

Potato plants with genetically engineered tropane alkaloid precursors

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Abstract

Main conclusion *Solanum tuberosum* tropinone reductase I reduced tropinone in vivo. Suppression of tropinone reductase II strongly reduced calystegines in sprouts. Overexpression of putrescine *N*-methyltransferase did not alter calystegine accumulation.

Calystegines are hydroxylated alkaloids formed by the tropane alkaloid pathway. They accumulate in potato (*Solanum tuberosum* L., Solanaceae) roots and sprouting tubers. Calystegines inhibit various glycosidases in vitro due to their sugar-mimic structure, but functions of calystegines in plants are not understood. Enzymes participating in or competing with calystegine biosynthesis, including putrescine *N*-methyltransferase (PMT) and tropinone reductases (TRI and TRII), were altered in their activity in potato plants by RNA interference (RNAi) and by overexpression. The genetically altered potato plants were investigated for the accumulation of calystegines and for intermediates of their biosynthesis. An increase in *N*-methylputrescine provided by *DsPMT* expression was not

sufficient to increase calystegine accumulation. Overexpression and gene knockdown of *StTRI* proved that *S. tuberosum* TRI is a functional tropinone reductase in vivo, but no influence on calystegine accumulation was observed. When *StTRII* expression was suppressed by RNAi, calystegine formation was severely compromised in the transformed plants. Under phytochamber and green house conditions, the *StTRII* RNAi plants did not show phenotypic alterations. Further investigation of calystegines function in potato plants under natural conditions is enabled by the calystegine deprived *StTRII* RNAi plants.

Keywords Calystegine · Genetic modification · Putrescine *N*-methyltransferase · *Solanum tuberosum* · Tropinone reductases

Abbreviations

Ds	<i>Datura stramonium</i>
PMT	Putrescine <i>N</i> -methyltransferase
RNAi	RNA interference
St	<i>Solanum tuberosum</i>
TRI (II)	Tropinone reductase I (II)

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Introduction

Calystegines are nortropane alkaloids with two to five hydroxyl groups found in Convolvulaceae (Tepfer et al. 1988; Schimming et al. 1998), Solanaceae (Nash et al. 1993; Dräger et al. 1994; Asano et al. 1995; Bekkouche et al. 2001; Biastoff and Dräger 2007), Moraceae (Asano et al. 1994a, b; Kusano et al. 2002), Brassicaceae (Brock et al. 2006), and Erythroxylaceae (Brock et al. 2005). Calystegines are formed by the tropane alkaloid pathway (Fig. 1), including putrescine *N*-methyltransferase (PMT)

and pseudotropine forming tropinone reductase (TRII). PMT produces *N*-methylputrescine, which is the first specific metabolite for tropane and nortropane alkaloids and for nicotine. TRII forms pseudotropine which, after *N*-demethylation, is the common precursor for the various calystegines. Tropine forming tropinone reductase (TRI) catalyzes a tropinone reduction competing with TRII. Tropine formation is essential in the course of the biosynthesis of the medicinal tropine alkaloids atropine and scopolamine that are not found in potato.

Similarities with monosaccharides are evident in the molecular structure of calystegines (Fig. 1). It is not surprising, therefore, that calystegines inhibit various glycosidases by occupying the substrate-binding site of the enzymes (Gloster et al. 2007). Many glycosidases catalyze glycosides cleavage by proton transfer from an acidic amino-acid residue onto the glycosidic oxygen. When calystegines are bound in the active site, the ring-bound nitrogen is protonated instead of the glycosidic oxygen, but without subsequent destabilisation (Borges de Melo et al. 2006; Gloster et al. 2006). Ring-bound nitrogen and several hydroxyl groups are common structural elements for many

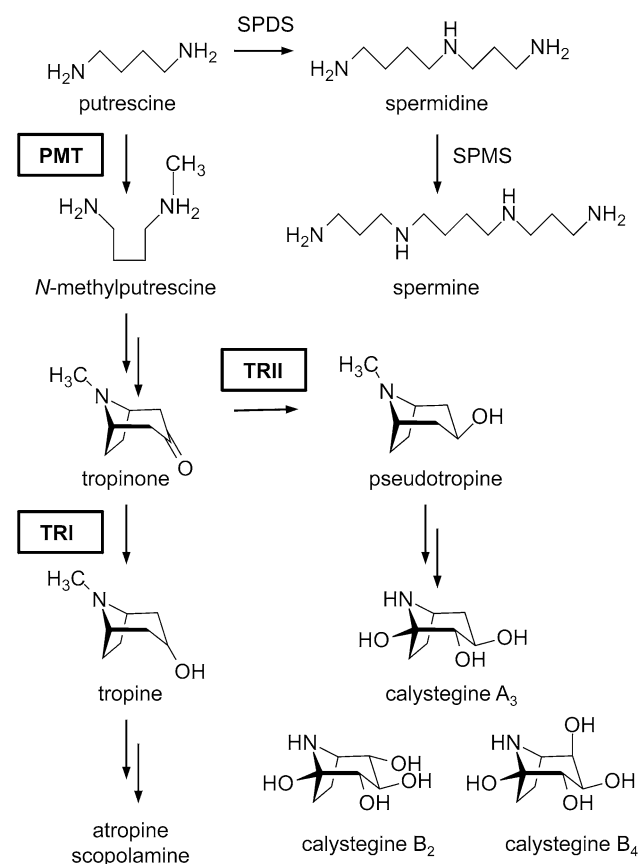


Fig. 1 Calystegine biosynthesis. *PMT* putrescine *N*-methyltransferase, *SPDS* spermidine synthase, *SPMS* spermine synthase, *TRI* tropine-forming tropinone reductase, *TRII* pseudotropine-forming tropinone reductase

