Running title: Role of GA and CK in potato tuber sprouting

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Reactivation of meristem activity and sprout growth in potato tubers require both cytokinin and gibberellin

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Reactivation of dormant meristems is of central importance for plant fitness and survival. Due to their large meristem size, potato tubers serve as model system to study the underlying molecular processes. The phytohormones cytokinins (CK) and gibberellins (GA) play important roles in releasing potato tuber dormancy and promoting sprouting, but their mode of action in these processes is still obscure. Here, we established an in vitro assay using excised tuber buds to study the dormancy-releasing capacity of GA and CK and show that application of GA$_3$ is sufficient to induce sprouting. In contrast, treatment with 6-benzylaminopurine induced bud break, but did not support further sprout growth unless GA$_3$ was administered additionally. Transgenic potato plants expressing Arabidopsis GA 20-oxidase or GA 2-oxidase to modify endogenous GA levels showed the expected phenotypical changes, and slight effects on tuber sprouting. The isopentyltransferase (IPT) from Agrobacterium tumefaciens and the Arabidopsis cytokinin oxidase/dehydrogenase 1 (CKX) were exploited to modify the amounts of CK in transgenic potato plants. IPT expression promoted earlier sprouting in vitro. Strikingly, CKX-expressing tubers exhibited a prolonged dormancy period and did not respond to GA$_3$. This supports an essential role of CK in terminating tuber dormancy and indicates that GA is not sufficient to break dormancy in the absence of CK. GA$_3$-treated wild-type and CKX-expressing tuber buds were subjected to a transcriptome analysis which revealed transcriptional changes in several functional groups including cell wall metabolism, cell cycle, auxin and ethylene signalling, denoting events associated with the reactivation of dormant meristems.
INTRODUCTION

Potato (*Solanum tuberosum*) is a staple food belonging to the three most important crops worldwide. Potato tubers are radially expanded stem axes formed by shortened internodes and nodes (“eyes”). They originate from underground stolons in a series of developmental processes comprising the cessation of growth at the apex, the swelling of the stolon by sub-apical radial growth and longitudinal cell divisions of the body (Xu et al., 1998). The latter process is accompanied by accumulation of starch and storage proteins and requires coordinated transcriptional and metabolic changes (Visser et al., 1994; Appeldoorn et al., 1999; Kloosterman et al., 2005; Kloosterman et al., 2008). After formation, potato tubers undergo a period of dormancy which is characterized by the absence of visible bud growth. It is generally accepted that the onset of dormancy coincides with the cessation of meristematic activity during tuber initiation (Burton, 1989). The length of the dormancy period depends on the genetic background and is affected by pre-harvest and post-harvest conditions (Sonnewald, 2001; Suttle, 2004a). With the onset of sprouting the tuber turns into a source organ supporting growth of the developing sprout. This is associated with structural and metabolic changes (Viola et al., 2007) as well as with major changes in gene expression pattern (Ronning et al., 2003; Campbell et al., 2008). Additionally, endogenous plant hormones play a critical role in regulation of dormancy and bud break (Hemberg, 1985; Suttle, 2004a).

Gibberellins (GA) and cytokinins (CK) are thought to be involved in release of dormancy (Smith and Rappaport, 1961; Turnbull and Hanke, 1985a), whereas abscisic acid (ABA) and ethylene have been associated with the onset and maintenance of tuber dormancy (Suttle, 1998b). While the role of ethylene in potato tuber dormancy is still obscure (Suttle, 2004a), the significance of ABA in regulation this process was first recognized by Hemberg (1949), while it was still known as β-inhibitor complex. Analyzing the ABA content of different potato varieties revealed a continuous decline in ABA during storage, but there was no correlation between the absolute ABA levels and the sprouting behavior of different potato varieties (Suttle and Hultstrand, 1994; Suttle, 1995; Biemelt et al., 2000). Therefore a threshold level of ABA for maintenance of dormancy could not be established. The observed decrease in ABA content during potato tuber storage is most likely due to activation of catabolism while dormancy advances (Destefano-Beltran et al., 2006).

So far, not much attention was paid to the role of indole-3-acetic acid (IAA) in controlling tuber dormancy and sprouting. Recently, Sorce and colleagues (2009) performed a detailed study to investigate how auxin influences tuber dormancy. Although the concentration of free
IAA in buds decreases during dormancy, additional immunolocalization experiments suggest that IAA might stimulate bud growth by enhancing early differentiation processes. Supporting this assumption, Faivre-Rampant et al. (2004) identified an auxin response factor gene (ARF6) as a marker for meristem re-activation in potato tubers.

Since their first isolation in 1935, GAs have been known to stimulate growth and their dormancy-terminating capacity in potato was first reported in the mid-1950s (Brian et al., 1955; Rappaport, 1956). Subsequent studies showed an increase of endogenous GA-like substances prior to or together with the onset of sprouting (Smith and Rappaport, 1961; Bialek and Bielinska-Czamecka, 1975), suggesting GA as a regulator of sprouting. Nevertheless, the role of GAs in dormancy regulation remains controversial, as quantitative analysis showed increased levels of GA₁ and GA₂₀, the predominant bioactive GA in potato and its immediate precursor, only in tubers that already exhibited actively elongating sprouts (Suttle, 2004b). Furthermore, manipulation of the endogenous GA-levels by ectopic expression of potato GA biosynthesis genes GA₂₀ox₁ and GA₂₀x₁ did not significantly alter tuber dormancy (Carrera et al., 2000; Kloosterman et al., 2007), indicating a role for GA in sprout growth rather than in dormancy release.

Cytokinins have been named for their ability to stimulate cell division and are involved in many growth and developmental processes. Early studies by Hemberg (1970) showed that both natural and synthetic CKs can rapidly induce sprouting in dormant tubers. Bioassays suggested an increase of endogenous CK levels before dormancy release, especially in the apical and lateral buds and the tissue surrounding them (Engelbrecht and Bielinska-Czamecka, 1972; van Staden and Dimalla, 1978). Evidence from immunological studies (Turnbull and Hanke, 1985b; Suttle, 1998a) confirmed an increase in bioactive CKs before dormancy release. Additionally, differences in sensitivity to applied CKs were observed in tuber tissues. At harvest and the beginning of the storage period, tubers were unresponsive to CK, but exhibited increasing sensitivity as dormancy progressed (Turnbull and Hanke, 1985b; Suttle, 2001). Suttle (2001) also found that this was not associated with changes in CK metabolism and hypothesized that CK signal perception and/ or transduction were influenced by the physiological status of the tuber.

On the cellular level, dormancy is most likely characterized by a G₁-phase arrest of the meristematic cells, as indicated by microdensitometry (Macdonald and Osborne, 1988) and flow-cytometry measurements (Campbell et al., 1996). Release from this arrest requires D-type cyclins (CycD) of which three groups have been isolated in Arabidopsis, CycD₁, CycD₂ and CycD₃ (Soni et al., 1995; Renaudin et al., 1996). During the cell division cycle, D-type
cyclins form complexes with cyclin-dependent kinases (CDKs) which then phosphorylate retinoblastoma-related (RBR) proteins. This releases E2F transcription factors and triggers G1-to-S-phase transition (reviewed in Horvath et al., 2003; Berckmans and De Veylder, 2009).

In this work we aimed at improving our understanding of the regulation of potato tuber dormancy and sprouting by GAs and CKs. Therefore, we have established an in vitro assay for synchronous induction of sprouting which enabled us to monitor developmental changes caused by GA or CK. Furthermore, we examined transgenic plants with increased or decreased levels of either phytohormone to investigate their impact on sprouting behavior. Finally, we performed microarray studies by applying our in vitro assay to transgenic plants with decreased CK levels and wild-type potato tubers which has allowed us to unravel the sequence of phytohormone action that leads to sprouting.

RESULTS

Treatment of excised potato tuber buds with gibberellic acid (GA₃) triggers synchronous sprouting.

Although potato tuber dormancy has been extensively studied, the coordinated fashion in which phytohormones regulate this process is little understood. This may be due to the non-synchronized sprouting of potato tubers, rendering kinetic studies during natural sprouting almost impossible. To circumvent this shortcoming and to synchronize sprouting, an in vitro sprout release assay was developed. The assay is based on isolated tuber buds which are placed on wet filter paper in closed petri dishes. Upon addition of different hormone solutions bud growth can be followed over time. Since GA₃ is known to support sprout growth (Suttle, 2004a), the initial assay was based on GA₃ applications.

To determine the optimal concentration of GA₃, different concentrations ranging from 5 µM to 100 µM were tested (Figure 1A). Regardless of the concentration tested, first signs of sprouting became visible three days after GA₃ treatment and after five days sprouts of 2 to 3 mm length could be clearly seen (Figure 1B). After 7 days, at least 95% of the buds treated with 25 µM, 50 µM or 100 µM GA₃ had sprouted (Figure 1A), the sprouts being thin and very elongated. A lower sprouting efficiency was observed for tuber buds treated with only 5 µM GA₃ (Figure 1A). Since there were essentially no differences in the ability to trigger sprouting between 25 µM and 100 µM GA₃, 50 µM was chosen for further experiments. Control tuber
discs treated with water showed no signs of sprouting during the period investigated (Figure 1B).

In the experiment shown in Figure 1, tuber discs were excised from tubers two weeks after harvest. Since a time-dependent increase in GA sensitivity during the dormancy period has been published (Suttle, 2004b), we also wanted to test whether or not GA$_3$ treatment can induce sprouting of discs taken from freshly harvested or deeply dormant tubers, and whether GA$_3$ responsiveness changes during storage. In all our experiments, incubation of tuber discs with 50 µM GA$_3$ was sufficient to break dormancy with similar kinetics. However, the efficiency was reduced in discs from freshly harvested tubers (Supplemental Figure 1A). Here, sprouting occurred in about 80% of tuber discs after six days, while almost all discs taken three or six weeks after harvest showed sprouting in the same time period. On the other hand, control tuber discs isolated from tubers stored for six weeks also started to sprout, although at a low rate (10%; Supplemental Figure 1A), indicating that these tubers had probably lost their endodormancy.

