

Dose- and tissue-specific interaction of monoterpenes with the gibberellin-mediated release of potato tuber bud dormancy, sprout growth and induction of α -amylases and β -amylases

Sonja Rentsch · Dagmara Podzimska · Antje Voegelé · Madeleine Imbeck · Kerstin Müller · Ada Linkies · Gerhard Leubner-Metzger

Received: 11 June 2011 / Accepted: 5 August 2011 / Published online: 20 August 2011
© Springer-Verlag 2011

Abstract Gibberellins (GA) are involved in bud dormancy release in several species. We show here that GA-treatment released bud dormancy, initiated bud sprouting and promoted sprout growth of excised potato tuber bud discs ('eyes'). Monoterpenes from peppermint oil (PMO) and *S*-(+)-carvone (CAR) interact with the GA-mediated bud dormancy release in a hormesis-type response: low monoterpene concentrations enhance dormancy release and the initiation of bud sprouting, whereas high concentrations inhibit it. PMO and CAR did, however, not affect sprout growth rate after its onset. We further show that GA-induced dormancy release is associated with tissue-specific regulation of α - and β -amylases. Molecular phylogenetic analysis shows that potato α -amylases cluster into two distinct groups: α -AMY1 and α -AMY2. GA-treatment induced transcript accumulation of members of both α -amylase groups, as well as α - and β -amylase enzyme activity in sprout and 'sub-eye' tissues. In sprouts, CAR interacts with the GA-mediated accumulation of α -amylase transcripts in an α -AMY2-specific and dose-dependent manner. Low CAR concentrations enhance the accumulation of α -AMY2-type α -amylase transcripts, but do not affect the α -AMY1-type transcripts. Low CAR concentrations also

enhance the accumulation of α - and β -amylase enzyme activity in sprouts, but not in 'sub-eye' tissues. In contrast, high CAR concentrations have no appreciable effect in sprouts on the enzyme activities and the α -amylase transcript abundances of either group. The dose-dependent effects on the enzyme activities and the α -AMY2-type α -amylase transcripts in sprouts are specific for CAR but not for PMO. Different monoterpenes therefore may have specific targets for their interaction with hormone signalling pathways.

Keywords α -Amylase · β -Amylase · Bud dormancy release · Carvone · Gibberellin · Hormesis · Monoterpenes · Peppermint oil · Post-harvest sprout inhibitors · Potato tuber sprouting · *Solanum*

Abbreviations

GA Gibberellin
CIPC Isopropyl *N*-(3-chlorophenyl)carbamate
CAR *S*-(+)-Carvone
PMO Peppermint oil
CON Control

S. Rentsch · D. Podzimska · A. Voegelé · M. Imbeck · A. Linkies (✉) · G. Leubner-Metzger (✉)
Faculty of Biology, Institute for Biology II,
Botany/Plant Physiology, Albert-Ludwigs-University,
Schänzlestr.1, 79104 Freiburg, Germany
e-mail: ada.linkies@biologie.uni-freiburg.de
URL: www.seedbiology.de

G. Leubner-Metzger
e-mail: gerhard.leubner@biologie.uni-freiburg.de

K. Müller
Department of Biological Sciences, Simon Fraser University,
8888, University Drive, Burnaby, BC V5A 1S6, Canada

Introduction

Potato (*Solanum tuberosum* L.) tubers are starchy storage organs that develop by subapical radial growth at the ends of stolons, which are diagravitropically growing underground shoots (Cuttler 1978; Leubner-Metzger and Amrhein 1993; Fernie and Willmitzer 2001). Potato tubers are therefore radially expanded shoot axes consisting of shortened internodes and 'eyes'. These eyes are nodes with their axillary buds. The tuber is usually dormant upon

harvest, which means that the bud meristems are in an arrested state and visible bud sprout growth (sprouting) is thereby blocked. Potato tuber dormancy is associated with down-regulated cell cycle genes and increased ABA contents in the bud meristems when compared with the non-dormant state (Destefano-Beltran et al. 2006a, b; Campbell et al. 2010). Bud dormancy release occurs after a period of tuber storage and is visible as post-harvest sprouting. The induction and maintenance of tuber bud dormancy seems to involve abscisic acid (ABA), whereas bud dormancy release seems to involve gibberellins (GA) and cytokinins (Biemelt et al. 2000; Suttle 2004; Destefano-Beltran et al. 2006a, b; Hartmann et al. 2011). However, these publications also show that changes in the endogenous hormone contents are not under all circumstances consistent with the changes in the dormancy status and bud sprouting. The control of tuber bud dormancy induction, maintenance and release therefore is mediated, at least in part, by changes in hormone signalling as it is also known for tree bud dormancy (Horvath et al. 2003; Rohde et al. 2007) and seed dormancy (Finch-Savage and Leubner-Metzger 2006; Holdsworth et al. 2008). It is for example clear that, although treatment with exogenous GA₁ or GA₃ causes potato tuber bud dormancy release as well as the initiation of sprouting and subsequent bud sprout growth (Clegg and Rappaport 1970; Davies and Viola 1988; Hartmann et al. 2011), the increase in the endogenous contents of bioactive GA₁ is not associated with bud dormancy release, but with subsequent bud sprout growth (Suttle 2004; Hartmann et al. 2011). GA-treatment also led to increased α -amylase enzyme activity and accumulation of reducing sugars (Clegg and Rappaport 1970). While initial bud sprouting seems not to depend on starch degradation, the maintenance of sprout growth probably depends on GA-mediated starch degradation (Biemelt et al. 2000; Suttle 2004).

Starch consists of simple α -1,4, α -1,6-linked glucose polymers organised to form semicrystalline, insoluble granules. This granular structure is relevant for the mechanisms of starch degradation, as many glucan-mobilising enzymes appear to be unable to act upon intact granules as substrate (Smith et al. 2005; Zeeman et al. 2010). Although several different types of enzymes are capable of releasing soluble glucans from purified starch granules *in vitro*, the only enzyme generally believed to achieve this *in planta* is the endo-acting α -amylase (α -1,4-D-glucan glucanohydrolase). Starch degradation proceeds differently in cereal endosperms and potato tubers as evident from the appearance of the granule surface. While cereal endosperm granules exhibit abundant channels leading from pores on the surface to the interior, potato tuber granules have few if any pores or channels running inwards from the surface. Potato tuber granules are highly resistant to enzymatic attack and it has been suggested that the type of

enzyme(s) responsible for attacking the starch granules differ between potato tubers and other well-known model systems like cereal endosperms. The relative importance of α -amylase in starch degradation may even differ within potato tubers, as inconsistent results were obtained for α -amylase accumulation by various groups (Clegg and Rappaport 1970; Davies and Viola 1988; Biemelt et al. 2000). In agreement with an important role for α -amylases in releasing soluble glucans from insoluble potato tuber starch granules, α -amylase transcripts and enzyme activity accumulate in 'sub-eye' tissue during storage and sprouting (Biemelt et al. 2000). Soluble glucans are further degraded by a battery of glucan phosphorylases and hydrolases for which the relative importance is a matter of a long-standing debate (Smith et al. 2005; Stensballe et al. 2008; Zeeman et al. 2010). Among them are exo-type β -amylases and α -glucosidases, which release maltose and glucose, respectively (Taylor et al. 1998; Kaplan et al. 2006). Low temperature potato tuber storage inhibits post-harvest sprouting, but can cause starch degradation by a process known as cold-induced sweetening. Specific isoforms of β -amylase and starch phosphorylase accumulate during this process, but their importance in the degradation is not known (Nielsen et al. 1997; Smith et al. 2005; Kaplan et al. 2006; Delaplace et al. 2009). Therefore, to prevent both cold-induced sweetening and post-harvest sprouting, an optimal temperature combined with means that control dormancy and/or inhibit visible bud sprouting are required for proper potato tuber storage.

CIPC [chlorpropham; isopropyl *N*-(3-chlorophenyl) carbamate] is the most effective post-harvest bud sprout inhibitor registered for use in potato tuber storage (Kleinkopf et al. 2003; Eshel et al. 2009; Campbell et al. 2010; Teper-Bamnolker et al. 2010). CIPC inhibits bud sprouting following loss of tuber dormancy by interfering with cell division; it modifies spindle formation by altering microtubule structure and thereby inhibits mitosis. The transcriptome analysis of Campbell et al. (2010) supports the view that CIPC does not act by prolongation of the dormant state, but by suppression of visible bud sprout growth. Alternative sprout inhibitors include essential oils extracted from plants like peppermint (*Mentha piperita*; PMO, peppermint oil), spearmint (*Mentha spicata*), or caraway (*Carum carvi*), as well as their volatile compounds (e.g. Oosterhaven et al. 1995a, b; Baydar and Karadoğan 2003; Kleinkopf et al. 2003; Eshel et al. 2009; Owlabi et al. 2010; Teper-Bamnolker et al. 2010). Volatile compounds from essential oils that inhibit sprouting include monoterpenes like menthol (major component of PMO) and the *R*-(-)- and *S*-(+)-carvone enantiomers (major components of spearmint and caraway essential oils, but not detectable in PMO). Although they also prevent visible sprouting rather than prolonging the dormant state, their