alkaloid-like glycosidase inhibitors, some of which are used as drugs against diabetes, e.g., miglitol (Campo et al. 2013). On the other hand, glycosidase inhibitors must be considered as potential toxins for mammals. If they are absorbed after ingestion, they can inhibit lysosomal glycosidases in human and animal cells. Sugar-mimic alkaloids, such as swainsonine and castanospermine, are plant-derived toxins for cattle, because they inhibit lysosomal glycosidases involved in glycoprotein processing (Elbein et al. 1981; Dibello et al. 1983; Saul et al. 1985) and cause symptoms similar to lysosomal storage diseases, such as bovine spongiform encephalitis (BSE) with vacuolated cells in different organs and neuronal damage (Molyneux et al. 1995; de Balogh et al. 1999). Armien and co-workers (Armien et al. 2007) reported an ataxia syndrome in cattle, whose occurrence was implicated with calystegine-containing *Ipomoea* species (Convolvulaceae) that grew in the area of poisoning events. The *Ipomoea* species that were investigated contain calystegines but also swainsonine, an alkaloidal glycosidase inhibitor with potent toxicity. Isolated calystegines were investigated for their toxicity in mice (Stegelmeier et al. 2008). After injection, they proved rather non-toxic on whole animals; however, histological examination revealed liver cell alterations with high doses (140 mg kg⁻¹ day⁻¹) of calystegine A₃. Swainsonine (10 mg kg⁻¹ day⁻¹), in comparison, exhibited mouse toxicity macroscopically and in organ sections, although mice as rodents are less sensitive toward glycosidase inhibitor toxicity and demand higher doses than ruminants, such as sheep and goats.

Calystegines occur in many plants used for human nutrition, mostly in vegetables from Solanaceae. Eggplant, green peppers, potatoes, and physalis berries are examples (Asano et al. 1997). Potato tubers (*Solanum tuberosum* L.) are a major carbohydrate source consumed worldwide. Calystegines accumulate in young tissues, such as potato sprouts, but they were also reported in the edible parts of the vegetables that are ingested. Obviously, they are non-toxic in general, and rare intoxications observed with potato consumption were attributed to the steroidal alkaloids in potatoes after inappropriate storage, e.g., solanine and chaconine (Barceloux 2009). Calystegines are not destroyed by boiling potatoes or other vegetables; they are heat-resistant. Non-toxicity of calystegines in food possibly results from non-absorption in the intestine. In Caco-2 cell cultures widely used as model system for intestinal transport, calystegines remained on the luminal side representing the intestinal lumen (Jockovic et al. 2013). Calystegine B₂, however, inhibited sucrase, one of the major intestinal enzymes responsible for carbohydrate digestion. Inhibition was visible in concentrations of calystegine B₂ achievable by a regular serving of potato tubers, indicating that tubers may possibly retard carbohydrate digestion in the diet (Jockovic et al. 2013).

Bacterial, plant, and mammalian glycosidases are among those affected by calystegines (Asano et al. 2001). In spite of their clearly defined biochemical actions, functions for calystegines in plants are poorly understood. Calystegine accumulation in subterranean plant organs may attract microorganisms that are beneficial for plant rhizosphere, such as *Rhizobia* (Reitz et al. 2000; Unno et al. 2015). *Rhizobium meliloti* that utilizes calystegines as carbon and nitrogen source (Tepfer et al. 1988) grows preferably in the rhizosphere of calystegine-producing plants (Goldmann et al. 1996; Guntli et al. 1999). For potato, the organ-specific accumulation was investigated in detail. Highest calystegine accumulation was found in below-ground organs, those are roots and sprouting tubers (Keiner and Dräger 2000). A role in plant defence was assumed as for many glycosidase inhibitors; however, a proof was not provided up to now. Investigation on endophytic fungi in leaves of calystegines accumulating plants revealed no growth reduction of fungal biomass and no inhibition of fungal invertase by calystegines. Plant invertase, however, was inhibited by calystegine B₂ (Höke and Dräger 2004). Different from defence, calystegines may be excreted into the soil by plant tissues and contribute to a rhizosphere beneficial for plant growth. Calystegines serve as carbon source for *Rhizobium meliloti* (Tepfer et al. 1988). *Rhizobia* were shown to increase in the rhizosphere of potato plants under macro-nutrient deficiency suggesting that by excreting compounds, potato plants may be able to create an altered rhizosphere and favour rhizobia growth (Unno et al. 2015).

As calystegines are compounds interesting for plant growth improvement and for human nutrition, it is important to investigate their role in potato growth and development. Gene expression of enzymes participating in or competing with calystegine biosynthesis was altered by RNA interference and by overexpression. The genetically altered potato plants were investigated regarding their accumulation of calystegines.

Materials and methods

Cloning of full-length and RNAi constructs, generation of transgenic plants

The coding regions of *Solanum tuberosum TRI* (*StTRI*, AJ307584) and *Datura stramonium PMT* (*DsPMT*, AJ583514) were amplified and cloned into pENTR/D-TOPO (Invitrogen). Full-length constructs were transferred via LR recombination (Invitrogen) to pGWB14 (Nakagawa et al. 2007). For cloning, the *StTRI* RNAi fragment primers (5'-GCTTATCATTTATCTCAAATTGC-3' and 5'-CACCGACTATTACTGCTGAAGCTTCTTC3') and the *StTRII*

RNAi fragment primers (5'-GCTTACCACTTATCTGTACTTGC-3' and 5'-CACCTGCAAC AACTGCTGCAAGTTC3') were used to amplify fragments from full-length genes *S. tuberosum TRI* and *TRII* (AJ292343). RNAi fragments of *StTRI* and *StTRII* were cloned into pENTR/D-TOPO (Invitrogen) and transferred to pHELLSGATE8 via LR recombination (Wesley et al. 2001). Leaves of potato plants (*Solanum tuberosum* cv *Désirée*) were transformed with *Agrobacterium tumefaciens* AGL0 (Lazo et al. 1991), containing the vectors with the constructs. Transgenic plants were regenerated from transformed and control potato leaves as described (Rocha-Sosa et al. 1989; Feltkamp et al. 1995). Potato cultivar *Désirée* was kindly provided by E. Tacke, Bioplant, Ebstorf, Germany.