Together these results show that GA$_3$ can trigger tuber sprouting even of deeply dormant tubers within three days of treatment in a synchronous fashion, as is schematically depicted in Figure 1C.

Over-expression of GA2ox and GA20ox from Arabidopsis thaliana affects plant growth and morphology, but has only weak impact on potato tuber sprouting

To further investigate the role of GA in potato tuber dormancy, we generated transgenic plants with an altered endogenous GA content. The genomic Arabidopsis clones coding for GA2oxI (GA2ox) (accession no. AJ132435) and GA20oxI (GA20ox) (accession no. X83379) were inserted into the pBinAR binary vector between the CaMV 35S promoter and the octopin synthase (OCS) terminator as described in Biemelt et al. (2004). These constructs were used to transform potato plants via Agrobacterium-mediated gene transfer. About 60 transgenic lines were obtained per construct. Since the transgenic potato lines expressing GA20ox exhibited elongated shoots and potato plants expressing GA2ox showed a dwarf phenotype as it is typical for increased or reduced GA content, respectively, pre-screening was based on phenotype. Expression of either transgene was confirmed by Northern blot analysis of the pre-screened lines and three highly expressing lines for each construct were selected for further characterization (Figure 2A). Transformants over-expressing GA20ox exhibited elongated stems and light green leaves with elongated petioles (Figure 2B). At harvest the stem height was about twice that of wild-type controls (Table 1) and correlated
with the abundance of GA20ox transcript. These plants formed many long stolons, but both the number of tubers and tuber yield were reduced in the highest expressing line (Figure 2C; Table 1).

In contrast, stem length of GA2ox-expressing potato plants was strongly reduced (Figure 2B) and the leaves were small, thick and dark green compared to wild type. Stolons of these plants were shorter than those of the untransformed controls (Figure 2E). On average, fewer tubers were formed leading to a significantly decreased tuber yield (Table 1).

To study the effect of altered GA content on potato tuber dormancy, tubers of five plants per line were stored in darkness at room temperature directly after harvest and sprouting behavior was monitored. Control tubers started to sprout nine weeks after harvest and reached 100% 13 weeks after harvest. Onset of sprouting was quite similar in transgenic GA20ox-expressing tubers. Only in the highest expressing line GA20ox-58 sprouting was slightly earlier as compared to wild type (Figure 3A). Conversely, sprouting onset was delayed in GA2ox-expressing tubers (Figure 3A). Tubers of lines GA2ox-27 and GA2ox-50 started sprouting ten weeks after harvest and reached a rate of about 60% sprouted tubers after 14 weeks. Line GA2ox-38 exhibited the strongest delay in sprouting with the first sprouts appearing three weeks later than in the wild type. Similar to the observed changes in stem growth, sprouts of GA20ox-expressing lines were strongly elongated and thinner, whereas sprouts formed by GA2ox-expressing tubers were shorter and thicker (Figure 3B).

Taken together, constitutive over-expression of GA20ox did not lead to a significantly altered dormancy period, whereas transgenic potato tubers expressing GA2ox showed a slightly prolonged rest period.

Gibberellin measurements confirm changes in endogenous GA levels in the transgenic lines

Although the strong growth phenotype of the transgenic plants indicated changes in GA content, only an undetermined effect on tuber dormancy could be observed. Therefore we aimed at confirming altered content of endogenous GAs in the transgenic potato lines.

Initially, we sought to determine the content of different GAs in buds of dormant and sprouting tubers. However, as published previously, GA levels were below the detection limit in tuber tissues (Morris et al., 2006). Therefore we determined endogenous GA levels in apical shoot tips of transgenic and wild-type plants. Consistent with previous reports (Van den Berg, 1995; Morris et al., 2006) the major bioactive GA detected in wild-type plants was GA1 (Figure 4). In addition, high levels of its precursor GA20 and its inactivation product GA8 were measured, as well as small amounts of the bioactive GA4. Strikingly, levels of all products of
the early 13-hydroxylation pathway, the main path of GA biosynthesis in potato plants (Van den Berg, 1995), such as GA44, GA19, GA20, GA1, GA29 and GA8 were reduced in GA20ox-expressing lines. Nevertheless, expression of GA20ox led to an increase in the amount of the bioactive GA4 and its inactivation product GA34 (Figure 4). This indicates that over-expression of the GA20ox in potato caused a shift to the 13-non-hydroxylation pathway emulating the major path in Arabidopsis (Sponsel et al., 1997; Coles et al., 1999).

Analysis of GA content in GA2ox-expressing potato plants revealed a clear decrease in the amount of GA1 compared to wild type and an increased content of GA29 and GA34, which are inactivation products of the 13-hydroxylation and 13-non-hydroxylation pathways, respectively (Figure 4). Together with the observed phenotypic changes this is consistent with a high GA2ox activity in these transgenic potato plants.

Since there was an obvious shift towards GA4 as major bioactive product in the GA20ox plants, we wanted to investigate whether GA4 treatment is capable of triggering tuber sprouting in our sprout release assay in a similar fashion to GA3 and GA1. Therefore, discs of wild-type tubers were treated with 50 µM GA1, GA3 or GA4. Water served as negative control. As shown in Supplemental Figure 2, all GAs tested induced tuber sprouting within three days. Even though GA1 and GA4 appeared to have slightly lower performance more than 90% of tuber discs had sprouted after six or seven days. Thus, the shift to the 13-non-hydroxylation pathway of GA biosynthesis in GA20ox-expressing lines would not account for the weak effect on tuber dormancy.

Expression of Arabidopsis GA20ox under control of the chimeric STLS1/CaMV35 promoter leads to early tuber sprouting

Another possible explanation for the small effect on sprouting in GA20ox tubers could be that expression of the transgene in buds was too low and the resulting production of bioactive GAs was not sufficient to induce sprouting. In another study, substantially higher expression of a target gene (E. coli pyrophosphatase) was obtained in potato tubers by using a chimeric STLS1 enhancer-CaMV 35S promoter compared to the unmodified CaMV 35S promoter (Hajirezaei and Sonnewald, 1999). Thus, in order to achieve elevated GA20ox expression in potato tubers, a construct was designed containing the GA20ox clone under control of the chimeric STLS1/CaMV 35S promoter and transformed into potato plants by Agrobacterium-mediated gene transfer. More than 50 transgenic plants (named STLS1/35S:GA20ox) were regenerated, transferred into the greenhouse and pre-screened for elongated shoot growth.
compared to untransformed wild-type plants. Expression of the transgene was confirmed for five lines by northern blotting (Supplemental Figure 3) and three highly expressing lines were selected for further analyses. After propagation in tissue culture five plants per lines were cultivated in the greenhouse. Tubers were harvested after about twelve weeks, stored in darkness at room temperature and sprouting behaviour was monitored. Five weeks after harvest both wild-type and STLS1/35S:GA20ox tubers were still dormant. However, eight weeks after harvest sprouting had already commenced in up to 10% of the transgenic tubers and three weeks later almost 80% of these tubers had developed long sprouts (Figure 5A, B). In contrast, first signs of visible sprouting in wild-type tubers were apparent after nine weeks and a rate of 62% sprouting was observed after twelve weeks (Figure 5A). However, almost all wild-type and transgenic tubers had started sprouting after thirteen weeks.

In order to compare the expression level of the GA20ox in tubers expressing the gene under control of the chimeric and the unmodified CaMV 35S respectively, a northern blot analysis was performed on samples taken during tuber storage. As shown in Supplemental Figure 4, there is a more constant expression of the GA20ox gene under control of the STLS1/CaMV 35S promoter during the rest period of potato tubers compared to the unchanged CaMV 35S promoter. This was especially marked after two and three months of storage when sprouting is initiated.

Together these results show that higher expression of GA20ox was obtained in potato tubers by using the chimeric STSL1/CaMV 35S promoter which led to an earlier onset of tuber sprouting. This supports the idea that GA is able to terminate tuber dormancy and promotes sprout outgrowth.

*Exogenously applied cytokinin breaks tuber dormancy in sprout release assays*

Subsequently, we wanted to study the role of CKs in tuber sprouting. Cytokinins were suggested to be the “primary factor” regulating the switch from a dormant to a non-dormant state (Turnbull and Hanke, 1985a). In their experiments the authors showed that CKs were able to trigger tuber sprouting, but this was dependent on “tissue sensitivity” (Turnbull and Hanke, 1985a).

To investigate the ability of CK to break tuber dormancy, we applied 6-benzylaminopurine (BAP) to excised tuber buds in the sprout release assay. Again discs containing a single bud were excised from tubers two weeks after harvest and different concentrations ranging from 5 µM to 100 µM BAP were tested. Similar to GA3-treated tuber discs, first signs of sprout induction became visible three days after BAP application (Figure 6). But in contrast to GA3,
BAP did not support further sprout outgrowth even after longer observation times. Soon after bud break, sprout growth was arrested at a length of ca. 0.5 – 1.5 mm (Figure 6B). Generally, treatment with 5 µM BAP results in the lowest frequency of induced bud break, with 25 µM, 50 µM and 100 µM BAP inducing increasingly higher rates of bud break, although by seven days these three dosages gave similar proportions of open buds (> 80%) (Figure 6A). The water controls remained dormant during the period investigated.

In order to investigate whether the sensitivity to BAP changes with tuber age, tuber discs from freshly harvested tubers and tubers stored for three or six weeks were sampled and incubated with 50 µM BAP (Supplemental Figure 1B). The response to application of BAP was comparable in tubers that had been stored for three or six weeks, resulting in bud break that was first detectable after three days. In contrast, sprouting efficiency was clearly decreased in tuber samples taken directly after harvest, where bud break occurred one day later. Five days after treatment about 35%, 80% or 90% of discs showed open buds in samples taken from freshly harvested, three or six weeks stored tubers, respectively. However, at the end of the experiment more than 80% of tubers showed broken dormancy regardless of the previous storage time (Supplemental Figure 1B).

Although differences in the kinetics of the BAP response were found, these results reveal that CK induces bud break, but it does not support further sprout growth during the endodormancy period. Notable sprout elongation was achieved only by an additional application of one droplet of 50 µM GA3 onto the bud (Supplemental Figure 5).