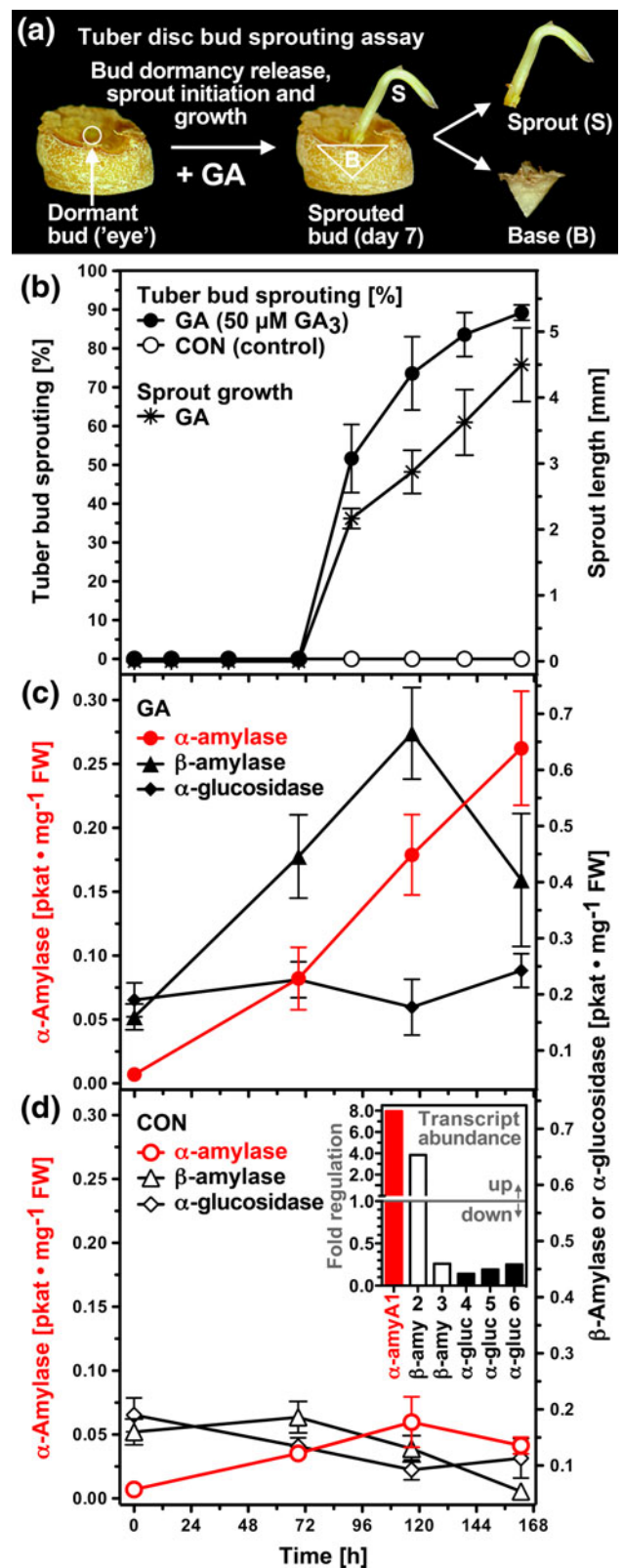
Fig. 1 The effect of gibberellin on potato tuber bud dormancy release, bud sprout initiation, sprout growth and on the expression of starch hydrolases. **a** Tuber discs containing dormant buds ('eyes') were excised from dormant tubers of *Solanum tuberosum* cv. Agria and shaken for 2 h in a solution without (CON, control) and with 50 μ M gibberellin A₃ added (GA). Tuber discs were subsequently incubated on wet filter paper for 1 week and dissected into sprout (S) and base (B) tissue for subsequent hydrolase analyses. Tuber disc base (B) tissue contains periderm ('skin') and proximal 'sub-eye' cortex tissue. **b** The effect of GA-treatment on tuber disc bud dormancy release, bud sprout initiation and sprout growth. Bud dormancy release and the initiation of bud sprouting were scored by calculating the percentage of tuber discs with visible sprouts (≥ 2 mm in length). Sprout growth was measured daily as sprout length. **c** The α -amylase, β -amylase, and α -glucosidase enzyme activities of sprouts from GA-treated tuber discs were analysed over time using specific assays. **d** The enzyme activities from unsprouted bud tuber disc tissue (CON) was measured for comparison. Mean values \pm SE ($n \geq 3$ tuber discs for activities, $n \geq 10$ tuber discs for sprouting). *Insert:* The relative transcript abundances of α -amylases, β -amylases and α -glucosidases in GA-treated tuber bud discs were obtained from the transcriptome analysis of Hartmann et al. (2011): Transcript abundance values are presented as fold regulation (day3/day0) for the α -AMY2-group α -amylase amyA1 (MICRO.10377.C2), the β -amylases MICRO.13368.C1 (2) and POACG68TP (3) and the α -glucosidases MICRO.856.C1 (4), SSBT006B09x.scf (5) and cSTA12F14TH (6)

mode of action differs from CIPC. Teper-Bamnlöcher et al. (2010) showed that *R*-carvone inhibits sprouting by causing cell membrane damage mainly at the meristem tips of the tuber buds. Essential oil and carvone can also cause necrosis of the tuber bud meristems, which in turn may inhibit bud sprouting of stored potato tubers. In our work presented here, we used a tuber disc bud sprouting assay to investigate how PMO and *S*-(+)-carvone (CAR) interact with the GA-mediated bud dormancy release and sprout growth.

Materials and methods

Plant material and potato tuber bud disc sprouting assay

Solanum tuberosum L. cv. Agria plants were grown using organic farming conditions on a field of Gregor Kapp, Füllinsdorf, close to Basel (Switzerland). Dormant potato tubers were harvested in September and stored, without applying any chemical treatment, at 8°C in the dark. Before use in experiments, tubers were washed with tap water, surface sterilised for 5 min in 1% (v/v) NaOCl, 0.02% (v/v) Tween 20 and subsequently rinsed for 20 min with tap water. For the sprouting assays, tuber bud ('eye') discs of 7-mm diameter were excised using a cork borer and cut to 5-mm height (Fig. 1). Ca. 15–20 bud discs were incubated for 2 h under shaking submerged in 30 ml of 0.02% (v/v) Tween 20 without (CON, control) or with 50 μ M gibberellin A₃ (GA₃, Sigma) added. Peppermint oil (PMO, Apotheke zum Rauracher, Riehen, Switzerland), *S*-(+)-



carvone (CAR, no. 435759, Sigma) or isopropyl *N*-(3-chlorophenyl)carbamate (CIPC, chlorpropham, Sigma) were added during this 2 h of incubation to the solution in

the concentrations indicated; the CIPC concentration was 0.05% (w/v). Tuber disc assignment to the different treatments was performed by considering that discs from different bud positions and tubers were distributed equally to each series. The tuber bud discs were subsequently incubated in Petri dishes on two layers of filter paper wetted with 0.1% (v/v) PPM (Preservative for Plant Tissue Culture Media, Plant Cell Technology, Manchester, UK). Petri dishes were sealed with parafilm and incubated in darkness. They were opened daily under sterile conditions to facilitate air exchange and the filter paper was regularly moistened. Bud dormancy release (visible bud sprouts >2 mm) and sprout growth (in mm length) were scored daily. At the times indicated, in most cases after 7 days, ‘tissue harvest’ (sprout, base) was performed as described in Fig. 1. The potato tubers used in the experiments were considered as dormant as there was no bud dormancy release (visible initial bud sprouting) of excised CON-discs for at least 7 days; at 8–10 days $\leq 10\%$ of excised CON-discs showed visible bud sprouting. Tissue pieces of usually 30- to 40-mg fresh weight were collected in 1.5-ml tubes and stored frozen until use.

Protein extraction and enzyme activity assays

Two stainless steel balls were added to each tissue sample and the tubes were frozen in liquid nitrogen. The tissues were pulverised by 6×30 s of shaking at a frequency of 30/s using a ball mill with intermitted refreezing them in liquid nitrogen. Addition of 120 μ l extraction buffer (50 mM MOPS pH 7.5, 20 mM $MgCl_2$, 2 mM $CaCl_2$, 1 mM EDTA, 3% (v/v) PEG-8000, 2% (w/v) PVP, 0.1% (v/v) β -mercaptoethanol), subsequent thawing and 15 s of shaking was used for homogenisation. After 45 min of centrifugation at 4°C and 17,608g, the supernatant was transferred to a new tube. This protein extract was used for quantifying the enzyme activities using assays with specific substrates for α -amylase (BNPG7, *p*-nitrophenyl-maltoheptasaccharide with chemically blocked non-reducing end, ‘Ceralpha reagent’ (R-AMHR4), Megazyme, Wicklow, Ireland), β -amylase (PNPG5, *p*-nitrophenyl-maltopentasaccharide, ‘Betamyl reagent’ (R-BAMR6), Megazyme) and α -glucosidase (PNPG1, *p*-nitrophenyl- α -D-glucopyranoside, no. N1377, Sigma). For each of these enzyme assays, a substrate master mix (SMM) was prepared by mixing 50 volumes of assay buffer (100 mM Mes-KOH pH 6.2, 1 mM EDTA, 0.1% (v/v) β -mercaptoethanol) plus 15 volumes of Ceralpha or Betamyl reagent (containing a thermostable α -glucosidase as assay coupling enzyme) for the α -amylase or β -amylase assay, respectively; for the SMM of the α -glucosidase assay, 35 vol assay buffer plus 30 vol 10 mM PNPG1 were mixed. To perform the enzyme activity assay, 10- μ l aliquots of the undiluted protein

extract was mixed with 65 μ l of SMM and incubated at 40°C. The assays were stopped at 0, 20 and 40 min by adding 180 μ l of 1% (w/v) Tris pH 11, and the released *p*-nitrophenol (yellow product) quantified photometrically at 405 nm. The enzyme activities were calculated based on the slopes of the obtained curves, which were corrected for assay background slopes from ‘no-extract controls’. The molar enzyme activity units (pkat) were calculated using a *p*-nitrophenol calibration curve, and the specific enzyme activities in pkat/mg fresh weight were determined. Mean values \pm SE were obtained from at least three biological replicates (protein extracts).

RNA extraction, cDNA cloning, sequence alignments and molecular phylogenetic analysis

Total RNA from potato tuber tissues was extracted using the RNeasy plant extraction kit (Qiagen) as described by Hermann et al. (2007). Subsequent RNA quality control, reverse transcription and cDNA cloning were done as described by Linkies et al. (2009). The StAMY1a1 and StAMY2a1 α -amylase cDNA sequences were cloned from GA-treated sprout tissue RNA, verified by sequencing of two independent clones each, and submitted to GenBank (accession number JK036076 and JK036077, respectively). For sequence analysis, the bioinformatics software Geneious 5.3.6 (Biomatters, Auckland, New Zealand) was used as described by Voegele et al. (2011). The MUSCLE alignment tool was used for alignments of the sequences shown in Fig. 4. The phylogenetic tree was constructed with PHYML (Guindon and Gascuel 2003), using the Jones–Taylor–Thornton substitution model and bootstrapped 100 times.