Plant cultivation and feeding experiments

Sterile potato plants (*Solanum tuberosum* cv *Désirée*) were grown in tissue culture in a phytochamber for 3 weeks under long-day conditions (16 h light, 140 μmol photons) at 22 °C. The plants were transferred into sterilized soil and grown in a phytochamber for 3–4 weeks under long-day conditions (16 h light, 140 μmol photons) at 18–20 °C and 70% relative humidity. For tropinone feeding, three mature leaves of a plant were cut off and put into a glass of 20 ml tropinone (5 mM, buffered with HCl to pH 7.4). Control leaves were placed in 20 ml water. Tropinone, 99% pure, (Sigma-Aldrich) did not contain tropine or pseudotropine (Keiner and Dräger 2000). When the liquids in the glass decreased, tropinone solution or water was refilled. Tropinone application was completed after 8 days in the phytochamber. The leaf stems (petioles) were cut off and discarded to reduce tropinone content in the samples, as a very high tropinone content interferes with the separation of tropinone, tropine, and pseudotropine by GC–MS. The leaf base was rinsed with water to remove residual outer tropinone and the whole leaf was frozen in liquid nitrogen for tropine and pseudotropine analysis.

Assessment of plant phenotypes

Plant phenotypes were monitored after sterile potato plants from tissue culture were transferred into soil in 110 mm diameter pots. Plant growth, colour, and shape of the leaves were monitored for the whole life cycle of the plant. The formation and shape of flowers were judged after 3- to 4-week-old plants were transferred into large pots (320 mm diameter) in the greenhouse. For tuber formation, 3- to 4-week-old plants were grown in the greenhouse and tubers were formed after further 8 weeks. Tubers were harvested and judged by weight, size, number, and shape. Representative samples of tubers were stored at 7 °C in the dark until sprouting initiated after 5 months. Equivalent tuber

samples were stored at 7 °C for 4 weeks and transferred to room temperature until sprouting initiated after another 2 months. Tuber sprouts (5–10 mm) were clipped off the tubers each week for 4 months and collected for analysis of gene expression and calyptegines.

RNA isolation and cDNA synthesis

Total RNA was isolated from potato leaves, roots, and tuber sprouts using the Trizol method (Chomczynski and Sacchi 1987). Samples containing 2 µg total RNA were treated with 1 U DNase (RNase-Free DNase Set, Qiagen) to avoid DNA contaminations. After 30 min incubation at 37 °C, 1 µl 25 mM EDTA was added and samples were heated to 65 °C for 10 min. The total RNA was transcribed into cDNA (RevertAid H Minus First Strand cDNA Synthesis Kit, Thermo Scientific) according to the manufacturers' instructions and diluted 1:10 with water.

Expression analysis (Northern blot, qRT-PCR)

Total RNA was isolated as described above. 20 µg of total RNA were separated on a formaldehyde gel and blotted to a positively charged nylon membrane (Roche). cDNA full-length fragments of *StTRI* (795 bp) and *DsPMT* (1135 bp) were labelled with α -³²P-dATP using the Megaprime DNA labelling system (Amersham Pharmacia Biotechnology). For hybridization, membrane and labelled probe were incubated in hybridization buffer (0.9 M NaCl, 50 mM NaH₂PO₄, 5 mM EDTA, 0.1% SDS, 0.1% PVP, 0.1% Ficoll, 0.1% BSA, 50% (v/v) formamide, 100 µg/ml denatured salmon sperm DNA) for 24 h at 42 °C. The membrane was washed in 50 ml 3× SSC, 0.5% (v/v) SDS at 65 °C three times.

Quantitative PCR was measured on an Mx3005P qPCR system (Agilent). Reactions were performed in triplicates using the Maxima Probe qPCR Master Mix (Thermo Scientific) and Roche Universal Probe Library, Probe #33 for amplification of *StTRI* (primers 5'-CTGAAGAAGTTTCA GCAGTAA-3' and 5'-GCCCATATAATTTGGCCTGTA A-3'), Probe #45 for *StTRII* (primers 5'-CAAAGGGG CAATGTTGTCTT-3' and 5'-TTTGGTTGCTCCATAAA CAGC-3'), and Probe #162 for the endogenous elongation factor *StEF1 α* (primers 5-CACTGCCAGGTCATCATC-3' and 5'-GTCGAGCACTGGTGCATATC-3'). Relative transcript levels were calculated using the MxPro qPCR software (Agilent).

Polyamine analysis

Putrescine, *N*-methylputrescine, spermidine, and spermine were extracted from potato leaves. Plant material was homogenized and extracted with 1.5 ml of 5% (v/v)

perchloric acid and incubated at 4 °C for 2 h in the dark. After centrifugation, the supernatant was used to determine free, soluble polyamines. 100 µl supernatant were derivatized with dansyl chloride by a modified method of Flores and Galston (1982). The extract was mixed with 100 µl saturated sodium carbonate and 400 µl dansyl chloride (7.5 mg/ml in acetone). The mixture was shaken in the dark at 60 °C for 60 min. After adding 100 µl proline (1 mg/ml) and incubation for 30 min in the dark, the dansylated polyamines were extracted in 500 µl toluol triply. The extracted organic phases were evaporated and resuspended in acetonitrile. For HPLC separation (LaChrom, Merck), a LiChrocart reverse phase C18 column, 5 µm particle size, 250 mm length, and 4 mm diameter (Merck) were used with an acetonitrile/water gradient from 10 to 90% at 40 °C and a flow rate of 1 ml/min. Detection was performed concurrently by a fluorescence detector (365 nm extinction, 510 nm emission) and diode array detector (220 nm). Dansylated putrescine, *N*-methylputrescine, spermidine, and spermine with retention times of 6.4, 8.0, 10.4, and 14.6 min were quantified by fluorescence detection. The limit of quantification was 5 ng/ml for each polyamine. *N*-methylputrescine was provided by T. Hashimoto, Nara Institute of Science and Technology, Nara, Japan. Putrescine, spermidine, and spermine were purchased from Sigma-Aldrich.