*Over-expression of IPT severely affects plant growth and morphology.*

To further investigate the role of CK in regulation of potato tuber dormancy, we aimed at generating transgenic potatoes with modified levels of active CKs. In order to increase endogenous CK content, plants were transformed with the *isopentenyltransferase (IPT)* gene (accession no. AF242881) from the *Agrobacterium tumefaciens* T-DNA, which encodes a rate-limiting enzyme, under control of the CaMV 35S promoter. Out of two rounds of transformation 15 transgenic plants could be regenerated. Three lines already displayed a typical CK responsive phenotype with bushy growth, small leaflets and inhibition of root growth (Figure 7B) in tissue culture. Two of the lines did not survive the transfer into soil. A third one (line 9) was characterized by strongly retarded growth, a loss of apical dominance, small leaves and a severe reduction in tuber yield under greenhouse condition (Figure 7B; Table II). Since only very few and small tubers were formed, these could not be further analyzed. However, another line (IPT-6) was obtained which resembled wild-type plants with
respect to growth and tuber yield. PCR analysis confirmed lower expression of the \textit{IPT} gene compared to the other lines (Figure 7A). Tubers were stored at room temperature in darkness and sprouting behavior was monitored. Sprout induction of \textit{IPT}-expressing tubers was not significantly earlier than in wild-type tubers (Figure 7C), but intriguingly, they formed thicker and longer sprouts as shown by cross sections through spouting tuber buds (Figure 7D). To further investigate whether \textit{IPT} expression leads to an earlier onset of sprouting, tuber discs of dormant tubers were excised and tested for their sprouting behavior using the in vitro sprout release assay. As described before, tuber buds derived from wild-type controls started to sprout three days after GA3 treatment. In contrast, transgenic tubers responded one day earlier to the GA3 treatment as shown in Figure 7E. About 25% of tuber discs had already started sprouting after two days, indicating that CKs may be important for initiation of tuber sprouting.

\textit{Transgenic potato plants expressing a cytokinin oxidase show an altered phenotype and a prolonged dormancy period.}

The catabolic enzyme CK oxidase/dehydrogenase (CKX) was shown to play a critical role in controlling CK levels in plants and was successfully exploited to reduce the endogenous content of CKs by means of over-expression in transgenic tobacco or Arabidopsis plants (Werner et al., 2001; Werner et al., 2003). We have chosen the Arabidopsis \textit{CKX}1 (\textit{CKX}) gene (At2g41510) for expression in potato plants to analyze the impact of decreased CK content on tuber dormancy. To this end the gene was amplified by PCR from Arabidopsis cDNA and cloned into the BinAR vector (Höfgen and Willmitzer, 1990). The construct was transformed into potato plants by \textit{Agrobacterium}-mediated gene transfer and a total of 12 transgenic plants were obtained. Based on northern blot analysis three lines with different expression levels of the transgene were selected for more detailed analysis, with CKX-4 exhibiting the strongest and CKX-11 the lowest expression of the \textit{CKX} gene (Figure 8A).

Plants were propagated in tissue culture and transferred into the greenhouse. Compared to wild type, transgenic lines showed reduced shoot growth and the degree of retardation in final shoot size correlated with the expression of the transgene (Figure 8A, B; Table II). Moreover, the plants produced a high number of fragile side shoots and had a lower number of leaflets per leaf. The strongest line, CKX-4 formed only single lanceolate leaves instead of the typical compound leaves (Figure 8C). With increasing \textit{CKX} expression tuber yield was reduced severely and only few, small, drop-like shaped tubers were formed (Table II, Figure 8D). Cross section through the tuber buds revealed that the size of the meristem is much smaller.
than that of wild-type tubers and that the two main vascular bundles were in close proximity to each other (Figure 8E). Interestingly, onset of sprouting of stored tubers was delayed five to eight weeks in CKX-expressing tubers compared to wild type and the delay correlated with the CKX transcript abundance (Figure 8A, F). Moreover, while sprouts of lines CKX-10 and CKX-11 developed normally after bud breakage, sprouts formed by strong expressing line CKX-4 remained diminutive and failed to achieve proper growth (data not shown), further supporting the assumption that CKs are important players for the re-activation and maintenance of meristematic activity during onset of tuber sprouting.

To further investigate the observed delay in sprouting of CKX-expressing tubers, we isolated buds from wild-type tubers and from of the strongest expressing line CKX-4 and performed a sprout release assay using 50 µM GA3. While sprouting could be induced in wild-type tuber buds as observed before, no signs of sprouting could be seen in transgenic tuber buds even when kept for a longer time (9 days) (Figure 9A). To confirm that this phenotype is due to a reduced level of CKs, CKX-expressing tuber discs were also incubated with 50 µM BAP. As shown in Supplemental Figure 6, BAP treatment of the transgenic tubers restored the wild-type phenotype.

In addition, tuber discs taken from the strongest GA20ox-expressing line 58 were also incubated with 50 µM BAP. While in wild-type tubers BAP treatment induced only bud break, it also promoted outgrowth of the sprout in tubers with increased GA levels (Figure 9B). Together these data led us to conclude that GA requires CKs to release tuber dormancy.

Microarray analysis of GA3-treated wild type and CKX-expressing tubers

In order to explore the cause for the lack of sprouting of CKX-expressing tubers after GA3 application, a transcript analysis was conducted using microarrays. To this end, dormant wild-type and CKX-4 tuber buds were subjected to a sprout release assay and transcript profiles were prepared from samples taken immediately (0 d) and after three days of GA3 treatment using the Agilent 4x44K array (Kloosterman et al., 2008). As before, wild-type tubers started sprouting three days after GA3 treatment and in about 30% of the samples little sprouts were already visible, indicating meristem re-activation and the beginning of sprout outgrowth (Figure 1). In contrast, no sign of sprouting could be seen in CKX-4 tubers.

Data analysis was performed as described in the “Materials & Methods” chapter using the GeneSpring GX7.3.1 software. Comparing expression profiles at 0 and 3 days after GA3 application, 4410 and 5629 features were found to be differentially expressed in wild-type and
CKX-4 tuber buds, respectively (Table 3, Tables S1, S2). Among them 2792 and 3218 features were up-regulated, while 1617 and 2411 were down-regulated in wild-type and CKX-4 tuber buds, respectively. The differentially regulated genes were grouped into 15 functional categories (Figure 10), with two groups, transcripts of unknown function and transcripts which could not be assigned to any other category, accounting for about 50% of the transcripts. With induction of sprouting in wild-type tubers, there was a clear increase in the percentage of transcripts falling into the functional groups “cell cycle activity and replication”, “cytoskeleton” and “cell wall”, which indicates increased cellular metabolism and cell proliferation during the re-activation of meristematic activity (Figure 10, Table S1). In contrast there was no increase or even a lower number of transcripts up-regulated in these categories in CKX-4 tuber buds after three days, reflecting the observation that no sprout growth has been initiated. Closer inspection of the data revealed that transcript quantity of most cell cycle regulators such as cyclins A, B, and D as well as the cyclin-dependent kinases CDK B was strongly increased in wild-type tubers, correlating with resumption of growth as illustrated using the MapMan tool in Figure 11. This was accompanied by an activated replication as indicated by a clearly increased expression of marker genes such as histone H4, histone H3 and deoxyuridine triphosphatase (dUTPase) (Brandstader et al., 1994; Senning et al., 2010) (Table S1). Strikingly, transcripts coding for components of the cytoskeleton that are necessary for cell division and expansion are predominantly increased in wild-type tuber buds three days after GA3 treatment. In addition, a number of genes controlling biosynthesis of cellulose (cellulose synthase) and genes involved in cell wall modification and loosening, such as polygalacturonases, pectin-(methyl)esterases and xyloglucan endotransglucosylase-hydrolases (XTH) were found to be induced exclusively in wild-type tubers (Tables S1, S2). This is in line with the fact that cell proliferation and expansion require cell wall biosynthesis and assembly as well as extensive modifications.

In relation to hormone signaling, most obvious differences between wild-type and CKX-expressing tubers were apparent for ethylene and auxin signaling pathways which were also visualized using the MapMan tool (Figure 12, 13). The entire ethylene signaling pathway appeared to be activated more strongly in CKX-4 tuber buds compared to wild type with the exception of the key transcription factor ETHYLENE-INSENSITIVE 3 (EIN3), expression of which is about six-fold down-regulated three days after GA3 treatment, probably due to the transcriptional activation of the F-Box proteins EIN3-BINDING F-BOX 1 and 2 (EBF1 and EBF2) which are in turn controlled by the activity of EIN5, a 5'-3’ exoribonuclease (recently reviewed by Stepanova and Alonso, 2009; Yoo et al., 2009). Expression of transcripts with
homology to *EBF1*, *EBF2* and *EIN5* was found to be two- to four-fold induced three days after GA$_3$ treatment of CKX-4 tubers, while there was a weaker induction in wild-type tubers with the exception of a gene coding for an ortholog of *At-EBF1* (Micro.1924.C1) (Figure 12, Tables S1, S2). Beside the *Ethylene Response Factors* (*ERF*) *EBF2* is the only gene directly activated by *EIN3* and *EIN3-like* (*EIL*) transcription factors, most likely as part of a rapid feedback regulatory loop. Among the *ERF*s, various ESTs with homology to *ERF5* from Arabidopsis or tomato showed strongly increased transcript abundance in the CKX-4 line in the sprout release assay when compared to the wild type (Figure 12; Tables S1, S2).

In wild type, we found a clear increase in transcripts coding for two different types of auxin biosynthetic enzymes, namely aldehyde oxidase(s) and flavin monooxygenase-like protein (*Yucca*-likes in Arabidopsis) three days after GA$_3$ incubation, while either their total expression level was lower or their induction was less pronounced in CKX-4 (Tables S1, S2). In accordance with a stimulated auxin biosynthesis there was a substantial increase in the expression of several of the primary auxin-responsive genes *SAUR* (small auxin up-regulated RNAs) and *GH3*-type (reviewed in Hagen and Guilfoyle, 2002) as well as of *auxin influx* and *PIN1*-like auxin efflux carrier(s) in wild-type as compared to CKX-4 tubers (Figure 13). Interestingly, the auxin response factor *ARF4* was induced up to 3.5-fold with onset of sprout outgrowth in wild-type tubers, while in CKX-4 *ARFs 2*, *10* and *19* were strongly transcriptionally activated by GA$_3$.

