid sequences (Maine 2009). RNA-mediated silencing is a powerful tool utilized for gene functional analysis, screening unknown genes and also genetic and nutritional value improvement of important crop plants (Angaji *et al*. 2010), e.g., the low-glutenin1 rice which was a relief to kidney patients unable to digest glutenin (Kusaba *et al*. 2003).

Although SR48 exhibited the lowest amount of *zep* mRNA at mature tuber stage, zeaxanthin amount in field-grown tubers of co-suppression derived SR47 was around 1.6 times higher. Morris *et al.* (2004) reported an inverse relationship between *zep* transcript level and total carotenoid content in a range of potato germplasm. Generally, this trend also applied to the two GE clones versus 'Baltica', but not when the two GE clones were compared to each other. This observation indicated that absolute carotenoid content in the two GE clones seems to rely, beside *zep* expression level, on additional factors which are unknown so far.

GE crops are currently created by random insertion of an engineered gene of the same or another organism. Unexpected changes in the expression of other genes in the engineered plant may occur especially when the target gene is part of a complex biosynthetic pathway. To evaluate the performance of GE crops to the non-transgenic counterpart plant, the natural varietal variation of conventional crops needs to be taken into account (Cheng *et al.* 2008). Here, we investigated *zep* expression in different tissues and growth stages of five conventional potato cultivars. From the results we could clearly see that besides the intended expression changes in tuber tissue, the differences of *zep* expression between GE and their conventional counterpart fall in the range of natural varietal variation. Similar conclusions were drawn in parallel projects of this study focusing on rhizosphere microbial communities (Weinert *et al.* 2009) and abundance and diversity of rhizobacteria with *in vitro* antagonistic activity (Weinert *et al.* 2010) carried out under same field conditions. Significant differences between the conventional counterpart clone 'Baltica' and two GE clones were detected mainly for *Actinobacteria*, *Betaproteobacteria* and *Streptomycetaceae*, yet only at one location in one year. Denaturing gradient gel electrophoresis (DGGE) analysis revealed that environment and plant genotype are more important factors affecting microbial community structure compared to genetic modification. In general, the differences observed between GE clones and the conventional counterpart clone 'Baltica' did not exceed natural cultivar-dependent variability (Weinert *et al.* 2009). In addition, 16S rRNA gene sequencing and antifungal metabolite analysis performed to 595 antagonists revealed that location and cultivar were important factors affecting the taxonomic composition of rhizobacteria. The effect of the genetic modification on the proportion of antagonists obtained did not exceed natural variability among the five commercial cultivars tested (Weinert *et al.* 2010).

In conclusion, the integrated *zep* gene in zeaxanthin-rich GE potatoes showed tuber-specific down-regulation under field conditions in three successive years. No significant changes of *zep* expression were found when comparing GE plants to their conventional counterpart in non-target tissues. The unchanged morphology and yield of GE plants certified the unaffected gene expression in non-tuber tissue in a macroscopical way. Furthermore, the minor changes of *zep* expression in non-target tissue under field conditions were within the natural cultivar variation.

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