Tropinone, tropine, and pseudotropine analysis

Tropinone, tropine, and pseudotropine were extracted from potato leaves. Homogenized plant material was extracted twice with distilled water by shaking for 1 min and incubation in an ultrasonic bath for 10 min. After centrifugation, the supernatant was concentrated to 1 ml, mixed with 100 µl 25% NH₃, and centrifuged. The extract was applied on a 1 g Extrelut[®] NT (Merck) column. After an exposure of up to 20 min, elution was performed by 2 × 3 ml chloroform and 3 ml of chloroform/methanol 85:15 (v/v). The eluate was dried by sodium sulphate, evaporated, and resuspended by ethyl acetate. The ethyl acetate samples were separated and quantified on a GC–MS 2010 system (Shimadzu) equipped with a FS-Supreme-5 ms column, 0.1 µm film thickness, (5% phenyl polysilphenylene-siloxane), 300 mm length, and 0.25 mm inner diameter (Chromatographie Service) at a constant flow rate of 1 ml/min helium. One µl sample was injected splitless at 110 °C and passed the temperature program: 60 °C (1 min), 10 °C/min up to 180 °C, 180 °C (1 min), 15 °C/min to 300 °C, and 300 °C (2 min). The mass spectrometer (QP2010S, Shimadzu) was set to the parameters: transfer line temperature 250 °C, ion source temperature 200 °C, EI ionisation 70 eV, and detector voltage 1.2 kV. Two scans per second were taken in the range of 50–350 amu. Retention

times were 6.9, 7.1, and 7.3 min for tropinone, tropine (Sigma-Aldrich), and pseudotropine, respectively (synthesized—as described in Nickon and Fieser 1952). Quantification limits were 1 µg/ml for tropinone and 5 µg/ml for tropine and pseudotropine.

Calystegine analysis

Potato sprout tissue was homogenized and extracted twice with 5 ml methanol/water 1:1 (v/v) by shaking and sonication for 10 min. After centrifugation, calystegine samples were purified and concentrated by a cation exchange column (Chromabond® SA, 6 ml/1000 mg, Macherey–Nagel). Solvent flow rate was set to 1 ml/min. After column conditioning with 5 ml methanol/water 1:1 (v/v), the sample was loaded and the column was washed with 10 ml water. Elution was performed with 4 ml 2 N ammonia and 1 ml water. The eluate was freeze-dried and silylated by an adapted procedure of Fleet et al. (1990). Standards and samples were dissolved in 30 µl anhydrous pyridine, mixed with 40 µl hexamethyl disilazane and 10 µl trimethyl chlorosilane, and incubated at 65 °C for 20 min. *n*-Hexane and azobenzol as internal standard were added to a final concentration of 200 ng/µl. GC–MS analysis (instrument and column as described in tropine and pseudotropine analysis) was performed with the temperature program: 50 °C (2.5 min), 20 °C/min to 160 °C, 160 °C (2 min), 5 °C/min to 240 °C, 240 °C (2 min), 10 °C/min to 330 °C, and 330 °C (2 min). Mass spectra in the EI mode with two scans per second were taken from 50 to 500 amu. Retention times were 11.0, 12.3, and 14.4 min for silylated calystegines A₃, B₄, and B₂. For calystegine quantification, castanospermine (Tocris Bioscience, Bristol, UK) was used as reference standard. The quantification limit was 10 µg/ml sample. Identity of calystegines was confirmed by comparison to calystegine reference standards, provided by N. Asano, Tohoku University, Kanazawa, Japan (Dräger 1995; Molyneux et al. 1996).

Statistical analysis

Experimental repetitions are indicated in the legends of each graph. Average values and standard deviations or standard deviations of the mean were calculated by and indicated for each experiment specifically.

Results

DsPMT expressing potato plants

Putrescine *N*-methyltransferase from *Datura stramonium* catalyzes the methylation of putrescine (Teuber et al.

2007). To assess the effect of increased levels of *N*-methylputrescine levels in potato, the coding region of *DsPMT* was cloned behind the 35S promoter in the binary vector pGWB14 (Nakagawa et al. 2007). Potato plants were transformed via *Agrobacterium tumefaciens*-mediated leaf disk transformation. The phenotypes of all regenerated plants did not differ from those of control plants (data not shown). Varying levels of *DsPMT* transcripts were detected in leaves of transgenic potato plants by Northern blotting (Suppl. Fig. S1). In control and empty-vector-transformed plant leaves, *PMT* transcripts were not detected. Five plant lines were selected which showed strongest *DsPMT* expression. In these plants, the soluble fraction of *N*-methylputrescine was analyzed, as well as the polyamines spermidine, spermine, and putrescine, the latter being the precursor of both, higher polyamines and of tropane alkaloids. *N*-methylputrescine was hardly detectable in non-transformed and empty vector control plants (Fig. 2a). This is in accordance with a screening for *N*-methylputrescine in potato organs that revealed the metabolite only in spouts of potato tubers (Stenzel et al. 2006). Leaves of *DsPMT* expressing plants contained *N*-methylputrescine up to 8 µg/g fresh weight (Fig. 2a). Yet, the considerable flux of putrescine into *N*-methylputrescine in *DsPMT* expressing plants did not impair the accumulation of putrescine and that of the derived spermidine and spermine (Fig. 2b–d). Possibly, the levels of polyamines are kept constant by anapleurotic reactions confirming the notion that these amines have vital regulatory functions in plants (Tiburcio et al. 2014). In three independent experiments, the levels of the tropane alkaloid precursors tropinone, tropine, and pseudotropine, which derive from *N*-methylputrescine, were at the detection limit in both *DsPMT* expressing and control plants (data not shown), suggesting that enhancing *N*-methylputrescine is not sufficient for enabling potato leaves to produce more tropane alkaloid precursors. Tropinone, tropine, and pseudotropine levels as metabolites, however, are mostly low in potato tissues, irrespective whether calystegines are accumulated (Richter et al. 2007).