Fewer differences between the genotypes were found in the expression of GA and CK biosynthetic and signaling genes. The amount of transcripts for several GA biosynthetic genes such as *ent-kaurene oxidase*, *GA2ox*, or *GA3ox* as well as of the *GA-receptor* declined in both genotypes three days after GA$_3$ application. These changes could be directly or indirectly caused by the GA$_3$ treatment. However, expression of *Gip1*-like genes was exclusively induced in wild-type tubers (Tables S1, S2). As expected from the genetic modification, expression of purine transport proteins as well as those of some response regulators (e.g. *ARR4* homologue) was lower in the CKX-4 line than in wild type, both before and after GA$_3$ treatment. In contrast, increased transcript amounts were found for *zeatin O*-xylosyltransferase(s), encoding an enzyme thought to be involved in CK homeostasis (Martin et al., 1993).

A higher percentage of transcripts coding for metabolic enzymes was up-regulated than down-regulated following GA$_3$ treatment of both wild-type and CKX-4 tuber buds, indicating an accelerated metabolic rate (Figure 10). Among them were a high number of ESTs that showed similar changes of expression in both genotypes and can therefore be found within the
population of 1867 overlapping transcripts as determined by Venn diagrams (Table 3). However, ESTs coding for enzymes of fatty acid biosynthesis were clearly induced in wild-type tubers, suggesting an increased demand for building blocks of plasma membranes, whereas a high fraction of them was down-regulated in CKX-4 tuber buds (Figure 10). Interestingly, a number of transcripts coding for transcription factors involved in maintenance of the shoot apical meristem (Carraro et al., 2006) were among the group of transcripts with similar changes in expression level in wild type and CKX-4, e.g. the homeobox transcription factors knotted1-like and knotted2 or those of the class III HD-ZIP family. In contrast, expression of putative transcription factors with homology to AINTEGUMENTA (ANT), PHANTASTICA (PHAN), GROWTH-REGULATING FACTOR3 (GRF3) or OVATE which have been associated with organ differentiation and (out)growth (Kim et al., 2003; Hackbusch et al., 2005; Carraro et al., 2006) was more strongly or exclusively induced in GA3-treated wild-type tuber discs. These data also reflect that in wild-type tubers meristematic activity is regained upon GA3 application followed by outgrowth of the sprout, while in CKX-4 tubers initiation and outgrowth of the sprout cannot be induced, probably due to the loss of activation of cell cycle activity and downstream differentiation processes.

DISCUSSION

GA is sufficient to break tuber dormancy and to stimulate sprouting

Although the dormancy-terminating capacity of GA was shown a long time ago (Brian et al., 1955) there is still a debate about its mode of action (Suttle, 2004a). Early studies showed an increase of endogenous GA-like substances prior to or together with the onset of sprouting as summarized in Suttle (2004a), while recent analyses using GC-MS showed increased levels of GA1, GA20 and GA19, the predominant bioactive GA in potato and its direct precursors, only in tubers with actively elongating sprouts (Suttle, 2004b). Furthermore, both antisense expression of the endogenous GA-biosynthesis gene GA20ox1 and over-expression of GA2ox1 resulted in reduced GA levels and dwarf phenotypes, but did not affect tuber dormancy (Carrera et al., 2000; Kloosterman et al., 2007). In contrast potato plants with higher expression of potato GA20ox1 had elongated shoots and tubers exhibited a decreased dormancy period (Carrera et al., 2000). Together these results suggested a role for GA in sprout growth rather than in dormancy release (Suttle, 2004a).

In this study we used an in vitro assay to stimulate sprouting of excised tuber buds using GA3. This triggered sprouting independent of tuber age although efficiency was reduced in freshly harvested, deeply dormant tubers. However, six days after treatment more than 80% of these
tuber discs had started sprouting. A slightly different result was obtained by Suttle (2004b)
who injected different GA species of the 13-hydroxylation pathway into tubers. Only the
bioactive GA$_1$ was sufficient to terminate dormancy of tubers stored for a short time, while
also its precursors GA$_{19}$ and GA$_{20}$ promoted sprouting of aged tubers, indicating that GA20ox
activity is rate-limiting. Our experiments differed from that of Suttle (2004b) since we applied
GA$_3$, which is less rapidly metabolized, to isolated tuber discs containing one bud. This may
result in more stable effects. Nevertheless, in all studies, treatment with bioactive GA species
was sufficient to terminate tuber dormancy and to stimulate sprout outgrowth.

To further substantiate our observation, transgenic potato plants with modified GA-
biosynthesis were generated by heterologous expression of the Arabidopsis GA20ox1 or
GA2ox1 genes, respectively under control of the CaMV 35S promoter. The observed slender
or dwarf phenotypes of the plants were consistent with that of previous reports for over-
expression of GA20ox or GA2ox genes in Arabidopsis, tobacco or potato plants (Coles et al.,
1999; Carrera et al., 2000; Biemelt et al., 2004; Kloosterman et al., 2007) and supported the
important role of GA for plant growth (Hedden and Phillips, 2000). Similar to transgenic
potato plants with increased expression of the endogenous GA20ox1 or GA2ox1 genes
(Carrera et al., 2000; Kloosterman et al., 2007), tuber yield was reduced in both groups of
transgenic plants in our study. However, in our experiments, length of dormancy was
extended in GA2ox tubers, while it remained almost unchanged in GA20ox tubers, opposite to
previous observations (Carrera et al., 2000; Kloosterman et al., 2007). As in the other studies,
we could confirm changes in levels of different GA species in the transgenic plants.
Interestingly, over-expression of the Arabidopsis GA20ox1 in potato plants caused a shift
towards the 13-non-hydroxylation pathway, which is the prevailing pathway in Arabidopsis
(Coles et al., 1999), indicated by increased amounts of GA$_4$ and GA$_{34}$. A similar observation
was made by Vidal et al. (2001) by ectopic expression of the citrus GA20ox1 in tobacco
which caused an accumulation of GA$_4$ at the expense of GA$_1$. However, the shift to GA$_4$ in
the transgenic potatoes would not account for the failure to induce tuber sprouting in these
lines, since the dormancy-releasing ability of GA$_4$ was found to be quite similar to that of GA$_1$
and GA$_3$. One possible explanation for the different and weak effects in the transgenic plants
might be that the amount of bioactive GA(s) must exceed a critical level in certain cell
populations to exert its function. Hence, the constitutive CaMV 35S promoter originally used
in this study might not be sufficiently active in stored tubers to achieve the required
eexpression level. In fact, higher and more stable expression of GA20ox was obtained in aged
tubers when driven by a chimeric STLS1 enhancer/ CaMV 35S promoter (Hajirezaei and
Sonnewald, 1999) compared to the unmodified CaMV 35S promoter. Interestingly, these transgenic tubers exhibited earlier induction of sprouting compared to wild-type tubers indicating that high expression of the transgene was necessary to cause an effect. However, this also demonstrates the requirement of specific promoters conferring strong expression in stored tubers.

Cytokinin is essential for bud break

Early studies have demonstrated that application of CKs results in termination of tuber dormancy. These studies also revealed an increasing sensitivity to the phytohormone during postharvest storage with tubers being insensitive immediately after harvest and for a period thereafter (Hemberg, 1970; Turnbull and Hanke, 1985a; Suttle, 1998a; Suttle and Banowetz, 2000). In a more recent study, Suttle (2008) showed that synthetic CKs such as N-2-(chloro-4-pyridyl)-N'-phenylurea and 1-(α-ethylbenzyl)-3-nitroguanidine are more effective in dormancy termination than the natural zeatin probably because they escape degradation by CKX, but they did not eliminate the initial resistance period. Applying BAP to excised tuber buds in our sprout release assay confirmed the ability of CKs to remove tuber dormancy. Moreover, we also observed a time-dependent increase in sensitivity to BAP treatment, but it could also release dormancy of freshly harvested tubers. Thus, the difference in the response to CK may be due to the different experimental systems, substances, and/ or potato cultivars used; but potato tubers may have also attained a certain metabolic competence before sprouting occurred (Sonnewald, 2001). In contrast to the previous studies, BAP induced only bud break, but did not support further sprout outgrowth. Sprout growth could be stimulated only after an additional dosage of GA3.

The importance of CK for initiating release of tuber dormancy became also evident from experiments with transgenic plants. In tubers harvested from transgenic potato plants expressing a bacterial 1-deoxy-d-xylose 5-phosphate synthase (DXS), sprout growth had already occurred at harvest to a length of about 1-2 mm (Morris et al., 2006). This was accompanied by an increased level of trans-zeatin riboside and isopentenyl adenosine produced in these transgenic tubers. Bud break was followed by a phase of growth arrest before further sprout growth could be detected, probably due to a hormonal and/ or metabolic imbalance. Furthermore, transgenic potato plants transformed with T-DNA from A. tumefaciens to increase CK biosynthesis displayed a variety of phenotypic changes, including premature sprouting (Ooms and Lenton, 1985). We exploited the IPT gene from A. tumefaciens to enhance endogenous CK levels. As reported before (Ooms and Lenton, 1985;
Macháčková et al., 1997) our transgenic potato plants hardly formed roots and exhibited a stunted, bushy phenotype with small leaflets. Hence, only one weakly IPT-expressing line formed enough tubers of similar size as the wild type to be suitable for further experiments. Strikingly, these tubers began to sprout one day earlier when used in a sprout release assay with 50 µM GA₃, even though there was no significant effect on sprouting behaviour when stored under normal conditions. Beside the transgenic IPT plants, we generated potato plants over-expressing the Arabidopsis CKXI gene to reduce the endogenous CK content. These plants also displayed considerable morphological and developmental changes. As reported for transgenic Arabidopsis and tobacco plants expressing the same gene (Werner et al., 2001; Werner et al., 2003), shoots of transgenic potato plants were retarded and leaf size and shape was changed. Remarkably, numbers of leaflets per leaf went down with increasing expression of the transgene to only single lanceolate leaves formed in the strongest line instead of the typical composite wild-type leaves. In this line, the tuber shape was changed and only few, droplet-like tubers were formed. These tubers exhibited a clearly prolonged dormancy period and, most interestingly for our study, tuber discs did not respond to exogenously applied GA₃. This result led us to conclude that GA requires the presence of CK to trigger tuber sprouting and that CK is essential for initiation of bud break. The assumption is confirmed by the observations that BAP application to excised wild-type tuber buds stimulated bud break, but not further sprout growth; however it was sufficient to induce sprouting of GA20ox-expressing tubers.