Semiquantitative RT-PCR (sqRT-PCR) for potato α -amylases

First-strand cDNA synthesis with 1 μ g of total RNA, 2.5 μ M oligo(dT)16 and 2.5 μ M random hexamers with the Superscript III reverse transcriptase kit (Invitrogen), as well as 18S rRNA primer and gel electrophoresis were as described by Hermann et al. (2007). The 20- μ l PCR reactions contained first-strand cDNA equivalent to 100-ng RNA, 0.2 μ l Taq polymerase and 2 μ l 10 \times reaction buffer (Genaxxon Bioscience, Ulm, Germany), 200 μ M dNTP mix, additional 1.5 mM of $MgCl_2$, and 0.2 μ M of each primer: FST2A-alpha and RST2A-alpha for amplifying a 0.79-kb α -AMY2-fragment, FST1A-alpha and RST1-alpha for amplifying a 0.62-kb α -AMY1-fragment, and the two 18S rRNA primers for amplifying a 0.45-kb rRNA-derived fragment (Fig. 5a). The optimal conditions for the sqRT-PCR with these three primer pairs were 25 cycles (annealing temperature: 58°C). Initial experiments with

different cycle numbers were conducted and showed that at 25–30 cycles, the PCR reactions are in the exponential amplification phase and have not reached saturation. Furthermore, competition control experiments between the three primer pairs were conducted that showed that either primer pair alone or any combination of two or three pairs yielded the same results. The gel band intensities of the 0.79-kb α -AMY2-, 0.62-kb α -AMY1- and the 0.45-kb rRNA PCR fragments were quantified as absorbance units using the Kodak 1D image analysis software. The relative values for each lane, α -AMY2/rRNA and α -AMY1/rRNA, were determined as a measure for the transcript abundances of α -AMY2- and α -AMY1-type α -amylases. Three independent experiments were performed each with at least two to three RNA samples (biological replicates) per treatment. Mean values \pm SE were calculated for each experiment; all three experiments support the findings presented in Fig. 5. The values presented in Fig. 5b are from one of the independent experiments and are based on at least two to three RNA samples (biological replicates) and three sqRT-PCR repetitions (technical replicates). Primer sequences (5' to 3') are: FST2A-alpha GTTAGAGCGATGGCGGACAT, RST2A-alpha TAGATGAACGGCTGTGTATGC, FST1A-alpha TATTCTGATGGCACRGGGAAT, RST1-alpha GAT TAGTGCTGAAATTCATCC, 18SrRNA-RRNA2 CGAG CTGATGACTCGCGCTTA, 18SrRNA-RRNA5 GAGTGG AGCC TGCGGCTTA.

Results

α - and β -amylase, but not α -glucosidase, enzyme activities increase during GA-induced potato tuber disc bud sprouting

In initial experiments, we compared the two cultivars Agria and Nicola of *S. tuberosum* regarding their suitability for our potato tuber disc bud sprouting assay. The European Cultivated Potato Database (<http://www.europotato.org>) assigns cultivars to dormancy period categories from 1 (very short) to 9 (very long). The two cultivars differ in that cv. Agria has a very long tuber dormancy (category 9) and sprouting resistance during post-harvest storage, while cv. Nicola has a medium tuber dormancy (category 5). In agreement with this, bud discs excised from cv. Agria tubers stored for up to 2 months and incubated without GA-treatment as described below ('CON') did not exhibit any visible meristem growth in excess of 2 mm during at least 8 days (see Fig. 1b and details in "Materials and methods"), while ca. 30% of the bud discs excised from cv. Nicola tubers showed visible sprouts >2 mm on day 4. Based on this observation, and in the style of the operational definition for tuber dormancy used by Campbell

et al. (2010), we classify the tubers of cv. Agria as dormant and those of cv. Nicola as non-dormant. The very long tuber dormancy and sprouting resistance during post-harvest storage as well as our results from these initial experiments qualifies the cultivar Agria as highly suitable for our tuber disc sprouting assay.

For our potato tuber disc bud sprouting assay, we used dormant tubers of *S. tuberosum* cv. Agria and excised 'eye' region discs of defined size and shape containing dormant bud meristems in their centres (Fig. 1). These excised tuber discs were incubated for 2 h submerged in a solution without ('CON', control) or with 50 μ M GA₃ added (GA) as described in "Materials and methods". Subsequent incubation on wet filter paper showed that the GA-treatment released bud dormancy and initiated bud sprouting (percentage of tuber discs with visible bud sprouts >2 mm) and subsequent sprout growth (sprout length of non-dormant tuber discs), which became first visible after 72–96 h (Fig. 1 a, b). After 7 days of incubation, bud sprouting had occurred in almost all GA-treated tuber discs. In contrast, and in agreement with the conclusion that the GA-treatment released bud dormancy and induced sprouting, none of the excised potato tuber bud discs of the CON-series exhibited bud sprouting (Fig. 1a). In dose–response experiments, we found that 1–100 μ M GA₃ was effective in our assay (data not shown), but 50 μ M GA₃ was an optimal concentration. This is in agreement with results by Hartmann et al. (2011), and Clegg and Rappaport (1970) have shown that 50 μ M of GA₃ is also in the optimal concentration range for promoting visible sprout growth and starch degradation as proposed from the increased release of reducing sugars in the tuber disc bud sprouting assay.

To investigate if GA induces the accumulation of starch-degrading enzymes, we used specific activity assays for α - and β -amylase and α -glucosidase (as described in "Materials and methods"). To investigate enzyme activities in a tissue-specific manner with the tuber disc bud sprouting assay, we dissected the sprouted discs into sprout (S) and base (B, contains periderm ('skin') and proximal 'sub-eye' cortex tissue) (Fig. 1a). Figure 1c shows that in GA-treated tuber disc sprouts, α -amylase accumulates 37.4-fold (days 7 compared to day 0), and β -amylase accumulates 4.2-fold (day 5) and 2.5-fold (day 7), while these enzyme activities remained roughly unchanged without GA-treatment, i.e. in the CON-series (Fig. 1d). In contrast to α - and β -amylase, roughly equal α -glucosidase activities were evident at any time in the sprouts of the GA-treated tuber bud discs (Fig. 1c) and no changes occurred compared to the CON-series (Fig. 1d). As for sprouts (Fig. 1a, d), GA also induced α - and β -amylases in base tissue, which was 21.7- and 2.2-fold (days 7 compared to day 0), respectively, but GA did not affect α -glucosidases in base tissue of sprouted potato tuber discs. In agreement

with these findings, a recent transcriptome analysis by Hartmann et al. (2011) showed that the transcript abundances of α - and β -amylase are up-regulated, while those of the three α -glucosidases are down-regulated in GA-treated tuber discs (see insert in Fig. 1d). We conclude that GA-treatment of excised tuber discs induces bud dormancy release and sprout growth. GA-induced sprout growth is associated with the accumulation of α - and β -amylases, but not α -glucosidases. This regulation by GA seems to be mediated, at least in part, at the level of the α - and β -amylase transcripts.

Peppermint oil and CAR interact with the GA-mediated bud dormancy release, initiation of bud sprouting, sprout growth and α - and β -amylase accumulation in a concentration-dependent manner

The effects of post-harvest bud sprout inhibitors like chlorpropham (CIPC), CAR and peppermint oil (PMO), containing as major monoterpenes (–)-menthol and (+)-menthone, but no CAR) have been investigated during long-term storage of entire potato tubers (see “Introduction”), but not regarding their interaction with GA-induced processes in tuber disc bud sprouting assays. Figure 2 shows that CIPC completely blocks the GA-mediated induction of visible sprout growth in our tuber disc bud sprouting assay. Higher concentrations of CAR (500 and 1,000 μ M) and PMO (500 and 1,000 μ l/l) inhibit the GA-mediated initiation of bud sprouting in that its onset is delayed in a concentration-dependent manner. Interestingly, in contrast to high concentrations, the GA-mediated initiation of bud sprouting was enhanced by low concentrations of CAR (100 μ M) and PMO (100 μ l/l), i.e. the onset of visible initial sprout growth was earlier in GA + 100CAR- and GA + 100PMO-treated tuber discs (Fig. 2a). In contrast to bud dormancy release and bud sprout initiation, CAR and PMO did not appreciably affect the rate of the GA-induced sprout growth after its onset, i.e. the slopes of the sprout growth curves are very similar for GA and all combined CAR and PMO concentrations except the high 1000-concentrations (Fig. 2b). We therefore conclude that the interaction of CAR and PMO with GA-mediated processes is concentration dependent and process specific. Low CAR/PMO concentrations promote and high concentrations inhibit the GA-mediated tuber disc bud dormancy release and bud sprout initiation, but they do not affect the rate of subsequent sprout growth.

We analysed if the post-harvest bud sprout inhibitors affect the accumulation of α - and β -amylase in our tuber disc bud sprouting assay. For this, we measured the enzyme activities in sprout (S) and base (B) tissues after 7 days of tuber disc incubation following the various treatments as presented in Fig. 3. In day-7 sprouts

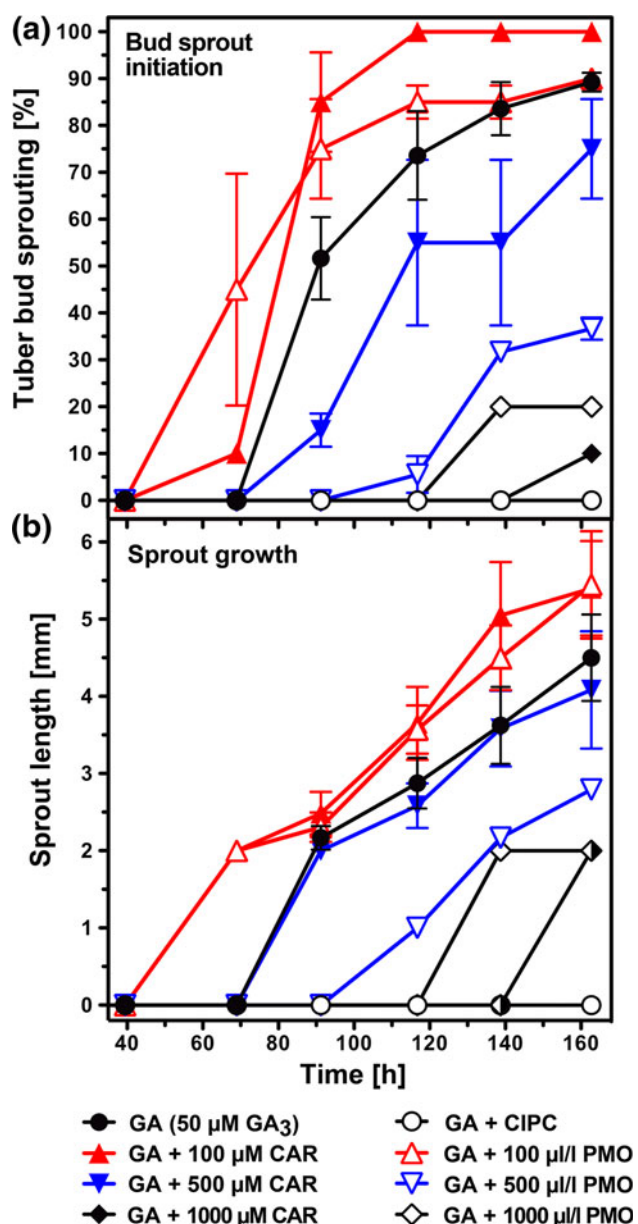


Fig. 2 The effects of *S*-(+)-carvone (CAR), peppermint oil (PMO) and CIPC on the kinetics of bud dormancy release (a) and sprout growth (b) of gibberellin-treated potato tuber bud discs. Excised tuber discs containing dormant buds (‘eyes’) from *Solanum tuberosum* cv. Agria and shaken for 2 h in a solution with 50 μ M gibberellin A_3 (GA) and CAR, PMO or CIPC added in the concentrations indicated in the legend and methods. Bud dormancy release and the initiation of bud sprouting was scored as visible initial sprout growth of ≥ 2 mm. Sprout growth after the initiation of bud sprouting was determined by daily measurements of the length. Mean values \pm SE of 10–20 tuber discs are presented