Overexpression and RNAi of *StTRI*

To analyze the function of *StTRI*, transgenic plants with reduced and enhanced expression of *StTRI* were generated. Overexpression of *StTRI* under the control of the 35S promoter was demonstrated by Northern blotting, and four lines with strong transcript signals were selected for further investigation (Suppl. Fig. S2). The effect of expression of an RNAi construct targeted against *StTRI* was quantified by qRT-PCR in leaves. Four lines with strongly suppressed *StTRI* expression were selected (Fig. 3). A possible effect of *StTRI* down-regulation on the expression of *StTRII* was

Fig. 2 Leaves of *DsPMT* overexpressing plants (*DsPMT*, lines B–I) contained higher levels of the tropane alkaloid precursor *N*-methylputrescine (NMP) (a), but non-altered levels of polyamines putrescine, spermidine, and spermine (b–d). NMP and polyamines are given as free, soluble compounds. WT wildtype, EV empty vector; B–I selected transformants overexpressing *DsPMT*. FW fresh weight. Error bars represent standard deviation. Asterisks indicate significant difference using Student's *t* test $***P \leq 0.001$, $n = 26$ (WT, EV), $n = 10$ (*DsPMT* overexpressing lines), two independent experiments

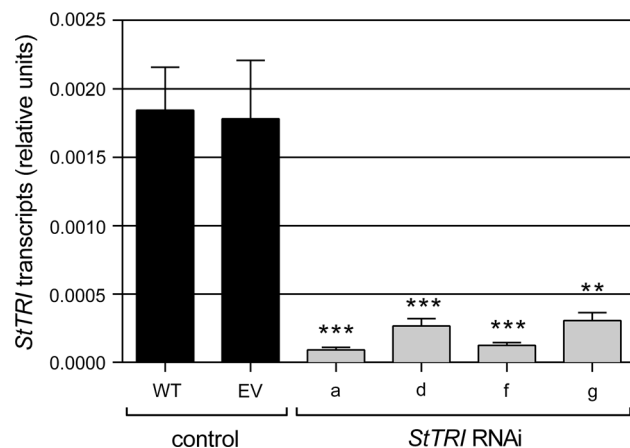
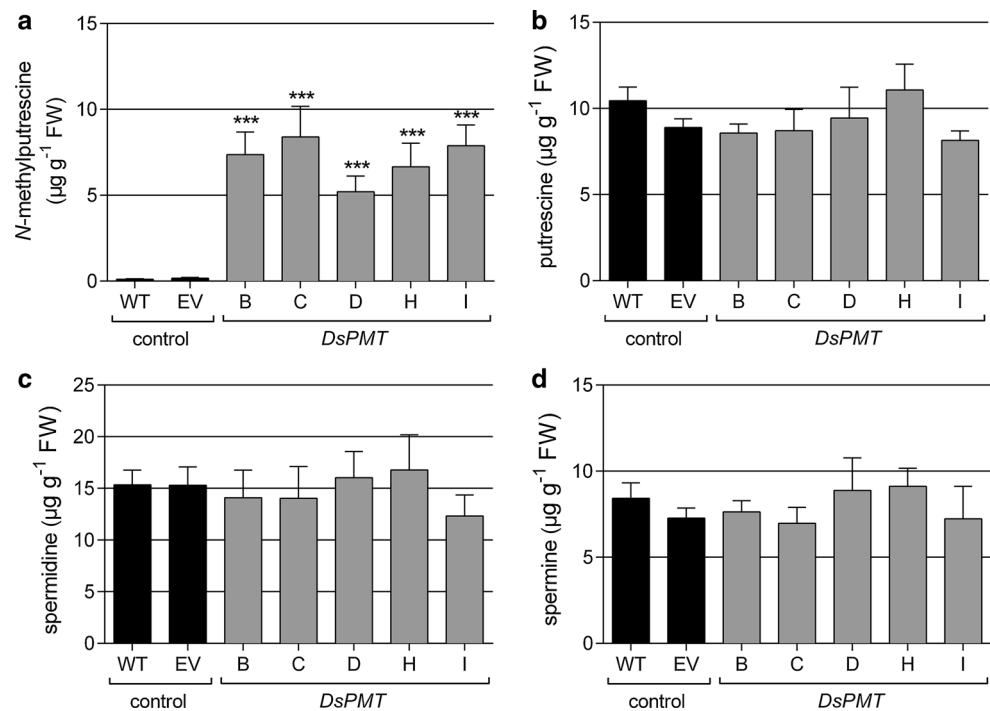


Fig. 3 Transcript levels of *StTRI* in leaves of *StTRI* RNAi lines (a–g) and control plants (WT wildtype, EV empty vector). RNA was isolated from leaves of 4-week-old potato plants, reverse transcribed, and analyzed for *StTRI* transcripts by quantitative PCR. *StEF1 α* expression was used as a reference. Error bars represent standard error of the mean. Asterisks indicate statistically different values compared with WT and EV by Student's *t* test, $**P \leq 0.01$; $***P \leq 0.001$; $n = 22$ (WT, EV), $n = 12$ (*StTRI* RNAi lines). Data are derived from two independent experiments

checked by qRT-PCR in potato sprouts and in roots, as these are the tissues of strongest *StTRII* expression (Keiner et al. 2002). No alteration of *StTRII* expression in *StTRI* RNAi plants was detectable in these tissues (Suppl. Fig. S3).