Cytokinins are characterized by their ability to stimulate cell division of plants in vivo and in vitro (see ref. in Francis and Sorrell, 2001; Werner et al., 2001). In the cell cycle, they regulate the G₁/S phase transition, the stage in which most cells of dormant buds are arrested, at least partially, by inducing CycD-type cyclins (Campbell et al., 1996; Francis and Sorrell, 2001; Horvath et al., 2003; Francis, 2007). Meanwhile there are numerous studies providing evidence for a CK – CycD3 connection. For instance, Soni et al. (1995) showed an induction of CycD3 by CK and Riou-Khamlichi et al. (1999) found elevated CycD3 transcript levels in Arabidopsis mutants with high CK content, while cycD3 mutants described by Dewitte et al. (2007) showed reduced CK response. Moreover, onset of tuber sprouting was found to be accompanied by a large increase in cell division (Campbell et al., 1996). Consistent with these results expression of almost all cell cycle regulators including cyclin D3 were found to be induced in wild-type tubers following GA₃-mediated sprouting, but not in transgenic tubers expressing CKX. Expression of genes controlling DNA replication such as histone H4 and the
dUTPase was also only activated in wild-type tubers when sprout growth commenced. Both genes were recently described as molecular markers defining the transition from a dormant to a sprouting tuber meristem (Senning et al., 2010). In addition, we found clear differences in the expression of genes involved in cell wall biosynthesis and modification, as well as of fatty acid biosynthetic enzymes, between wild-type and CKX-expressing tubers; these genes are involved in processes necessary to initiate cell division and growth. More of these genes were activated by GA3 treatment of wild-type than of CKX-4 tubers, reflecting that sprout growth had commenced in wild type, but not in the transgenic tubers.

Interestingly, cross sections through sprouting IPT-expressing tubers indicated an increased meristem size, whereas the tuber meristem of CKX-expressing plants was smaller and had less developed vasculature. Similarly, Werner et al. (2001; 2003) observed smaller SAMs in CKX-expressing tobacco and Arabidopsis plants and suggested that CKs are not only required to maintain cell division, but might also be involved in cell differentiation. Evidence for the role of CK in cell differentiation is also provided by transgenic potato plants expressing the IPT gene under control of the UNUSUAL FLORAL ORGANS (UFO) promoter from Arabidopsis (Ingram et al., 1995; Deveaux et al., 2003). These plants formed flower-like structures at the tip of various organs, such as side shoots, stolons or potato tuber buds (Supplemental Figure 7) indicating that the meristem identity was changed by increased CK content in the boundaries of the shoot meristems.

In accordance with this assumption, a strongly increased expression of putative transcription factors controlling organ differentiation and (out)growth as e.g. ANT, PHAN, GRF3 or OVATE (Kim et al., 2003; Hackbusch et al., 2005; Carraro et al., 2006) was seen in wild-type tuber discs as opposed to CKX discs, indicating that organ differentiation is not promoted in CK-deficient tubers. Moreover, clear differences in the response to GA3 were found between both genotypes with respect to auxin biosynthesis and signaling. Recent work elucidated an essential role for auxin in primordium initiation and outgrowth of lateral organs (reviewed in Carraro et al., 2006; Leyser, 2009). Thus, expression of auxin-responsive SAUR and GH3 genes was dampened in CKX-4 tubers after GA3 treatment when compared to wild type. Interestingly, expression of ESTs with homology to ARF2 was two- to four- fold induced by GA3 in CKX-expressing tubers. In Arabidopsis, ARF2 was described as a general repressor of cell division (Schruff et al., 2006). In contrast, transcript amounts of ARF4 homologues and of auxin transport proteins accumulated in sprouting wild-type buds. The latter proteins are important for establishment of an active auxin transport and in particular PIN proteins.
generate high local auxin maxima necessary to initiate organ outgrowth (Reinhardt et al., 2000; Friml et al., 2003; Carraro et al., 2006; Leyser, 2006). In addition, at least two auxin biosynthesis genes appeared to be transcriptionally activated in wild-type tubers. This is in line with results of Sorce et al. (2000), who documented a positive correlation between the concentration of IAA and the end of tuber dormancy. In a recent, more detailed study the same authors reported a progressive decrease in free IAA content during the dormancy period (Sorce et al., 2009). In addition they performed immunolocalization studies that showed an accumulation of the hormone in the apical meristem and the vascular tissue beneath the dormant tubers, whereas in sprouting tubers, IAA was localized mainly in the primordia. From these results Sorce et al. (2009) postulated that auxin supports early developmental processes that underlie dormancy break. This assumption is also strengthened by Faivre-Rampant et al. (2004), who found that expression of ARF6 was strongly up-regulated at dormancy release, especially in the developing vasculature as shown by in situ hybridization. Hence, ARF6 was discussed as a marker for meristem activation (Faivre-Rampant et al., 2004). In our transcript profiling experiment, expression of ARF6 was not changed in the wild type which might be due to the time points investigated (after 3 days sprouting had already commenced), but it was clearly decreased in CKX-expressing tubers. However, our data support an important role for auxin in onset of tuber sprouting, possibly by stimulation of vascular tissue differentiation and determination of leaf primordia formation of the newly developing sprout via directed auxin transport and distribution. This activation seems to be mediated by GA3 and dependent on a functional CK metabolism and signaling pathway (Figure 14).

Beside auxin, transcripts coding for components of the ethylene signaling pathway were found to be differentially expressed in GA3-treated wild-type and CKX1-4 tubers. Thus, expression of transcripts with homology to ERF1 and ERF5 from tomato was more strongly activated in CKX-4 tubers than in wild type, which could also be seen for EBF1 and EBF2 homologues. EBF1 and EBF2 are F-Box proteins that target the central transcription factors EIN3 and EIL1 to proteasome-dependent degradation (recently summarized by Stepanova and Alonso, 2009; Yoo et al., 2009). EIN3 and EIL1 activate expression of ERF1 and other primary responsive genes which induce expression of secondary response genes by binding to the GCC box in the promoter (Solano et al., 1998). Interestingly, the transcript of the potato EIN3 homologue accumulated in untreated CKX-4 tubers and its expression decreased following GA3 treatment. This might be caused by the activation of EBF1 and EBF2 seen in CKX-4 tubers. Expression of both genes was found to be induced by ethylene and might
provide an additional layer of regulation (see Stepanova and Alonso, 2009; Yoo et al., 2009). On the other hand, a M KK9-like EST was transcriptionally induced in CKX-4 lines, but not in wild-type tubers in response to GA₃. M KK9 is part of a positively acting signalling cascade and may stabilize EIN3. However, it needs to be considered that almost nothing is known about ethylene signalling in potato and even in Arabidopsis many fundamental questions still remain to be addressed (Stepanova and Alonso, 2009). Nevertheless, ethylene has been shown to play a pivotal role in initiation and maintenance of tuber dormancy, but conflicting results have been published concerning its impact on terminating the rest period (Suttle, 1998b; 2004a; Suttle, 2009). Hence, transient treatment with ethylene could hasten dormancy release while continuous treatment resulted in sprout growth inhibition (Rylski et al., 1974). Application of ethylene inhibitors to developing microtubers resulted in a dose-dependent increase in premature sprouting (Suttle, 1998b). Conversely, several publications described an increased rate of ethylene production as sprouting commenced (see ref. in Suttle, 2004a). Inspection of our array data also revealed an increased expression of several ESTs coding for the biosynthetic genes 1-aminocyclopropane-1-carboxylic acid (ACC) synthase and ACC oxidase (Table S1, S2) upon GA₃ treatment, with different ESTs being induced in wild type and CKX-4. This induction may indicate increased ethylene formation. However, its perception and signal transduction is clearly different in both genotypes and our data suggest that ethylene signaling negatively influences sprout outgrowth. Together with stimulated auxin biosynthesis, transport and signaling pathway, a dampened ethylene response might trigger cell differentiation and finally sprout outgrowth upon GA₃ application (Figure 14). Induction of sprout growth also requires energy which is initially provided by available soluble sugars and amino acids (Sonnewald, 2001) (Figure 14). However, re-activation of meristematic activity is clearly dependent on the availability of CKs as is evident from transgenic lines with increased expression of the CK-degrading enzyme CKX in which sprouting cannot be induced by GA₃ treatment. This was also reflected by the suppressed cell proliferation and differentiation in these transgenic tubers.

In summary, our data points to CK as an essential component controlling dormancy release. GA requires CK to stimulate resumption of meristematic activity, but is sufficient to support sprout growth once bud break has occurred.
MATERIALS AND METHODS

Plant material and growing conditions

Potato plants (*Solanum tuberosum* cv. Solara) were obtained from Bioplant, Ebstorf, Germany. Plants were propagated in tissue culture under a 16 h light – 8 h dark period on Murashige and Skoog medium (Murashige and Skoog, 1962) containing 2% sucrose. Plants used for the analyses were cultivated in the greenhouse in individual pots (diameter 20 cm, depth 15.5 cm) at 50% humidity with 16 h supplemental light (150 µmol quanta m⁻² s⁻¹) and 8 h darkness. The temperature regime followed the light - dark cycle with 21°C and 18°C. After harvest, tubers were stored at room temperature in darkness.