(Fig. 3a), GA caused 6.3- and 24.2-fold higher α - and β -amylase activities, respectively, compared to the untreated CON-series tuber discs. While simultaneous treatment with GA + CIPC completely blocked this GA effect, the GA-induction was not appreciably affected or

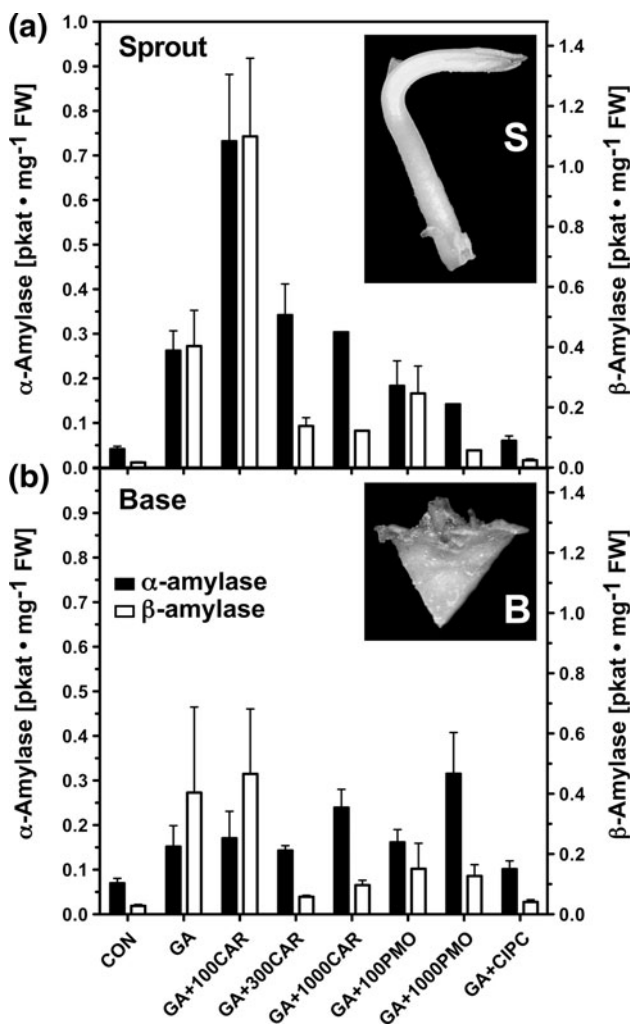


Fig. 3 The effects of gibberellin (GA), *S*-(+)-carvone (CAR), peppermint oil (PMO) and CIPC on the enzyme activities for α - and β -amylase in specific tissues of tuber bud discs from *Solanum tuberosum* cv. Agria. Tuber bud discs were treated for 2 h as described in the methods without (CON) or with 50 μ M gibberellin A₃ (GA) and, as indicated 100 or 1,000- μ M CAR, 100 or 1,000 μ l/l PMO or CIPC. The enzyme activities were quantified in sprout (unsprouted bud tissue for CON and GA + CIPC) (a) and base (b) tissues excised from bud discs after 7 days of incubation (see “Materials and methods”). Mean values \pm SE ($n \geq 3$)

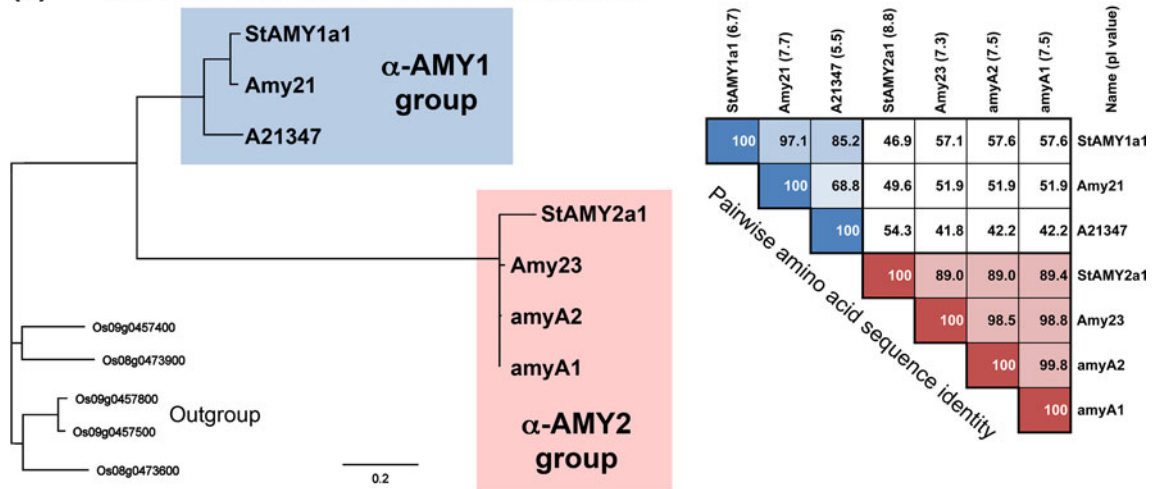
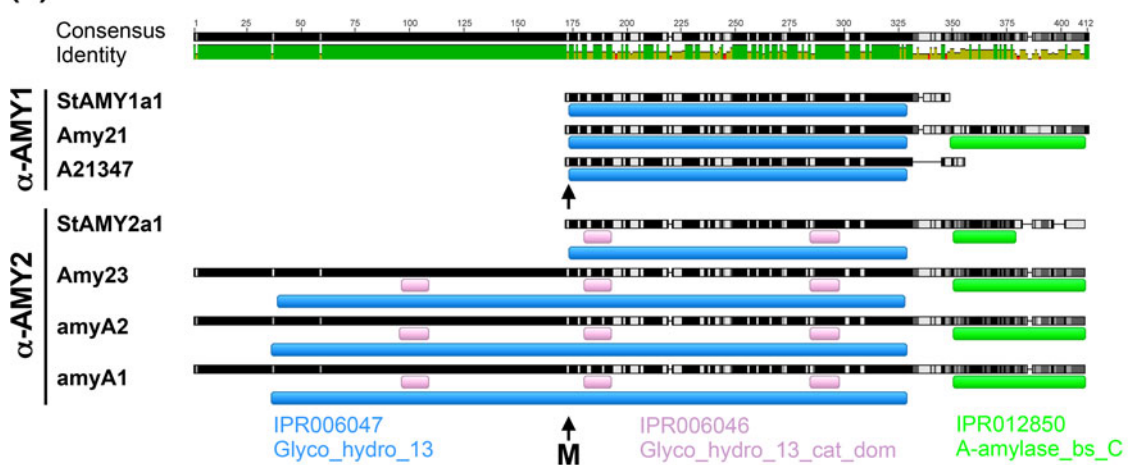
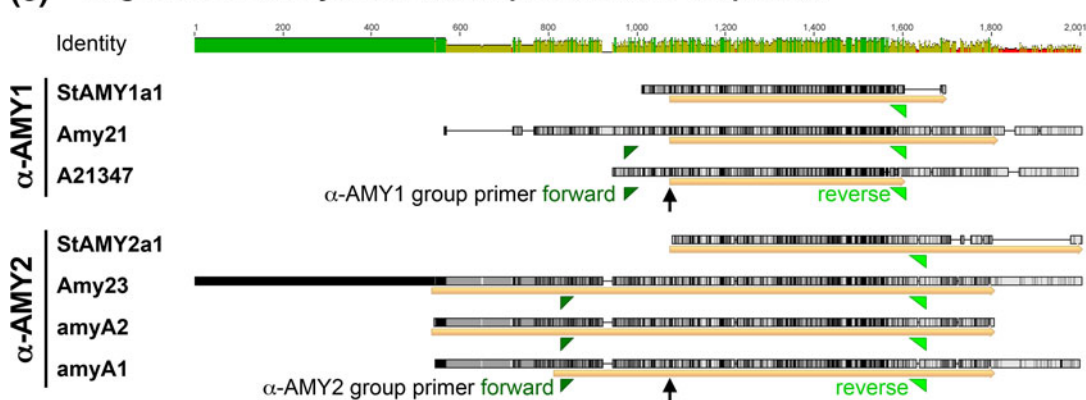
partially inhibited by 1,000- μ M CAR, as well as by 100 or 1,000 μ M PMO. In contrast to PMO and to the high CAR concentration, the GA-induction of α - and β -amylase was enhanced by the low (100 μ M) CAR concentration: GA + 100CAR caused 17.7- and 65.8-fold higher α - and β -amylase activities, respectively, compared to CON; 100- μ M CAR therefore enhanced the GA-mediated increase in α - and β -amylase activities in day-7 sprouts ca. threefold (Fig. 3a).

Treatment with GA also caused α - and β -amylase accumulation in day-7 base tissues and this induction was inhibited by CIPC (Fig. 3b). In contrast to sprouts, 100- μ M CAR did not enhance the GA-mediated α - and β -amylase

accumulation in base tissue. Neither CAR nor PMO appreciably affected the GA-induction of α -amylase, and β -amylase was either not affected or partially inhibited (Fig. 1b). Taken together, α - and β -amylase accumulation in our tuber disc bud sprouting assay is regulated in a tissue-specific manner, and the two tissues differ in GA-sensitivity and regarding the effects of CAR and PMO. The most striking effect is found in growing sprouts where low concentrations, but not high concentrations, of CAR enhanced the GA-induced α - and β -amylase accumulation ca. threefold. As this low-dose effect was only evident in S (not in B) and only with CAR (not with PMO), it is therefore a dose-, tissue- and monoterpene-specific interaction with the GA-induction of the amylase activities in the sprouts.