Potato plants with suppressed and increased *StTRI* expression were used to explore the activity of StTRI in vivo. Tropinone was applied to leaves of the transformed

plant lines and both reduction products, tropine and pseudotropine, were monitored. When no external substrate was applied, tropinone concentration in potato tissues was very low, and reduction products of potentially elevated enzyme activity did not accumulate. Upon exogenous tropinone application, the formation of tropine increased in *StTRI* overexpressing leaves, suggesting that StTRI protein was synthesized in the tissues and that in vivo StTRI is an active tropine-forming tropinone reductase (Fig. 4a). Correspondingly, in *StTRI* RNAi leaves, only traces of tropine were detected after tropinone feeding. Levels of tropinone (visible in the gas chromatograms) also varied somewhat from leaf to leaf, and the reduction product levels thus may reflect not only the enzyme activity in situ, but also the transport and local availability of tropinone. All tropinone-fed leaves contained higher concentrations of pseudotropine than of tropine (Fig. 4b). Compared with hardly detectable tropine and pseudotropine levels in untreated leaves (data not shown), tropinone feeding strongly increased pseudotropine levels in control and transformed leaves. Together with the tropine levels, the results show that StTRI is a functional tropine-forming enzyme in vivo, but the pseudotropine-forming StTRII is the stronger tropinone reductase in potato leaves producing more products.

Knockdown of *StTRII*

To elucidate StTRII function in vivo, *StTRII* expression was suppressed by RNAi. Transgenic potato plants expressing an RNAi construct targeted against *StTRII* were

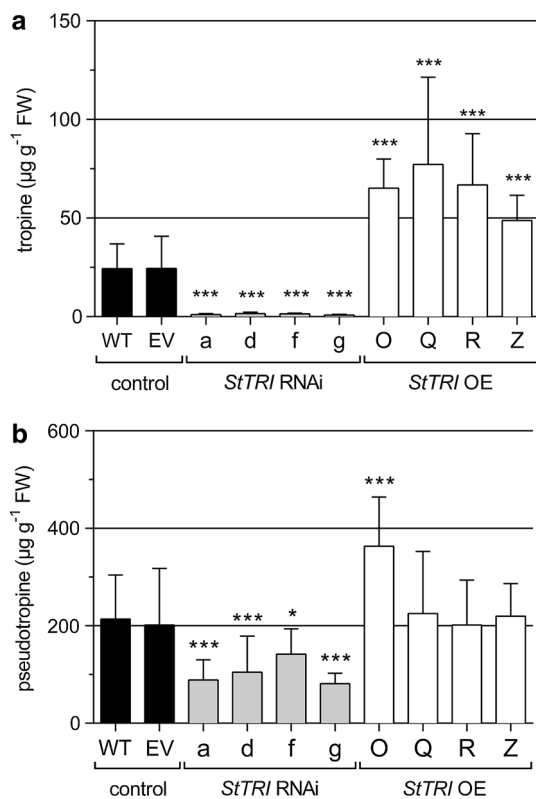


Fig. 4 Tropine (a) and pseudotropine (b) levels after feeding leaves of 4-week-old potato plants with 5 mM tropinone for 8 days. Control leaves were from wildtype (WT) and empty vector-transformed (EV) plants. Transformed plants were suppressed for *StTRI* expression (*StTRI* RNAi, lines a–g) or overexpressed *StTRI* (*StTRI* OE, lines O–Z). FW fresh weight. Error bars represent standard deviation, $n = 24$ (WT, EV), $n = 12$ (*StTRI* RNAi and *StTRI* OE lines). Asterisks indicate statistically different values compared with WT and EV by Student's *t* test, $*P \leq 0.05$; $**P \leq 0.01$; $***P \leq 0.001$; $n = 22$ (WT, EV), $n = 12$ (*StTRI* RNAi lines). Data are derived from two independent experiments

analyzed for alterations in *StTRII* expression in roots and sprouts by qRT-PCR. All lines showed a significant reduction in *StTRII* transcript levels; however, in sprouts of lines l and n, transcripts levels were reduced only threefold (Fig. 5).

To test for reduced TRII enzyme activity in tissues, potato leaves were supplemented with tropinone and both reduction products, tropine and pseudotropine, were measured. While tropine levels were not different among *StTRII* RNAi and control lines, pseudotropine increased strongly in control lines but remained below tropine levels in the *StTRII*-RNAi lines (Fig. 6). Thus, reducing the expression of *StTRII* correlated with a strongly reduced ability to synthesize pseudotropine from exogenously applied tropinone. These results suggest that, in vivo, *StTRII* is the main enzyme catalyzing the synthesis of pseudotropine from tropinone.

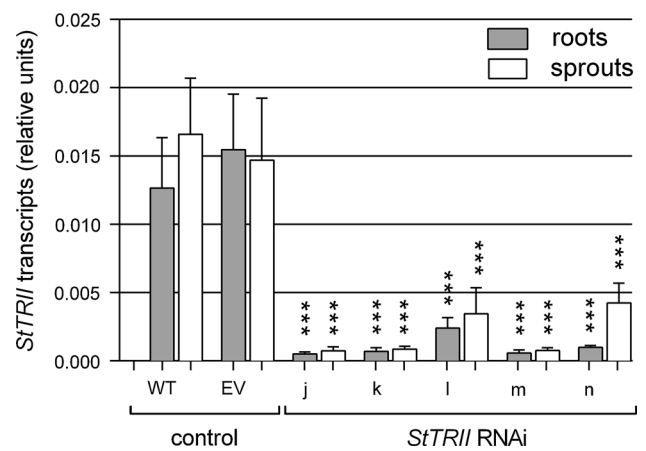


Fig. 5 Reduced transcript levels of *StTRII* in roots and sprouts of *StTRII* RNAi lines (j–n) compared with control plants (WT wildtype; EV empty vector). RNA was isolated from leaves of 4-week-old potato plants, reverse transcribed and analyzed for *StTRII* transcripts by quantitative PCR. *StEF1 α* expression was used as a reference. Error bars represent standard deviation. Asterisks indicate statistically different values compared with WT and EV by Student's *t* test, $***P \leq 0.001$; $n = 20$ (WT, EV), $n = 8$ (*StTRII* RNAi lines). Data are derived from two independent experiments