Plasmid construction and plant transformation

Standard procedures were performed as described in Sambrook et al. (1989). The cloning of the Arabidopsis *gibberellin 20-oxidase* (*GA20ox*) (accession no. X83379; At *GA20ox1*) and *gibberellin 2-oxidase* (*GA2ox*) (accession no. AJ132435; At *GA2ox1*) into the BinAR vector (Höfgen and Willmitzer, 1990) containing the CaMV 35S promoter and the octopin synthase polyadenylation signal was described in Biemelt et al. (2004). To express the *GA20ox* under control of the chimeric STLS1/CaMV 35 promoter, a ca. 1300bp fragment of the STLS1 promoter was amplified by PCR using the primers L700-5’ 5’-agaattcgcggccgcccattccttaaaaattccc-3 and L700-3’ 5’-gaattcctgctctcactacttagtatg-3’. The fragment was sub-cloned into pCR 2.1 vector (Invitrogen) and subsequently inserted into the binary construct 5’- upstream of the CaMV 35S promoter using EcoRI restriction sites.

The *isopentenyltrasferase* (*IPT*) clone (accession no. AF242881) originating from the *Agrobacterium tumefaciens* Ti plasmid pTi15955 (Accession no. AF242881) was kindly provided by T. Schmülling (FU Berlin, Germany) as a T-DNA sub-clone in the pUC9 vector. This vector was used as a template to amplify the *IPT* gene by PCR using the primers IPT-5’ 5’-ggatccatggacctgcatctaattttc-3’ and IPT-3’ 5’-gtcgacctaatacattccgaacgg-3’. The *Arabidopsis thaliana* cytokinin oxidase/dehydrogenase 1 (*CKX*) gene (accession no. NM_129714, At2g41510, At *CKX1*) was amplified from Arabidopsis leaf cDNA using the following primers: CKX1-5’ 5’-ggatccatggacctgcatctaattttc-3’ and CKX1-3’ 5’-gtcgacctaatacattccgaacgg-3’. The resulting PCR fragments were sub-cloned into the pCRblunt vector (Invitrogen). The respective fragments were excised using Asp718 and SalI (*IPT*) or BamHI and SalI (*CKX*) restrictions sites and inserted into the binary vector BinAR (Höfgen and Willmitzer, 1990).
The binary constructs were transformed into *Agrobacterium tumefaciens*, strain C58C1, carrying the virulence plasmid pGV2260 (Deblaere et al., 1985). Transformation of potato plants was performed as described by (Rocha-Sosa et al., 1989).

Sprout Release Assay (in vitro tuber sprouting assay)

Discs of 5 mm height containing one bud each were excised from potato tubers using a Korkbohrer size 4 (Ø 8 mm). Discs were washed three times for 15 min in sterile-filtered buffer containing 20 mM 2-(N-morpholino)ethanesulfonic acid (MES), 300 mM D-mannitol and 5 mM ascorbic acid, pH 6.5. Discs were incubated with 5 to 100 µM gibberellic acid (GA3), 5 to 100 µM 6-benzylaminopurine (BAP) or sterile water for 5 minutes and subsequently placed in petri dishes lined with moist filter paper. Petri dishes were sealed with tape and stored in darkness under tissue culture conditions. The filter paper was regularly moistened by adding sterile water. Sprouting behavior of tuber discs was scored daily.

RNA isolation, RT-PCR and Northern blot analysis

Isolation of total RNA was essentially performed as described in Logemann et al. (1987). For reverse transcription (RT)–PCR, cDNA was synthesized from 10 µg of total RNA as described in (Biemelt et al., 2004). An aliquot was applied to PCR using the gene-specific primers IPT-5’ and IPT-3’. Ubiquitin was used as a loading control using primers described in Kloosterman et al. (2005).

For Northern blot analysis 20 - 30 µg of total RNA were separated on 1.5% formaldehyde-containing agarose gels and blotted onto nylon membranes (GeneScreen, NEN, Boston, USA) by capillary blotting overnight. The membranes were pre-hybridized and hybridized at 65°C. cDNA fragments of At-GA2ox1, At-GA20ox1, At-CKX1 were used as probes and radioactively labeled with [32P]dCTP by means of the High Prime Kit (Roche, Basel, Switzerland). After stringent washing, radioactive membranes were exposed to X-Ray films (Kodak) over night at -70°C. Hybridization with a cDNA fragment of the small subunit of ribulose 1,5-bisphosphate carboxylase (*RbcS*) (Acc. No X02353) served as loading control.

Sample Preparation and microarray hybridization

For transcript profiling an in vitro sprout experiment with tubers from wild-type and CKX-4 plants pre-stored for ca. one week was performed using 50 µM GA3. Samples were taken
directly and three days after treatment by pooling eight tuber discs per sample. To reduce the portion of parenchyma in the samples, size of the tuber discs was further reduced by cutting the eyes with Korkbohrer size 2 (⌀ 4 mm). Total RNA from three replicates each was isolated as described above and purified using RNeasy Mini Spin Columns (QIAGEN, Valencia, CA) following manufacturer’s protocol. RNA quantity was measured with the ND-100 Spectrophotometer v3.3.0 (NanoDrop Technologies). RNA integrity was verified using an Agilent RNA 6000 Nano Chip on an Agilent 2100 BioAnalyzer (vB.02.03 BSI307) as recommended by manufacturer’s protocol (Agilent RNA 6000 Nano Assay Protocol2).

Sample labeling and preparation for microarray hybridization was performed as described in the one-color microarray-based gene expression analysis protocol provided by Agilent including the one-color RNA spike-in kit (v5.0.1, 2006; Agilent Technologies, Santa Clara). Slides were scanned on the Agilent Microarray Scanner with extended dynamic range (XDR) at high resolution (5 µm). Data sets were extracted by the feature extraction software package (v9.5.3.1/ Agilent Technologies) using a standard protocol.

Microarray data analysis

For data analysis, text-files generated by the feature extraction software, were imported into GeneSpring GX 7.3.1 (Silicon Genetics, Palo Alto, CA, USA). Three normalization steps were applied: (i) data transformation: measurements less than 5.0 were set to 5.0; (ii) per chip: normalization to 50th percentile; (iii) per gene: the signal of each feature was normalized to the median of its value across the dataset. Features passing the quality check (flags present) and showing changes in expression levels equal or more than 2-fold were selected for further analysis. A volcano plot was applied to identify statistically significant (p-Value ≤ 0.05; equal variances assumed), more than two-fold differentially expressed genes between two conditions including the Benjamini-Hochberg multiple test correction. Annotations were taken from POCI online tool (http://pgrc.ipk-gatersleben.de/poci). Functional assignment was done based on annotations, GO-terms or by homology search against the Arabidopsis genome. Heat maps for selected pathways were generated using the MapMan tool (https://www.gabipd.org/projects/MapMan/).

Measurement of gibberellin contents

To analyze GA contents apical shoot tips of potato plants comprising the first five leaves were harvested, frozen in liquid nitrogen, freeze-dried and stored at -80°C until analyzed. Approximately 500 mg (dry weight) milled leaf material were extracted in the presence of $^2$H-labelled internal standards (5 - 25 ng µl$^{-1}$) and $^3$H-labelled GA$_1$, GA$_4$, 16, 17-dihydroGA$_{19}$,
and GA20 (50000 dpm each) as markers, at 4°C overnight in 100 ml of 80% (v/v) aqueous methanol. The extracts were purified, fractionated and subjected to quantitative analysis by combined gas chromatography-mass spectrometry with selected ion monitoring as described in Griffiths et al. (2006).

Imaging

Images of tuber disc cross sections were captured with a Leica DFC480 digital camera on a Leica MZ16F stereomicroscope. Images were processed with Adobe Photoshop software.

Accession numbers: Sequence data from this article can be found in EMBL/ GenBank data libraries: At GA20ox1 (X83379) (At4g25420.1); At GA2ox1 (AJ132435) (At1g78440); IPT (AF242881); At CKX1 (NM_129714) (At2g41510); STLS1 (X04753). The transcriptome data discussed here have been deposited at ArrayExpress (E-MEXP-2921).

SUPPLEMENTAL DATA

Supplemental Figure 1. In vitro sprouting assay with potato tuber discs excised at different time points after harvest.

Supplementary Figure 2. In vitro tuber sprouting of wild-type tubers using different gibberellin species.

Supplemental Figure 3. Expression of Arabidopsis GA20ox in transgenic potato plants under control of the chimeric STSL1/CaMV 35S promoter.

Supplemental Figure 4. Expression level of GA20ox gene caused by different plant promoters during tuber storage.

Supplemental Figure 5. Induction of growth in BAP-treated tuber discs by application of GA3.

Supplemental Figure 6. In vitro tuber sprouting assay of CKX-4 tuber buds treated with BAP.

Supplemental Figure 7. Phenotype of potato plants expressing IPT under control of the UFO promoter from A. thaliana.

Supplemental Data Set 1. Differentially expressed transcripts found in the comparison of wild type tuber buds immediately and 3d after GA3 treatment.

Supplemental Data Set 2. Differentially expressed transcripts found in the comparison of At CKX-4 tuber buds immediately and 3d after GA3 treatment.
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FIGURE LEGENDS

Figure 1: In vitro sprouting assay using different concentrations of gibberellic acid.
Two weeks after storage tuber discs containing one bud each were isolated and incubated for
5 min with 5, 25, 50, 100 µM gibberellic acid (GA_3) and H_2O as control. The tuber discs were
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(A) The percentage of sprouted tuber buds after application of different GA_3 concentrations
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(A) Sprouting behaviour of wild type (WT), GA2ox and GA20ox expressing tubers was
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Gibberellins were isolated from the shoot apex of growing plants (leaf no. 1 - 5) and analysed
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Insert: Schematic drawing of the changes in GA-metabolism due to expression of GA2ox (black circles) or GA20ox (grey circles) in potato plants. GA12 is the common precursor for all GAs. The 13-hydroxylation pathway leads to GA1; whereas the 13-non-hydroxylation pathway leads to GA4 as major bioactive forms. Bioactive GA-species are underlined.