S-(+)-carvone interacts with the GA-induced accumulation of potato α -amylase group-2 transcripts, but does not affect group-1 transcripts in a concentration-dependent manner

Two major classes of cereal grain α -amylases are known and accumulate in the endosperm in a GA-dependent manner. These are known as the α -AmyA (cereal ‘high-pI’ α -amylase isoforms) and α -AmyB (cereal ‘low-pI’ α -amylase isoforms) classes for all known cereals; the wheat and rice multigene families can also be divided into three subfamilies (Mitsui and Itoh 1997; Leubner-Metzger 2007). Based on primers designed for conserved regions of cereal α -amylases and the five potato α -amylases cDNA sequences from public databases in 2006 (<http://www.ncbi.nlm.nih.gov>), we cloned two distinct α -amylase cDNAs from sprout RNA extracts of GA-treated tuber discs. They were named *StAMY1a1* and *StAMY2a1*, and have been submitted to GenBank (accession numbers JK036076 and JK036077, respectively). Transcripts of these two α -amylases are therefore expressed in GA-treated tuber discs bud sprouts of *S. tuberosum* cv. Agria. A recent transcriptome analysis by Hartmann et al. (2011) showed that the amyA1 α -amylase is also induced in GA-treated tuber discs (see insert in Fig. 1d). Sequence alignment and molecular phylogenetic analysis show that *StAMY1a1*, *StAMY2a1* and the known potato α -amylases cluster apart from the rice sequences fall into two groups (Fig. 4a): *StAMY1a1* belongs to the potato α -AMY1 group, whereas *StAMY2a1* and *amyA1* belong to the potato α -AMY2 group. Pairwise comparisons show that the potato α -AMY2 group contains four ‘high-pI’ (7.3–8.8) proteins with 89–99.8% amino acid sequence identity, while the three potato α -AMY1 group members are more diverse (Fig. 4a). Based on their domains, these proteins are members of the glycosyl hydrolase family 13 (Fig. 4b) and contain as evident from the barley α -amylase crystal structure the

(a) Molecular phylogeny of potato α -amylases(b) Alignment of α -amylase deduced protein sequences(c) Alignment of α -amylase cDNA sequences and PCR primers

domains important for catalytic activity (Pujadas and Palau 2001; Bozonnet et al. 2007). With the exception of two cDNA clones (StAMY1a1, A21347), all potato sequences also contain the C-terminal 'A-amylase_bs_C' domain

(Fig. 4b) typical for α -amylases (Pujadas and Palau 2001). There is a conserved nucleotide triplet coding for a stop codon in all of the nucleotide sequences, but due to insertions or deletions in the α -AMY1 group sequences,

◀ **Fig. 4** Molecular phylogenetic analysis of the potato α -amylase gene family reveals clustering into two distinct groups: α -AMY1 and α -AMY2. **a** The members of the two potato α -amylase groups α -AMY1 and α -AMY2 (phylogenetic tree on the *left*) differ among each other as presented for the pairwise comparison (table on the *right*) of their protein sequence identities and pI values (in *brackets* behind the names). Note that based on these values the α -AMY2 group contains highly conserved members as based on the sequence identities of the ‘high-pI’ proteins. **b** Alignment of deduced α -amylase amino acid sequences and characteristic domains as defined by InterPro database. ‘M’ and the *arrow* designate a conserved methionine in α -AMY1- and α -AMY2-proteins, which for the α -AMY1-proteins is the N-terminus. **c** Alignment of α -amylase cDNA sequences and the deduced open reading frames (*long arrows*). The positions for the group-specific primers for the semi-quantitative RT-PCR are indicated as triangles. Note that the forward primer for the α -AMY2-group is located within the coding sequence, while for the α -AMY1-group it is located in a conserved region of the 5′ untranslated cDNA region; the small arrow indicates the position of the encoded conserved methionine and the ca. 100-bp sequence downstream of the arrow does not allow the design of a forward primer that distinguishes between the two α -amylase groups. Accession numbers to GenBank for the potato α -amylases: *StAMY1a1* (JK036076), *Amy21* (M81682, identical with A21345), *StAMY2a1* (JK036077), *Amy23* (M79328, identical with A21341), *amyA2* (GU134783), *amyA1* (GQ406048, identical with A21343 and with MICRO.10377.C2 of Hartmann et al. 2011)

there is a functional stop codon in case of *StAMY1a1* and A21347, which therefore do not possess the C-terminal ‘A-amylase_bs_C’ domain (Fig. 4b). This non-catalytic C-terminal domain, known to be present in many, but not all, α -amylase is thought to modify the affinity for starch granule binding (Pujadas and Palau 2001; Bozonnet et al. 2007). High affinity depends on a conserved tyrosine in this domain, which is also present in all potato α -AMY2 group proteins. The potato α -AMY2 group is further distinguished from the α -AMY1 group in that the α -AMY2 group members contain a tripartite ‘Glyco_hydro_13’ domain (Fig. 4b). Interestingly, a conserved methionine (M in Fig. 4b) of the α -AMY2 group proteins corresponds to the N-terminal methionine (the start) of the α -AMY1 group proteins. Based on this finding we assume that, although the deduced protein sequence of *StAMY2a1* starts with methionine, the *StAMY2a1* cDNA is 5′ truncated and the N-terminal protein part is therefore missing. Our analysis shows that the potato α -AMY2 group members are highly similar in protein domains, pI, and amino acid sequence, but are distinct from the potato α -AMY1 group.

Having established that there are two distinct potato α -amylase groups, α -AMY1 and α -AMY2, and that transcripts of both groups are expressed in sprouts from GA-treated tuber discs of the bud sprouting assay, we wanted to further investigate their transcript expression patterns. To do this, we designed group-specific primers and conducted semi-quantitative RT-PCR (sqRT-PCR) analysis as described in “Materials and methods”. Figure 4c shows that the high sequence similarities between the α -AMY2

cDNAs allow the design of α -AMY2 group-specific sqRT-PCR primers that will amplify all known α -AMY2, but will not amplify α -AMY1 transcripts. Based on the high sequence similarity within the α -AMY2 group, these α -AMY2 primers will quite likely amplify all α -AMY2-group transcripts. The opposite amplification pattern is expected for the α -AMY1 group-specific primers, but as this group is more heterogeneous the primers may not amplify all α -AMY1 group transcripts. Taken together, the sequence comparisons of the potato α -amylases allow the design of group-specific primers that can be used for the comparative analysis of transcript expression pattern between the α -AMY2 and α -AMY1 groups.

Figure 5a shows that our sqRT-PCR was established in a way that a ca. 0.8-kb α -AMY2, a ca. 0.6-kb α -AMY1 and a ca. 0.45-kb rRNA PCR fragment were co-amplified in single-tube assays. The relative transcript abundances of α -AMY2 and α -AMY1 could therefore be directly calculated for each individual RNA sample and could be normalised using the abundance of the rRNA as internal control for the RNA amounts. In-gel quantification of the different PCR bands combined with normalisation, based on the corresponding rRNA signal, delivered relative α -AMY2 and α -AMY1 transcript abundances. Interestingly, when GA-treated sprout RNA samples were compared with the treatment ‘GA plus 100- μ M CAR’, an enhancing effect on the α -AMY2 transcript abundances was obtained (Fig. 5a), whereas the α -AMY1 transcripts were not affected. α -AMY2 transcript abundances were ca. twofold higher in GA + 100CAR when compared to GA alone, whereas α -AMY1 transcript were not affected. This low CAR concentration also enhanced α -amylase enzyme activity accumulation (Fig. 3a) and promoted bud dormancy release (Fig. 2a). To further investigate this finding, an independent experiment was conducted that in addition to a low CAR concentration also contained a higher CAR concentration. Figure 5b shows that this experiment verified the enhancing effect of a low CAR concentration on the α -AMY2 transcript abundances (GA + 100CAR), while a higher CAR concentration had no effect (GA + 300CAR). The quantification of the sqRT-PCR results obtained from this experiment is presented in Fig. 5c: In sprouts, the α -AMY2 transcript abundances of GA + 100CAR were 1.8-fold compared to GA alone, but only 1.1-fold for GA + 300CAR. In contrast, there was no such effect of CAR on the α -AMY1 transcript abundances in sprouts. Furthermore, there was no effect of any CAR concentration on the α -AMY2 or α -AMY1 transcript abundances in the base tissue (Fig. 5c). Taken together, these findings show that CAR interacts in a concentration-dependent and tissue-specific manner with the GA-induction of α -AMY2 genes, but does not affect the transcript abundances of the α -AMY1 genes.