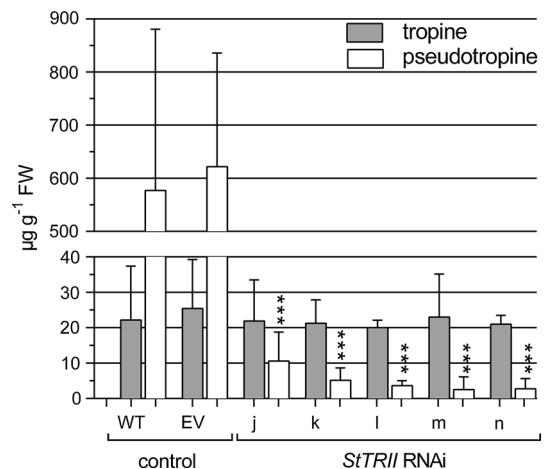


Fig. 6 Tropine and pseudotropine levels after feeding leaves of 4-week-old potato plants with 5 mM tropinone for 8 days. Control leaves were from wildtype (WT) and empty vector-transformed (EV) plants. Transformed plants were suppressed for *StTRII* expression (*StTRII* RNAi, lines j–n). FW fresh weight. Error bars represent standard deviation, $n = 10$ (WT, EV), $n = 5$ (*StTRII* RNAi). Asterisks indicate statistically different values compared with WT and EV by Student's *t* test, $***P \leq 0.001$. Data are derived from two independent experiments

Phenotypes and calystegine levels

For all transgenic lines, plant phenotypes, growth, and development, and the accumulation of calystegines in potato sprouts were monitored. Potato plants regenerated from transformed calli did not differ in phenotype or

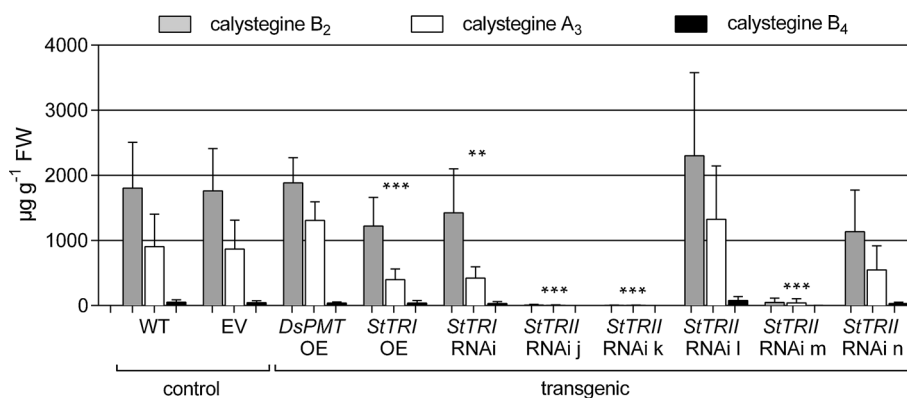


Fig. 7 Calystegine levels in sprouts of *DsPMT* and *StTRI* overexpressing lines (*DsPMT* OE, combined lines B, C, D, H, I as in Fig. 2; *StTRI* OE, combined lines O, Q, R, Z as in Fig. 4), *StTRI* RNAi and *StTRII* RNAi lines (*StTRI* RNAi, combined lines a, d, f, g as in Fig. 4; *StTRII* RNAi, combined lines j, k, l, m, n as in Fig. 5), control plants from wildtype (WT) and empty vector (EV). FW fresh weight. Error

bars represent standard deviation, $n \geq 20$ (control plants, *DsPMT* OE, *StTRI* OE, *StTRI* RNAi), $n \geq 12$ (*StTRII* RNAi). Asterisks indicate statistical difference of the sum of all three calystegines B₂, A₃, B₄ between WT/EV and the transgenic lines by Kruskal–Wallis test $**P \leq 0.01$; $***P \leq 0.001$. Data are derived from two to three independent experiments per construct

growth and development from wild-type plants and from plants transformed with empty vector. All plants formed tubers after 8 weeks of cultivation. Potato tubers from all transgenic lines were compared. Differences in tuber size, shape, or number between control and transgenic plants were not observed. After 5 months of tuber dormancy at 7 °C or after 4 weeks at 7 °C and 2 months tuber storage at room temperature, all tubers began to sprout simultaneously.

Tuber sprouts were investigated for their calystegine accumulation. The major calystegines in tubers of the cultivar *Désirée* used in this study were calystegines B₂, A₃, and, at lower levels, calystegine B₄ (Fig. 1). This is in accordance with the pattern of calystegines reported from other potato cultivars (Keiner and Dräger 2000; Friedman et al. 2003). Levels of calystegines varied considerably between transformed lines (Fig. 7). Sprouts from wild-type plants and from those transformed with empty vector accumulated up to 3 mg total calystegines per gram FW, corresponding to earlier findings in other cultivars (Keiner and Dräger 2000). Transformed lines expressing *DsPMT* did not show significantly higher calystegine concentrations. *N*-methylputrescine is essential for the biosynthesis of tropinone (Hashimoto et al. 1989), and tropinone is incorporated into calystegines (Scholl et al. 2001); however, in potato, *N*-methylputrescine levels in leaves do not appear to be rate-limiting for calystegine accumulation in sprouts.