Figure 5: Impact of GA20ox expression under control of the chimeric STLS1/CaMV 35S promoter on potato tuber sprouting and sprout development.
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Two weeks after storage tuber discs were isolated and incubated for 5 min with 5, 25, 50 or 100 μM 6-benzyladeninepurine (BAP) and H2O as control. Tuber discs were kept in darkness under tissue culture conditions and scored daily for visible sprouting.
(A) The percentage of sprouted tuber buds after incubation with different BAP concentrations or water over an eight days period. The graphs represent the mean ± SD of 3 independent experiments (n = 25). (B) Cross sections through tuber buds before and three and five days after treatment with either 50 μM BAP and water. The black bars represent 1 mm. (C) Schematic drawing of the in vitro tuber sprouting experiment using BAP.

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(A) PCR analysis of IPT expressing potato plants (lines 6, 7, 8, 9). Five micrograms of total RNA isolated from leaves were reverse-transcribed using oligo dT primers and RevertAid[TM] H minus reverse transcriptase. An aliquot of first strand cDNA was amplified with gene-specific primers for IPT and ubi3, respectively. (B) Phenotypic alteration of transgenic potato plants. From left to right: wild type (WT); IPT lines 6, 7, 8 and 9. Pictures were taken eight weeks after transfer to the greenhouse (wild type, IPT lines 6 and 9) and from plants propagated in tissue culture (lines 7, 8) six weeks after cutting. (C) Sprouting behavior of IPT-expressing line 6 (IPT-6) compared to wild type (WT). Tubers were stored at...
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(A) Northern blot analysis of CKX (lines 4, 10, 11) expressing potato plants. Thirty micrograms of total RNA isolated from leaves were loaded per lane and probed with At CKX1. Hybridization with the small subunit of Rubisco (RbcS) was used as loading control. (B) Phenotypic alteration of transgenic potato plants ca. eight weeks after transfer into the greenhouse. From left to right: two wild-type plants (WT), CKX lines 4, 10 and 11. (C) Effect on leaf morphology and (D) on tuber formation. (E) Cross sections of wild type (WT) and CKX expressing (line 4; CKX-4) tubers at harvest. Freshly harvested tubers were hand-sectioned and imaged on a stereomicroscope. Arrowheads indicate vascular strands. The black bars represent 1 mm. (F) Sprouting behavior of CKX-expressing lines (4, 10, 11) in comparison to wild type (WT) monitored for 24 weeks after harvest until 100% sprouting was reached. Tubers were stored at room temperature in darkness (n = 25-48).

Figure 9: In vitro tuber sprouting of transgenic lines expressing CKX or GA₂₀ox treated with GA₃ or BAP
(A) Cross sections through buds of wild-type (WT) and CKX-4 tubers treated with water (MQ) or 50 µM GA₃ after six and nine days (d). (B) Cross sections through buds of wild-type (WT) and GA₂₀ox-58 tubers treated with water (MQ) or 50 µM BAP six days after treatment. The bars represent 1 mm.

Figure 10: Functional assignment of transcripts differentially expressed in wild type and
CKX expressing tuber buds during the sprout release assay.

Transcripts differentially expressed 3d after GA$_3$ treatment in buds of wild-type or CKX-4 tubers were classified into functional groups. Bars illustrate the percentage of transcripts within various categories based on total numbers. The category “unknown” containing about 35% of differentially expressed transcripts was not shown to increase clarity (See Supplemental Table S1 and S2 for more detailed information).

**Figure 11: Expression of cell cycle genes in wild-type and CKX-4 tubers after GA$_3$-mediated tuber sprouting.**

Expression data of differentially expressed ESTs representing genes of the cell cycle are shown on the left-hand panel and a schematic overview of the mitotic cell cycle, modified after Inze and Veylder (2006), is shown on the right. Color bars as displayed by the MapMan Tool were copied to and aligned in Powerpoint. The color scale next to the panel indicates transcript levels with red representing an increase and blue representing a decrease in transcript levels. The colors saturate at eightfold changes.

At the G1/ S phase transition, CDK inhibitory protein ICK dissociates from CDKA-CYCD complexes and is degraded via the proteasome pathway. Phosphorylation of CDKA then activates the CDKA-CYCD complex which initiates phosphorylation of the retinoblastoma-related protein (RBR), releasing the E2F-DP1 complex. This complex promotes transcription of genes required for progression into and through S-phase where DNA-replication takes place.

G2/ M-phase transition is accompanied by a marked increase in transcription of both A- and B-type CDKs and A- and B-type cyclins. CDKA/ B-CYCA/ B complexes are activated by removal of the inhibitory phosphate by CDC25 and phosphorylation by CAK (CDK-activating kinase), both enzymes acting on the CDK part of the complex. The reverse reaction, inhibitory phosphorylation of CDK, is catalyzed by kinase WEE1 and promotes endoreduplication. During mitosis, CDC20- and CCS25-activated APC degrades cyclins A and B leading to the transition from mitosis into G1 phase.

Abbreviations: CYC – cyclin; CDK – cyclin dependent kinase; CAK – CDK activating kinase; ICK – CDK inhibitory protein; APC – anaphase-promoting complex; RBR – Retinoblastoma-related protein; DP – docking protein; CDC – cell division cycle.

**Figure 12: Expression of ethylene signaling genes in wild-type and CKX-4 tubers after GA$_3$-induced tuber sprouting.**
Expression data of differentially expressed ESTs representing genes of the ethylene signaling pathway are shown on the left-hand panel and a schematic overview of this pathway as assumed from knowledge in *Arabidopsis thaliana* (Kendrick and Chang, 2008; Yoo et al., 2009) is shown on the right. Color bars as displayed by the MapMan Tool were copied to and aligned in Powerpoint. The color scale next to the panel indicates transcript levels, with red representing an increase and blue representing a decrease in transcript levels. The colors saturate at fourfold changes.

Without ethylene binding, the ethylene receptor complexes in the ER membrane activate CTR1. RTE1 acts as a negative regulator of ethylene response by regulating ethylene receptor ETR1. CTR1 is proposed to be a MKKK that activates a cytosolic kinase signaling cascade leading to phosphorylation of residues Thr592 and Thr546 of the transcription factors EIN3 and EIL1 in the nucleus as well as the inhibition of membrane-bound protein EIN2. Phosphorylated EIN3 and EIL1 interact with F-Box proteins EBF1 and -2 resulting in constant degradation of the transcription factors and thus suppression of ethylene signaling. EIN5/XRN4 indirectly influences transcript levels of EBF1 and -2. Upon binding of ethylene, CTR1 is inactivated which leads to inactivation of the CTR1-MAPK signaling and activation of another kinase signaling cascade involving M KK9, MPK3 and -6 and probably an as yet unknown M KK K. This cascade leads to the phosphorylation of residues Thr176 and Thr176 of EIN3 and EIL1, assumingly resulting in increased stability because of a reduced interaction with EBF1 and -2. Membrane-bound protein EIN2 also positively influences EIN3 accumulation through a mechanism that has not been elucidated yet. EIN3/EIL1 activates ethylene responses both directly and indirectly through expression of the transcriptional activator ERF1.

Abbreviations: CTR1 – Constitutive Triple Response 1; EBF - EIN3-Binding F-Box protein; EIL1 – EIN3-like 1; EIN – Ethylene Insensitive; ERF – Ethylene Response Factor; ERS – Ethylene Response Sensor; ETR – Ethylene Response; MKKK – Mitogen-activated protein (MAP) kinase kinase kinase; MKK – MAP kinase kinase; MPK – MAP protein kinase; RBX – RING-Box protein; RTE1 – Reversion to Ethylene Sensitivity 1; SCF complex – SKP1-Cullin-F-Box complex; SKP1 – S-phase kinase associated protein 1 ; XRN4 – Exoribonuclease 4

**Figure 13: Expression of auxin transport and signaling genes in wild-type and CKX-4 tubers after GA3-mediated tuber sprouting.**

An overview of auxin transport proteins is shown on the left, the auxin signaling pathway is
shown on the right as derived from *Arabidopsis thaliana* (Mockaitis and Estelle, 2008; Weijers and Friml, 2009). Expression data of differentially expressed ESTs representing genes of auxin transport and signaling are shown in the grey panel. Color bars as displayed by the MapMan Tool were copied to and aligned in Powerpoint. The color scale above the panel indicates transcript levels, with red representing an increase and blue representing a decrease in transcript levels. The colors saturate at fourfold changes.

Auxin transport: IAA is the most abundantly occurring auxin in nature. It can enter the cell in its protonated form either by diffusion through the plasma membrane or facilitated by AUX1 permeases. Inside the cell, IAAH is deprotonated and trapped due to the change in pH. Auxin efflux is performed by two types of transporters, PGP1/PGP19 ABC transporters and PIN proteins. PGP1/19 usually has a nonpolar localization whereas PIN proteins show a polar localization which determines the orientation of the auxin flow within the cell.

Auxin signaling: Without IAA, ARF transcription factors bound to AREs in the promoters of auxin-responsive genes and interact with Aux/IAA proteins which in turn recruit the transcriptional co-repressor TPL to prevent gene expression. When accumulating in the nucleus, IAA binds to Aux/IAA proteins and the TIR1 subunit of the SCFTIR1 complex, targeting Aux/IAA for degradation via the 26S proteasome and releasing ARF transcription factors from inhibition. The activity of the SCFTIR1 complex can be regulated by addition and removal of ubiquitin-like protein RUB1 through the action of conjugating (AXR1, RCE1 and ECR1) and de-conjugating (CSN) enzymes. ARFs released from inhibition act as monomers or dimers to promote expression of auxin-responsive genes, e.g. those coding for SAUR and GH3 proteins.