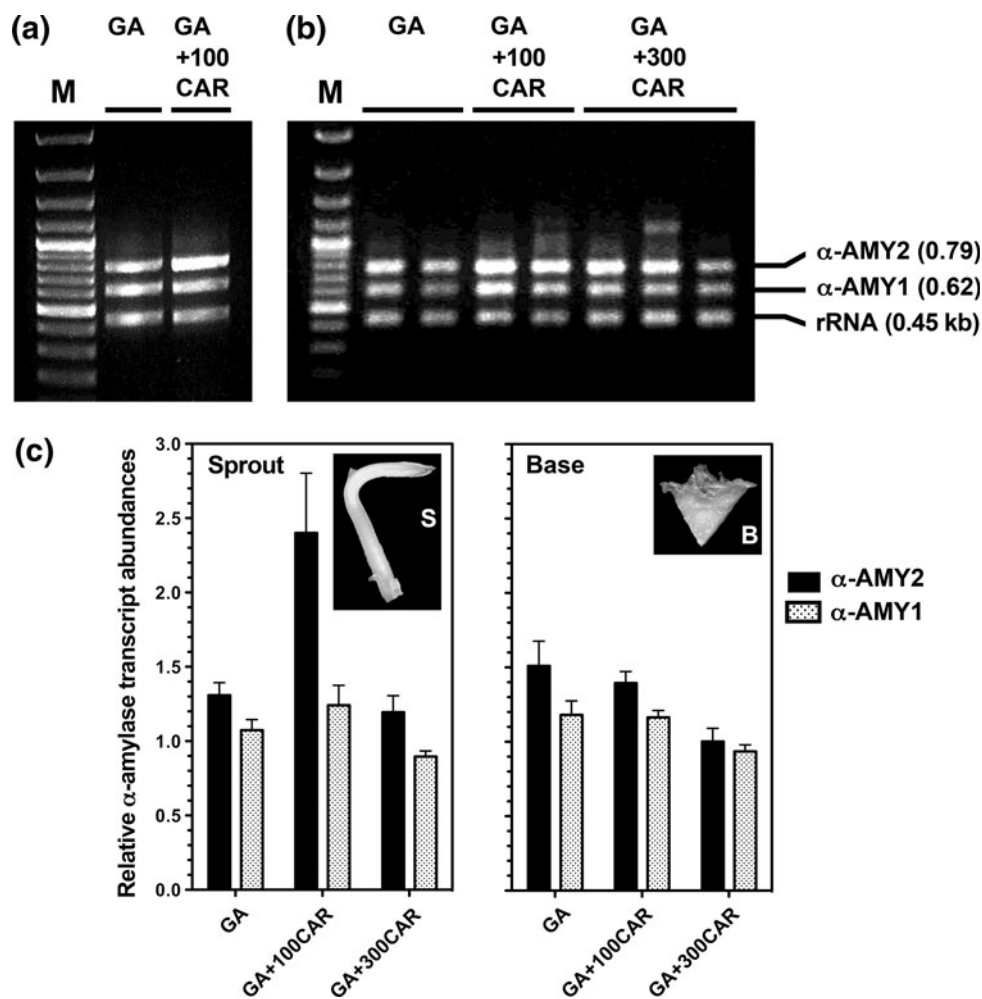


Fig. 5 RT-PCR analysis of the dose-dependent effects of *S*-(+)-carvone (CAR) on the gibberellin-mediated induction of α -AMY2- and α -AMY1-type α -amylase transcripts in specific tissues of tuber bud discs from *Solanum tuberosum* cv. Agria. Tuber bud discs were treated for 2 h with 50 μ M gibberellin A₃ (GA) and, as indicated, with 100- or 300- μ M CAR, and subsequently incubated for 7 days. RNA was isolated from sprout (S) and base (B) tissues and semi-quantitative RT-PCR with three primer pairs was conducted as described in the methods. **a** The two group-specific α -amylase primer pairs amplified a 0.8-kb α -AMY2- and a 0.6-kb α -AMY1-fragment, while the rRNA primer pair amplified a 0.45-kb fragment. Compared to the GA-treated RNA sample from sprouts, the sprout sample treated with ‘GA + 100CAR’ shows a ca. twofold enhanced abundance of the α -AMY2-band, while the α -AMY1- and rRNA-bands are similar in intensity between the two samples. **b** An independent

experiment with sprout RNA confirmed that low CAR treatment (‘GA + 100CAR’) caused enhanced α -AMY2-bands compared to ‘GA’, while this effect was not evident for α -AMY1. It showed in addition that the enhancing effect on the α -AMY2-bands is not evident with high CAR treatment (‘GA + 300CAR’). **c** Quantification of the gel band intensities of the 0.79-kb α -AMY2-, 0.62-kb α -AMY1-, and the 0.45-kb rRNA PCR fragments. The relative values for each lane, α -AMY2/rRNA and α -AMY1/rRNA, were determined as a measure for the transcript abundances of α -AMY2- and α -AMY1-type α -amylases. The mean values \pm SE presented are from the experiment shown partly in **b** and are based on at least two to three RNA samples (biological replicates) and three sqRT-PCR repetitions (technical replicates). Note that 25 cycles were used in the sqRT-PCR and that this was verified to be in the exponential phase as described in detail in “Materials and methods”

Discussion

Monoterpenes from essential oils interact with the GA-mediated tuber bud dormancy release in a concentration-dependent and hormesis-type manner: high concentrations inhibit, and low concentrations enhance the induction of visible sprouting

The molecular mechanisms by which post-harvest potato tuber bud sprout inhibitors act differ between CIPC and

monoterpenes (e.g. Oosterhaven et al. 1995a, b; Baydar and Karadoğan 2003; Kleinkopf et al. 2003; Eshel et al. 2009; Campbell et al. 2010; Owolabi et al. 2010; Teper-Bamnolker et al. 2010). In contrast to CIPC, the inhibition of post-harvest tuber sprouting by monoterpenes from essential oils appears not to be by interference with the cell cycle, as *S*-carvone (CAR) has no effect on flow cytometric histograms of potato cell nuclei (Oosterhaven et al. 1995a). An increase in bioactive GA contents was not associated with potato tuber dormancy release during post-harvest

storage, but was clearly associated with subsequent visible sprout growth (Oosterhaven et al. 1995b; Suttle 2004). However, it is clear from several publications (e.g. Clegg and Rappaport 1970; Davies and Viola 1988; Hartmann et al. 2011) that GA-treatment causes tuber bud dormancy release, initiates bud sprouting and promotes subsequent sprout growth as well as starch degradation. This is in agreement with conclusions that GA and ABA signalling, as well as the GA/ABA ratios, are important as is known for tree bud sprouting (Horvath et al. 2003; Rohde et al. 2007), seed germination of eudicots (Finch-Savage and Leubner-Metzger 2006; Holdsworth et al. 2008; Linkies et al. 2009; Bassel et al. 2011; Voegelé et al. 2011) and monocots (Barrero et al. 2009; Gerjets et al. 2010), and for the induction of α -amylases in the endosperm of germinated cereal grains (e.g. Mitsui and Itoh 1997; Lovegrove and Hooley 2000; Leubner-Metzger 2007). Our work shows that essential oil monoterpenes interact with the GA-mediated tuber bud dormancy release, bud sprout initiation, subsequent sprout growth and α -amylase gene expression, and suggests that GA signalling components are molecular targets.

In agreement with published work (Clegg and Rappaport 1970; Hartmann et al. 2011), we show that GA-treatment induces dormancy release, initiates visible sprouting as well as α - and β -amylase induction by using a potato tuber disc bud sprouting assay. This assay was used by us to study the effect of PMO and the monoterpene CAR on these inter-related processes. Monoterpenes are major constituents of essential oils and PMO contains 40.5–48.4% menthol, 14.6–22.1% menthone, ca. 6% 1,8-cineole, ca. 2% limonene, plus other monoterpenes, but no carvone (Rohloff 1999; Dimandja et al. 2000; Maffei et al. 2001). In contrast to PMO, spearmint oil contains 37.7% carvone plus other relative percentages for menthol, menthone, limonene and other monoterpenes. Carvone is also a major constituent of caraway oil (54.9% *S*-carvone). The essential oils including those from peppermint (our work), spearmint (Teper-Bamnolker et al. 2010) and other species (Owolabi et al. 2010), as well as carvone, menthol, menthone, limonene and other monoterpenes are known to act as potato tuber bud sprout inhibitors (e.g. Vaughn and Spencer 1991; Oosterhaven et al. 1995a, b; Baydar and Karadoğan 2003). *S*-carvone (CAR) has the promising potential as a commercial sprout inhibitor, and also *R*-carvone and menthol are highly active if applied to potato tubers in storage (Oosterhaven et al. 1995a, b; Eshel et al. 2009; Teper-Bamnolker et al. 2010). We demonstrate using a potato tuber disc bud sprouting assay that simultaneous 2-h treatment with GA plus high-dose CAR or PMO effectively inhibits bud dormancy release as scored by initial sprout growth during the subsequent incubation, almost as it is the case for CIPC (Fig. 2). In contrast to high

concentrations, and as an unexpected surprise, low-dose CAR or PMO concentrations enhanced the effect of the GA-treatment on bud dormancy release. That GA causes bud dormancy release in the tuber discs sprouting assay has already been shown before (Clegg and Rappaport 1970; Hartmann et al. 2011), but these authors did not study the effect of monoterpenes. Our monoterpene results are also in agreement with recent work by Teper-Bamnolker et al. (2010) on entire tubers during storage. These authors did not use tuber discs sprouting assay and GA-treatment, but showed that treatment of stored potato tubers with a low-dose *R*-carvone promoted visible sprouting, while high doses inhibited sprouting and were phytotoxic, causing necrosis.

Such a dose–response phenomenon which is characterised by a low-dose stimulation and a high-dose inhibition is known as hormesis (e.g. Stebbing 1982; Duke et al. 2006; Calabrese and Blain 2009). Some substances, although toxic in higher doses, can be stimulatory or even beneficial at low doses. Dose–response concepts like the widely accepted threshold models (Bradford and Trewavas 1994) are probably insufficient to describe such bi-phasic pattern, but the acceptance of hormesis as a viable dose–response concept is still a matter of debate in different disciplines from biomedicine to plant biology (e.g. Duke et al. 2006; Calabrese et al. 2007; Kendig et al. 2010). Examples for hormesis-type responses from plant biology include the herbicide glyphosate, which at low doses can stimulate crop growth (Duke et al. 2006; Velini et al. 2008), low-dose stimulation of pollen germination and tube growth by cadmium (Xiong and Peng 2001), as well as the promotion of *Arabidopsis* hypocotyl growth by low-dose treatment with the plant hormone ethylene, which at higher doses inhibits growth (Pierik et al. 2006). Allelochemicals, including monoterpenes, produced by donor plants are proposed to mediate growth responses of target plants, but very little is known about the underlying mechanisms (Inderjit and Duke 2003). It is proposed that low doses of allelochemicals may act by stimulating mild stress responses, while high doses are phytotoxic. Examples for hormesis-type dose responses of putative allelochemicals are in most cases just descriptive and include effects on plant growth (An 2005; Duke et al. 2006), as well as α -amylase activity accumulation in germinating seeds (Lovett et al. 1989). We found a hormesis-type response of CAR and PMO on the GA-mediated tuber disc bud dormancy release, i.e. low-dose of CAR or PMO enhance dormancy release and initiate bud sprouting, while high doses inhibit it. This effect was specific for the dormancy release, as CAR or PMO did not affect the rate of subsequent sprout growth after its onset. In contrast to our finding that low or high dose (except for the very high and thereby phytotoxic dose GA + 1000CAR in Fig. 2b) of

CAR or PMO did not affect the rate of sprout growth from GA-treated tuber bud discs, Oosterhaven et al. (1995a) found in experiments that did not involve GA-treatment that CAR inhibits the rate of sprout growth from stored tubers. In addition to the hormesis-type response of CAR and PMO on the GA-mediated tuber disc bud dormancy release, we found that they had concentration-dependent and tissue-specific effects on the expression of α - and β -amylases as discussed in the subsequent sections.

GA-mediated potato tuber bud sprouting is associated with tissue-specific differences in the expression pattern of α -amylases important for the initial attack of potato tuber starch granules and subsequent starch degradation by β -amylases and other enzymes

While contradicting results were obtained for phosphorytic starch degradation of sprouting potato tubers (as discussed in Clegg and Rappaport 1970; Biemelt et al. 2000; Smith et al. 2005), the situation is clearer for the amylolytic enzymes, i.e. for α - and β -amylases, transcript and enzyme activity accumulated in ‘sub-eye’ tissue during storage and sprouting (Biemelt et al. 2000). Furthermore, although the temporal and spatial patterns may differ, α - and β -amylases accumulate eventually in potato tubers during post-harvest storage/sprouting and cold sweetening (Davies and Viola 1988; Smith et al. 2005; Kaplan et al. 2006). We found that α - and β -amylase transcripts and enzyme activities increase in GA-treated potato tuber bud discs in association with bud sprout growth. In agreement with this, by using a similar tuber discs bud sprouting assay, Hartmann et al. (2011) showed by transcriptome analysis that GA-treatment caused α - and β -amylase transcript accumulation, and Clegg and Rappaport (1970) showed that GA-treatment promoted α -amylase activity accumulation. In agreement with the findings for tubers during storage (Biemelt et al. 2000), we found using the tuber discs bud sprouting assay that there were GA-related and tissue-specific distinct patterns for the α - and β -amylase regulation. In sprouts and base (sub-eye) tissues, GA-treatment was required for the α - and β -amylase activity accumulation (Figs. 1, 3). Our results are in agreement with the proposal of Biemelt et al. (2000) that initial sprout growth does not depend on starch degradation, but that maintenance of sprout growth may depend on starch degradation. Our finding that GA-treatment was required for the α - and β -amylase activity accumulation, combined with the importance of GA for sprout growth (Suttle 2004), supports the view that starch degradation is important for the maintenance of sprout growth.