When *StTRI* gene expression was suppressed, the effect on calystegine accumulation was not prominent. Higher availability of tropinone, when *StTRI* gene expression is reduced, and enhanced formation of pseudotropine, that may result in a higher calystegine accumulation, were not evident (Fig. 7). Inversely, it was tested whether ectopic and overexpression of *StTRI* might reduce tropinone

availability to the extent that the flux through pseudotropine was reduced and resulted in lower calystegine levels. However, only a slight reduction in calystegine levels was observed.

A strong reduction of calystegines, however, appeared in the *StTRII* RNAi lines j, k, and m (Fig. 7). This suggests that endogenous pseudotropine levels were reduced in these *StTRII* RNAi lines not only in detached leaves (Fig. 6), but also in sprouts. Lines l and n accumulated calystegines; in line l, calystegine accumulation was comparable to wildtype (Fig. 7). This correlates with higher *StTRII* transcript levels, compared with the other RNAi lines, in sprouts (Fig. 5). Taken together, the reduction of *StTRII* expression correlates with a decreased ability to reduce tropinone to pseudotropine and to synthesize calystegines. These results strongly argue for a role of *StTRII* in calystegine formation in potato sprouts.

Discussion

N-Methylputrescine is the first specific metabolite for calystegine formation (Biastoff et al. 2009), but an increase of *N*-methylputrescine in leaves provided by *PMT* expression was not sufficient to increase calystegine accumulation in potato sprouts. This is different from tobacco, where increased *PMT* activity augmented nicotine content (Sato et al. 2001). In *Atropa belladonna*, a tropane alkaloid accumulating plant, *PMT* overexpression did not result in increased atropine or scopolamine formation, and calystegine levels equally were not enhanced (Rothe et al. 2003). Obviously, further steps downstream of *N*-methylputrescine in the subsequent biosynthetic pathway are rate-limiting for tropane alkaloid accumulation.

StTRI overexpressing plants proved that StTRI is a functional tropinone reductase in vivo, but its role in potato metabolism is not clear. Tropine does not normally accumulate in potato tissues, possibly due to high StTRII activity that prevents tropinone accumulation and tropinone availability for StTRI. The two enzymes are synthesized in different tissues (Kaiser et al. 2006) and may have different access to tropinone. StTRs, like many short chain dehydrogenases and ketone reductases (Oppermann et al. 2003), accept a range of substrates (Keiner et al. 2002; Kaiser et al. 2006). It remains a question, under which metabolic or stress conditions StTRI will exert tropinone reducing activity in potato tissues. Irrespective whether StTRI was genetically enhanced or repressed, it did not interfere with tropinone reduction by StTRII.

Decrease of *StTRII* expression reduced pseudotropine accumulation and calystegine levels considerably, but not all *StTRII* RNAi lines were equally affected. It is evident from feeding tropinone to wildtype and transformed potato leaves that StTRII is a very active enzyme that is not rate-limiting for calystegine formation. Our results suggest that only if TRII activity is suppressed below a certain threshold, pseudotropine levels become limiting and calystegine accumulation is severely decreased.

The transgenic potato lines provided in this study with various levels of calystegines did not reveal differences in sprouting time or intensity. Potato tuber sprouting demands carbohydrate relocation, in particular degradation of starch and formation of sucrose for translocation into the sprouts (Farre et al. 2001; Hajirezaei et al. 2003). These processes might be responsive to calystegine-induced glycosidase inhibition, but transgenic tubers obtained in this study did not differ phenotypically when storage and sprouting were observed under controlled conditions (Suppl. Fig. S4). It must be considered, however, that tuber sprouting in field agriculture is influenced by many parameters, which are not encountered by isolated tubers that are maintained in a controlled atmosphere.

Sprouting tubers in the soil are subjected to a number of infections and pests. *Rhizoctonia solani*, for example, is a soil-borne fungus that attacks tuber sprouts (Lahlali and Hijri 2010) and also roots and stolons of potato plants by forming pores on the subterranean organ surface (Chamoun et al. 2014). The density and virulence of *Rhizoctonia solani* are influenced by the plant rhizosphere. Soil microorganisms able to reduce *Rhizoctonia* growth, such as *Bacterium subtilis*, are, therefore, used as biocontrol agents against *Rhizoctonia* infections, and furthermore, beneficial microorganisms for potato cultivation are intensively searched for (Lahlali and Hijri 2010; Ghyselinck et al. 2013). Among the microorganisms that induce a beneficial rhizosphere for potato plants are *Rhizobia* (Reitz et al. 2000; Unno et al. 2015), which

catabolize calystegines (Tepfer et al. 1988). It is hypothesized that calystegines are important metabolites for potato plant health in the field, for example by inhibiting growth of pathogenic microorganisms, such as *Rhizoctonia*. The generation of transgenic potato plants with reduced *StTRII* expression thus should permit functional analyses of calystegines with regard to sprout development, pest resistance, and stress tolerance.

Greenhouse cultivation of transgenic potato plants may permit to compare the rhizosphere microbiome in pots with *StTRII* RNAi and wild-type plants. Addition of growth-supportive and pathogenic microorganisms to the pots and observation of their development in the presence and absence of calystegine-producing potato plants may support the assumption of a function of calystegines in the rhizosphere. Potato sprouts and potato roots contain steroidal glycoalkaloids (Friedman and Dao 1992; Asano et al. 1996; Friedman et al. 2003), which may also be present in the rhizosphere of potato plants. It will be interesting to investigate whether these alkaloids with membrane penetrating properties influence the microbiome and whether they interact with calystegines. Subsequent experiments ideally should involve governmentally approved field trials to judge the performance of calystegine-free plants under highly diverse conditions. Exposure to potential pathogens and predators and the presence of a more natural and potentially highly complex rhizosphere may provide further hints for the function of calystegines in potato plants.

Author contribution statement BD and SR conceived and designed research. NK designed and conducted experiments and analyzed data. NK contributed new analytical tools. NK and BD wrote the manuscript. All authors read and approved the manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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