Abbreviations: AUX1 – Auxin 1; ADP – adenosine diphosphate; ARE – Auxin Responsive Element; ARF – Auxin Response Factor; ATP – adenosine triphosphate Aux/IAA – auxin/indole-3-acetic acid protein; AXR1 – auxin resistant 1; CSN – COP9 Signalosome; ECR1 – E1 C-terminal Related 1; PGP – P-Glycoprotein; PIN – PIN-formed; PM – plasma membrane; RBX – RING-Box protein; RCE1 – RUB1-conjugating enzyme; RUB1 – Related to Ubiquitin; SAUR – Small Auxin-upregulated RNA; SCF complex – SKP1-Cullin-F-Box complex; SKP1 – S-phase kinase associated protein 1; TIR1 – Transport Inhibitor Response 1; TPL – TOPLESS; Ub - Ubiquitin

Figure 14: Schematic representation of postulated phytohormonal events which induce potato tuber bud breakage and sprouting.
Tables

**Table I.** Phenotypic characteristics of transgenic potato plants expressing either a *GA20ox* or a *GA2ox* gene from *A. thaliana*. Parameters were determined from soil-grown plants at harvest. Results are shown for three independent experiments and represent the mean ± SE of 19 - 21 plants. Statistically significant differences from the wild type were determined using one-tailed t-tests assuming unequal variance and are indicated by asterisks (*P ≤ 0.05).

<table>
<thead>
<tr>
<th></th>
<th>Stem height (cm)</th>
<th>Tuber yield (g per plant)</th>
<th>Number of tubers per plant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type (Solara)</td>
<td>55.2 ± 3.0</td>
<td>166.3 ± 7.6</td>
<td>7.8 ± 0.5</td>
</tr>
<tr>
<td><strong>GA20ox</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GA20ox-5</td>
<td>102.4 ± 3.5</td>
<td>166.6 ± 5.3</td>
<td>7.2 ± 0.5</td>
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<tr>
<td>GA20ox-15</td>
<td>99.8 ± 3.0</td>
<td>135.0 ± 4.9</td>
<td>7.0 ± 0.5</td>
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<tr>
<td>GA20ox-58</td>
<td>120.4 ± 5.9</td>
<td>116.3 ± 9.4</td>
<td>4.5 ± 0.3</td>
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<tr>
<td><strong>GA2ox</strong></td>
<td></td>
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</tr>
<tr>
<td>GA2ox-27</td>
<td>38.2 ± 4.3</td>
<td>94.9 ± 6.4</td>
<td>3.7 ± 0.3</td>
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<tr>
<td>GA2ox-38</td>
<td>35.9 ± 3.5</td>
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<td>GA2ox-50</td>
<td>59.1 ± 4.4</td>
<td>80.5 ± 8.3</td>
<td>4.1 ± 0.4</td>
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**Table II.** Phenotypic characteristics of transgenic potato plants expressing either a *cytokinin oxidase* (*CKX*) gene from *A. thaliana* or an *isopentyltransferase* (*IPT*) gene from *Agrobacterium tumefaciens*. Parameters were determined from soil-grown plants at harvest. Results are shown for three independent experiments and represent the mean ± SE of 25 - 40 plants. Statistically significant differences from the wild type were determined using one-tailed t-tests assuming unequal variance and are indicated by asterisks (*P ≤ 0.05).

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<th>Number of tubers per plant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type (Solara)</td>
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<td>176.7 ± 10.4</td>
<td>7.6 ± 0.4</td>
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<tr>
<td><strong>CKX</strong></td>
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<tr>
<td>CKX-4</td>
<td>39.2 ± 0.8</td>
<td>20.0 ± 0.7</td>
<td>6.5 ± 0.6</td>
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<td>CKX-10</td>
<td>46.5 ± 1.2</td>
<td>136.1 ± 5.1</td>
<td>3.9 ± 0.4</td>
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<tr>
<td>CKX-11</td>
<td>55.3 ± 1.3</td>
<td>168.0 ± 2.7</td>
<td>5.8 ± 0.3</td>
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<tr>
<td><strong>IPT</strong></td>
<td></td>
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<tr>
<td>IPT-6</td>
<td>54.2 ± 1.1</td>
<td>170.2 ± 3.9</td>
<td>7.2 ± 0.2</td>
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<tr>
<td>IPT-9</td>
<td>11.1 ± 0.9</td>
<td>10.7 ± 3.4</td>
<td>2.8 ± 0.6</td>
</tr>
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</table>
Table III. Number of transcripts significantly (P ≤ 0.05) regulated in wild-type and CKX1-4 tuber buds during the GA3-mediated sprout release assay.

*aTranscripts regulated in both, wild type and CKX1-4 tuber buds, identified by Venn diagram.
*bNumber of up- and down-regulated transcripts identified by K-Means clustering.

<table>
<thead>
<tr>
<th>Comparison</th>
<th>≥2-fold regulated transcripts</th>
<th>Overlapping transcripts(^a)</th>
<th>3d GA3 UP(^b)</th>
<th>3d GA3 DOWN(^b)</th>
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<td>WT 0d vs. 3d GA3</td>
<td>4410</td>
<td>1867</td>
<td>2793</td>
<td>1617</td>
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<tr>
<td>CKX1-4 0d vs. 3d GA3</td>
<td>5629</td>
<td></td>
<td>3218</td>
<td>2411</td>
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Figure 12: Expression of ethylene signaling genes in wild-type and CKX-4 tubers after GA₃-induced tuber sprouting.

Expression data of differentially expressed ESTs representing genes of the ethylene signaling pathway are shown on the left-hand panel and a schematic overview of this pathway as assumed from knowledge in *Arabidopsis thaliana* (Kendrick and Chang, 2008; Yoo et al., 2009) is shown on the right. Color bars as displayed by the MapMan Tool were copied to and aligned in Powerpoint. The color scale next to the panel indicates transcript levels, with red representing an increase and blue representing a decrease in transcript levels. The colors saturate at fourfold changes.

Without ethylene binding, the ethylene receptor complexes in the ER membrane activate CTR1. RTE1 acts as a negative regulator of ethylene response by regulating ethylene receptor ETR1. CTR1 is proposed to be a MKKK that activates a cytosolic kinase signaling cascade leading to phosphorylation of residues Thr⁵⁹² and Thr⁵⁴⁶ of the transcription factors EIN3 and EIL1 in the nucleus as well as the inhibition of membrane-bound protein EIN2. Phosphorylated EIN3 and EIL1 interact with F-Box proteins EBF1 and -2 resulting in constant degradation of the transcription factors and thus suppression of ethylene signaling. EIN5/XRN4 indirectly influences transcript levels of EBF1 and -2. Upon binding of ethylene, CTR1 is inactivated which leads to inactivation of the CTR1-MAPK signaling and activation of another kinase signaling cascade involving MKK9, MPK3 and -6 and probably an as yet unknown MKKK. This cascade leads to the phosphorylation of residues Thr¹⁷⁶ and Thr¹⁷⁶ of EIN3 and EIL1, assumingly resulting in increased stability because of a reduced interaction with EBF1 and -2. Membrane-bound protein EIN2 also positively influences EIN3 accumulation through a mechanism that has not been elucidated yet. EIN3/EIL1 activates ethylene responses both directly and indirectly through expression of the transcriptional activator ERF1.

Abbreviations: CTR1 – Constitutive Triple Response 1; EBF - EIN3-Binding F-Box protein; EIL1 – EIN3-like 1; EIN – Ethylene Insensitive; ERF – Ethylene Response Factor; ERS – Ethylene Response Sensor; ETR – Ethylene Response; MKKK – Mitogen-activated protein (MAP) kinase kinase kinase; MKK – MAP kinase kinase; MPK – MAP protein kinase; RBX – RING-Box protein; RTE1 – Reversion to Ethylene Sensitivity 1; SCF complex – SKP1-Cullin-F-Box complex; SKP1 – S-phase kinase associated protein 1; XRN4 – Exoribonuclease 4
Figure 13: Expression of auxin transport and signaling genes in wild-type and CKX-4 tubers after GA3-mediated tuber sprouting.

An overview of auxin transport proteins is shown on the left, the auxin signaling pathway is shown on the right as derived from Arabidopsis thaliana (Mockaitis and Estelle, 2008; Weijers and Friml, 2009). Expression data of differentially expressed ESTs representing genes of auxin transport and signaling are shown in the grey panel. Color bars as displayed by the MapMan Tool were copied to and aligned in Powerpoint. The color scale above the panel indicates transcript levels, with red representing an increase and blue representing a decrease in transcript levels. The colors saturate at fourfold changes.

Auxin transport: IAA is the most abundantly occurring auxin in nature. It can enter the cell in its protonated form either by diffusion through the plasma membrane or facilitated by AUX1 permeases. Inside the cell, IAAH is deprotonated and trapped due to the change in pH. Auxin efflux is performed by two types of transporters, PGP1/PGP19 ABC transporters and PIN proteins. PGP1/19 usually has a nonpolar localization whereas PIN proteins show a polar localization which determines the orientation of the auxin flow within the cell.

Auxin signaling: Without IAA, ARF transcription factors bound to AREs in the promoters of auxin-responsive genes and interact with Aux/IAA proteins which in turn recruit the transcriptional corepressor TPL to prevent gene expression. When accumulating in the nucleus, IAA binds to Aux/IAA proteins and the TIR1 subunit of the SCFTIR1 complex, targeting Aux/IAA for degradation via the 26S proteasome and releasing ARF transcription factors from inhibition. The activity of the SCFTIR1 complex can be regulated by addition and removal of ubiquitin-like protein RUB1 through the action of conjugating (ARF1, RCE1 and ECR1) and de-conjugating (CSN) enzymes. ARFs released from inhibition act as monomers or dimers to promote expression of auxin-responsive genes, e.g. those coding for SAUR and GH3 proteins.

Abbreviations: AUX1 – Auxin 1; ADP – adenosine diphosphate; ARE – Auxin Responsive Element; ARF – Auxin Response Factor; ATP – adenosine triphosphate Aux/IAA – auxin/indole-3-acetic acid protein; AXR1 – auxin resistant 1; CSN – COP9 Signalosome; ECR1 – E1 C-terminal Related 1; PGP – P-Glycoprotein; PIN – PIN-formed; PM – plasma membrane; RBX – RING-Box protein; RCE1 – RUB1-conjugating enzyme; RUB1 – Related to Ubiquitin; SAUR – Small Auxin-upregulated RNA; SCF complex – SKP1-Cullin-F-Box complex; SKP1 – S-phase kinase associated protein 1; TIR1 – Transport Inhibitor Response 1; TPL – TOPLESS; Ub - Ubiquitin.
Figure 14: Schematic representation of postulated phytohormonal events which induce potato tuber bud breakage and sprouting.