We found that monoterpenes interact with the GA-induced bud dormancy release in a hormesis-type manner and that the low-dose CAR treatment also causes a ca. threefold

enhanced α - and β -amylase accumulation in day-7 sprouts, while the high-dose CAR treatment either does not appreciably affect the enzyme activity (α -amylase) or inhibits it (β -amylase). Although both enzymes show an enhanced response to low-dose CAR treatment and a reversion of this by high-dose CAR treatment, a classical bi-phasic (clear inhibition by high-dose) hormesis-type response was only evident for β -amylase. The enhanced response to low-dose CAR of α - and β -amylase in growing sprouts was, however, not evident for PMO. This demonstrates that the dose-dependent effect is specific for CAR and not a general stress response to monoterpene treatments (PMO does not contain CAR, but other monoterpenes contain CAR; see above). It further supports the view that the enhancement of the α - and β -amylase expression above the GA-induced levels is not causally required for the initiation of visible sprouting, as the low-dose treatment with CAR as well as with PMO both cause earlier onset of sprout growth. The enhanced response by low-dose CAR of the α - and β -amylase activities is furthermore tissue specific, as no enhancement of the GA-mediated accumulation occurs in the base (sub-eye) tissue. Taken together, these findings suggest that the regulation of α - and β -amylase activities is distinct regarding GA-requirement, tissue specificity, as well as the dose-dependent and molecule-specific effects by monoterpenes. This suggests that specific genes of these enzymes may be involved and are regulated in a distinct manner.

S-(+)-carvone confers a concentration-dependent interaction with the GA-induced transcript abundances of group-2 (α -AMY2) α -amylases in potato tuber bud sprouts, but does not affect the expression of the group-1 α -amylases (α -AMY1)

We further analysed if the enhanced low-dose response of CAR is also evident on the level of the α -amylase transcripts and can be assigned to specific genes, we cloned potato sprout α -amylase cDNAs. The obtained sequences were compared with all known potato α -amylases and the molecular features, characteristic domains and sequence similarities are described in detail in “Results”. The molecular phylogenetic analysis demonstrated that the known potato α -amylases cluster into two distinct groups (Fig. 4a). The potato α -AMY2 group members are highly similar in cDNA and amino acid sequences, protein domains and all have a high-pI value. They are clearly distinct from the potato α -AMY1 group which appears to be more diverse. Transcripts of α -AMY2 and α -AMY1 group are expressed in sprout and base tissues of GA-treated tuber bud discs as shown by us with cDNA cloning (*StAMY1a1*, *StAMY2a1*, our work) and sqRT-PCR amplification, and by transcriptome analysis (amyA1, Hartmann

et al. 2011). The two distinct groups of potato α -amylases allowed the design of group-specific primers for the comparative analysis of the α -AMY2 and α -AMY1 transcript expression pattern by sqRT-PCR. The sqRT-PCR assay that we established allowed the simultaneous amplification of α -AMY2-, α -AMY1- and rRNA-bands in single-tube reactions, and could therefore be used for the direct comparison of the relative transcript abundances for the two distinct potato α -amylase groups.

We found that in sprouts, CAR interacts with the GA-induced accumulation of α -AMY2 transcripts in an enhanced low-dose response manner, while it did not affect the α -AMY1 transcript abundances (Fig. 5). Low-dose CAR treatment caused ca. twofold higher α -AMY2 transcript abundances in sprouts, while high-dose CAR treatment had no effect. This promoting effect of low-dose CAR treatment was sprout specific as it was not evident in the base tissue. We conclude from these findings that CAR confers a tissue- and concentration-specific interaction with the GA-induction of the α -AMY2 genes, but not the α -AMY1 genes. We assume that α -amylase activity increase by low-dose CAR treatment is due to translation from de novo synthesised α -AMY2 transcripts. We propose that the observed enhanced low-dose response for the α -amylase is achieved by specific enhancement of α -AMY2 gene transcription. This low-dose enhancement is therefore not a general stress response, but seems to directly interact with a transcription factor that binds and regulates specifically the α -AMY2, and not the α -AMY1 genes. It is furthermore specific for CAR as other monoterpenes present in PMO like menthol, menthone, 1,8-cineole, and limonene do not elicit this low-dose response.

In contrast to the specific enhanced low-dose response of α -AMY2 genes to CAR, the hormesis-type response for the interaction with the GA-mediated release of tuber bud dormancy was evident for CAR and PMO. It is therefore not specific to a certain monoterpene. This may be achieved by interacting with a more general component of the GA signalling pathway. A known GA-related mechanism for the dormancy release of seeds (Leubner-Metzger 2002, 2003), tree buds (Rinne et al. 2001, 2011) and potato tuber buds (Viola et al. 2007) is by the opening of plasmodesmata-related symplastic connections that allow cell-to-cell communication, metabolite flow and shifts in sink–source relationships between tissues. In contrast to low-dose treatment, high-dose treatment with monoterpenes can be phytotoxic in that it damages membranes and causes necrosis (Oosterhaven et al. 1995b; Teper-Bamnlker et al. 2010), alters the cytoskeleton and inhibits cell division (Inderjit and Duke 2003; Kriegs et al. 2010), and causes massive changes at the transcriptome level (Godard et al. 2008). Major conclusions from this transcriptome analysis are that treatment of Arabidopsis seedlings with

the monoterpenes myrcene and ocimene caused transcriptome changes for which the categories stress, membranes and transcription factors (TFs), including several hormone-related TFs, are clearly over-represented. The monoterpene treatment also induced several β -amylases, which is in agreement with the finding that β -amylase and starch breakdown have roles in responses to abiotic stress (Kaplan et al. 2006). Regarding the β -amylases, an interesting finding of Godard et al. (2008) is that their induction seems to be monoterpene specific: While myrcene treatment increased the transcript abundances of the β -amylases At4g15210 and At5g18670 ca. three- to four-fold, the fold induction with ocimene was only weak (1.1–1.3-fold). The finding that β -amylase transcript abundances are regulated in a monoterpene-specific manner is in agreement with our finding that β - and α -amylase activities are regulated by CAR, but not by the PMO-monoterpenes. Taken together, we found dose-, tissue-, gene- and monoterpene-specific interaction with GA signalling during potato tuber bud dormancy release and sprout growth. High monoterpene doses act via general stress-type response pathways, are phytotoxic and decrease the GA-mediated responses. The concentration-dependent interaction of monoterpenes with the expression of β - and α -amylase genes during this process is monoterpene specific and we propose that it is mediated by interaction with specific components of the GA signalling pathway that enhance the GA-mediated responses.

Acknowledgments Our work was funded by grants from the Deutsche Forschungsgemeinschaft (Grant DFG LE720/6 and MU3114/1-1) to G.L.-M. and K.M., respectively, and the Wissenschaftliche Gesellschaft Freiburg to A.L., A.V. and Kai Graeber (licences for Geneious bioinformatics software) which are gratefully acknowledged. We also acknowledge awards related to this work to S.R. by the Faculty of Biology in Freiburg, and to M.I. in the national competition ‘Schweizer Jugend forscht’ in Switzerland. For further information see: ‘The Seed Biology Place’ - <http://www.seedbiology.de>.

References

- An M (2005) Mathematical modelling of dose–response relationship (hormesis) in allelopathy and its application. *Nonlinearity Biol Toxicol Med* 3:153–172
- Barrero JM, Talbot MJ, White RG, Jacobsen JV, Gubler F (2009) Anatomical and transcriptomic studies of the coleorhiza reveal the importance of this tissue in regulating dormancy in barley. *Plant Physiol* 150:1006–1021
- Bassel GW, Lan H, Glaab E, Gibbs DJ, Gerjets T, Krasnogor N, Bonner AJ, Holdsworth MJ, Provart NJ (2011) Genome-wide network model capturing seed germination reveals coordinated regulation of plant cellular phase transitions. *Proc Natl Acad Sci USA* 108:9709–9714
- Baydar H, Karadoğan T (2003) The effects of volatile oils on in vitro potato sprout growth. *Potato Res* 46:1–8
- Biemelt S, Hajirezaei M, Hentschel E, Sonnewald U (2000) Comparative analysis of abscisic acid content and starch

- degradation during storage of tubers harvested from different potato varieties. *Potato Res* 43:371–382
- Bozonnet S, Jensen MT, Nielsen MM, Aghajari N, Jensen MH, Kramhøft B, Willemoës M, Tranier S, Haser R, Svensson B (2007) The ‘pair of sugar tongs’ site on the non-catalytic domain C of barley α -amylase participates in substrate binding and activity. *FEBS J* 274:5055–5067
- Bradford KJ, Trewavas J (1994) Sensitivity thresholds and variable time scales in plant hormone action. *Plant Physiol* 105:1029–1036
- Calabrese EJ, Blain RB (2009) Hormesis and plant biology. *Environ Pollut* 157:42–48
- Calabrese EJ, Bachmann KA, Bailer AJ, Bolger PM, Borak J, Cai L, Cedergreen N, Cherian MG, Chiueh CC, Clarkson TW et al (2007) Biological stress response terminology: Integrating the concepts of adaptive response and preconditioning stress within a hormetic dose–response framework. *Toxicol Appl Pharmacol* 222:122–128
- Campbell MA, Gleichsner A, Alsbury R, Horvath D, Suttle J (2010) The sprout inhibitors chlorpropham and 1,4-dimethylnaphthalene elicit different transcriptional profiles and do not suppress growth through a prolongation of the dormant state. *Plant Mol Biol* 73:181–189
- Clegg MD, Rappaport L (1970) Regulation of bud rest in tubers of potato, *Solanum tuberosum* L: VI. Biochemical changes induced in excised potato buds by gibberellic acid. *Plant Physiol* 45:8–13
- Cuttler EG (1978) Structure and development of the potato plant. In: Harris PM (ed) *The potato crop*. Chapman Hall, London, pp 70–152
- Davies HV, Viola R (1988) The effect of gibberellic acid on starch breakdown in sprouting tubers of *Solanum tuberosum* L. *Ann Bot* 61:689–693
- Delaplace P, Fauconnier ML, Sergeant K, Dierick JF, Oufir M, van der Wal F, America AH, Renaut J, Hausman JF, du Jardin P (2009) Potato (*Solanum tuberosum* L.) tuber ageing induces changes in the proteome and antioxidants associated with the sprouting pattern. *J Exp Bot* 60:1273–1288
- Destefano-Beltran L, Knauber D, Huckle L, Suttle J (2006a) Chemically forced dormancy termination mimics natural dormancy progression in potato tuber meristems by reducing ABA content and modifying expression of genes involved in regulating ABA synthesis and metabolism. *J Exp Bot* 57:2879–2886
- Destefano-Beltran L, Knauber D, Huckle L, Suttle JC (2006b) Effects of postharvest storage and dormancy status on ABA content, metabolism, and expression of genes involved in ABA biosynthesis and metabolism in potato tuber tissues. *Plant Mol Biol* 61:687–697
- Dimandja J-MD, Stanfill SB, Grainger J, Patterson DG (2000) Application of comprehensive two-dimensional gas chromatography (GC \times GC) to the qualitative analysis of essential oils. *J High Resol Chromat* 23:208–214
- Duke SO, Cedergreen N, Velini ED, Belz RG (2006) Hormesis: is it an important factor in herbicide use and allelopathy? *Outlooks Pest Manag*: 29–33. doi:10.1564/16feb10
- Eshel D, Orenstein J, Tsror L, Hazanovsky M (2009) Environmentally friendly method for the control of sprouting and tuber-borne diseases in stored potato. *Acta Horticult* 830:363–368
- Fernie AR, Willmitzer L (2001) Molecular and biochemical triggers of potato tuber development. *Plant Physiol* 127:1459–1465
- Finch-Savage WE, Leubner-Metzger G (2006) Seed dormancy and the control of germination. *New Phytol* 171:501–523
- Gerjets T, Scholefield D, Foulkes MJ, Lenton JR, Holdsworth MJ (2010) An analysis of dormancy, ABA responsiveness: after-ripening and pre-harvest sprouting in hexaploid wheat (*Triticum aestivum* L.) caryopses. *J Exp Bot* 61:597–607
- Godard KA, White R, Bohlmann J (2008) Monoterpene-induced molecular responses in *Arabidopsis thaliana*. *Phytochemistry* 69:1838–1849
- Guindon S, Gascuel O (2003) A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. *Syst Biol* 52:696–704
- Hartmann A, Senning M, Hedden P, Sonnewald U, Sonnewald S (2011) Reactivation of meristem activity and sprout growth in potato tubers require both cytokinin and gibberellin. *Plant Physiol* 155:776–796
- Hermann K, Meinhard J, Dobrev P, Linkies A, Pesek B, Heß B, Machackova I, Fischer U, Leubner-Metzger G (2007) 1-Aminocyclopropane-1-carboxylic acid and abscisic acid during the germination of sugar beet (*Beta vulgaris* L.): a comparative study of fruits and seeds. *J Exp Bot* 58:3047–3060
- Holdsworth MJ, Bentsink L, Soppe WJJ (2008) Molecular networks regulating *Arabidopsis* seed maturation, after-ripening, dormancy and germination. *New Phytol* 179:33–54
- Horvath DP, Anderson JV, Chao WS, Foley ME (2003) Knowing when to grow: signals regulating bud dormancy. *Trends Plant Sci* 8:534–540
- Inderjit, Duke SO (2003) Ecophysiological aspects of allelopathy. *Planta* 217:529–539
- Kaplan F, Sung DY, Guy CL (2006) Roles of β -amylase and starch breakdown during temperature stress. *Physiol Plant* 126:120–128
- Kendig EL, Le HH, Belcher SM (2010) Defining hormesis: evaluation of a complex concentration response phenomenon. *Int J Toxicol* 29:235–246
- Kleinkopf GE, Oberg NA, Olsen NL (2003) Sprout inhibition in storage: current status, new chemistries and natural products. *Am J Potato Res* 80:317–327
- Kriegs B, Jansen M, Hahn K, Peisker H, Samajova O, Beck M, Braun S, Ulbrich A, Baluska F, Schulz M (2010) Cyclic monoterpene mediated modulations of *Arabidopsis thaliana* phenotype: effects on the cytoskeleton and on the expression of selected genes. *Plant Signal Behav* 5:832–838
- Leubner-Metzger G (2002) Seed after-ripening and over-expression of class I β -1,3-glucanase confer maternal effects on tobacco testa rupture and dormancy release. *Planta* 215:959–968
- Leubner-Metzger G (2003) Functions and regulation of β -1,3-glucanase during seed germination, dormancy release and after-ripening. *Seed Sci Res* 13:17–34
- Leubner-Metzger G (2007) Samendormanz und Keimungskontrolle: Gene, Umweltfaktoren und Klimawandel. *Vorträge für Pflanzenzüchtung* 72:87–104
- Leubner-Metzger G, Amrhein N (1993) The distribution of hydroxycinnamoylputrescines in different organs of *Solanum tuberosum* and other Solanaceous species. *Phytochemistry* 32:551–555
- Linkies A, Müller K, Morris K, Turečková V, Cadman CSC, Corbineau F, Strnad M, Lynn JR, Finch-Savage WE, Leubner-Metzger G (2009) Ethylene interacts with abscisic acid to regulate endosperm rupture during germination: a comparative approach using *Lepidium sativum* and *Arabidopsis thaliana*. *Plant Cell* 21:3803–3822
- Lovegrove A, Hooley R (2000) Gibberellin and abscisic acid signalling in aleurone. *Trends Plant Sci* 5:102–110
- Lovett JV, Ryuntyu MY, Liu DL (1989) Allelopathy, chemical communication, and plant defense. *J Chem Ecol* 15:1193–1202
- Maffei M, Camusso W, Sacco S (2001) Effect of *Mentha* \times *piperita* essential oil and monoterpenes on cucumber root membrane potential. *Phytochemistry* 58:703–707
- Mitsui T, Itoh K (1997) The α -amylase multigene family. *Trends Plant Sci* 2:255–261
- Nielsen TH, Deiting U, Stitt M (1997) A β -amylase in potato tubers is induced by storage at low temperature. *Plant Physiol* 113: 503–510
- Oosterhaven K, Hartmans K, Scheffer J (1995a) Inhibition of potato sprout growth by carvone enantiomers and their bioconversion in sprouts. *Potato Res* 38:219–230

- Oosterhaven K, Poolman B, Smid EJ (1995b) *S*-carvone as a natural potato sprout inhibiting, fungistatic and bacteristatic compound. *Ind Crop Prod* 4:23–31
- Owolabi MS, Lajide L, Oladimeji MO, Setzer WN (2010) The effect of essential oil formulations for potato sprout suppression. *Nat Prod Commun* 5:645–648
- Pierik R, Tholen D, Poorter H, Visser EJ, Voesenek LA (2006) The Janus face of ethylene: growth inhibition and stimulation. *Trends Plant Sci* 11:176–183
- Pujadas G, Palau J (2001) Evolution of α -amylases: architectural features and key residues in the stabilization of the $(\beta/\alpha)_8$ scaffold. *Mol Biol Evol* 18:38–54
- Rinne PLH, Kaikuranta PM, van der Schoot C (2001) The shoot apical meristem restores its symplastic organization during chilling-induced release from dormancy. *Plant J* 26:249–264
- Rinne PL, Welling A, Vahala J, Ripel L, Ruonala R, Kangasjarvi J, van der Schoot C (2011) Chilling of dormant buds hyperinduces FLOWERING LOCUS T and recruits GA-inducible 1,3- β -glucanases to reopen signal conduits and release dormancy in *Populus*. *Plant Cell* 23:130–146
- Rohde A, Ruttink T, Hostyn V, Sterck L, Van Driessche K, Boerjan W (2007) Gene expression during the induction, maintenance, and release of dormancy in apical buds of poplar. *J Exp Bot* 58:4047–4060
- Rohloff J (1999) Monoterpene composition of essential oil from peppermint (*Mentha × piperita* L.) with regard to leaf position using solid-phase microextraction and gas chromatography/mass spectrometry analysis. *J Agric Food Chem* 47:3782–3786
- Smith AM, Zeeman SC, Smith SM (2005) Starch degradation. *Annu Rev Plant Biol* 56:73–98
- Stebbing AR (1982) Hormesis - the stimulation of growth by low levels of inhibitors. *Sci Total Environ* 22:213–234
- Stensballe A, Hald S, Bauw G, Blennow A, Welinder KG (2008) The amyloplast proteome of potato tuber. *FEBS J* 275:1723–1741
- Suttle JC (2004) Involvement of endogenous gibberellins in potato tuber dormancy and early sprout growth: a critical assessment. *J Plant Physiol* 161:157–164
- Taylor MA, George LA, Ross HA, Davies HV (1998) cDNA cloning and characterisation of an α -glucosidase gene from potato (*Solanum tuberosum* L.). *Plant J* 13:419–425
- Teper-Bamnolker P, Dudai N, Fischer R, Belausov E, Zemach H, Shoseyov O, Eshel D (2010) Mint essential oil can induce or inhibit potato sprouting by differential alteration of apical meristem. *Planta* 232:179–186
- Vaughn S, Spencer G (1991) Volatile monoterpenes inhibit potato tuber sprouting. *Am J Potato Res* 68:821–831
- Velini ED, Alves E, Godoy MC, Meschede DK, Souza RT, Duke SO (2008) Glyphosate applied at low doses can stimulate plant growth. *Pest Manag Sci* 64:489–496
- Viola R, Pelloux J, van der Ploeg A, Gillespie T, Marquis N, Roberts AG, Hancock RD (2007) Symplastic connection is required for bud outgrowth following dormancy in potato (*Solanum tuberosum* L.) tubers. *Plant Cell Environ* 30:973–983
- Voegele A, Linkies A, Müller K, Leubner-Metzger G (2011) Members of the gibberellin receptor gene family *GID1* (*GIBBERELLIN INSENSITIVE DWARF1*) play distinct roles during *Lepidium sativum* and *Arabidopsis thaliana* seed germination. *J Exp Bot*. doi:10.1093/jxb/err214
- Xiong ZT, Peng YH (2001) Response of pollen germination and tube growth to cadmium with special reference to low concentration exposure. *Ecotoxicol Environ Saf* 48:51–55
- Zeeman SC, Kossmann J, Smith AM (2010) Starch: its metabolism, evolution, and biotechnological modification in plants. *Annu Rev Plant Biol* 61:209–234