RESEARCH PAPER

Ethanol breaks dormancy of the potato tuber apical bud

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Received 28 January 2005; Accepted 8 June 2005

Abstract

Growing potato tubers or freshly harvested mature tubers have a dormant apical bud. Normally, this dormancy is spontaneously broken after a period of maturation of the tuber, resulting in the growth of a new sprout. Here it is shown that in in vitro cultured growing and maturing tubers, ethanol can rapidly break this dormancy and re-induce growth of the apical bud. The in vivo promoter activity of selected genes during this secondary growth of the apical bud was monitored, using luciferase as a reporter. In response to ethanol, the expression of carbohydrate-storage, protein-storage, and cell division-related genes are rapidly down-regulated in tuber tissue. It was shown that dormancy was broken by primary but not by secondary alcohols, and the effect of ethanol on sprouting and gene expression in tuber tissue was blocked by an inhibitor of alcohol dehydrogenase. By contrast, products derived from alcohol dehydrogenase activity (acetaldehyde and acetic acid) did not induce sprouting, nor did they affect luciferase reporter gene activity in the tuber tissue. Application of an inhibitor of gibberellin biosynthesis had no effect on ethanol-induced sprouting. It is suggested that ethanol-induced sprouting may be related to an alcohol dehydrogenase-mediated increase in the catabolic redox charge [NADH/(NADH + NAD⁺)].

Key words: AGPase, alcohol, alcohol dehydrogenase, cell cycle.

Introduction

Growing tubers and freshly harvested mature potato tubers do not sprout, even when environmental conditions are favourable. This dormancy of the apical bud of the tuber (also called tuber dormancy) has advantages and disadvantages: for the plant it is advantageous to survive a period unfavourable for growth, while for man it is favourable when plant material needs to be stored for a certain period of time. However, dormancy is disadvantageous when growth is required soon after tubers have been harvested.

Dormancy has been well studied in seeds and it has been shown that gibberellins (GAs) can break seed dormancy (e.g. tomato seeds: Groot and Karssen, 1987; Arabidopsis seeds: Koornneef and Van der Veen, 1980). Moreover, for seeds it has been shown that besides GAs, a large number of non-hormonal organic and inorganic chemicals (e.g. ethanol) can also affect dormancy (e.g. red rice: Cohn et al., 1989; oat: Corbineau et al., 1991). Based on studies with various inhibitors, it has been suggested that the stimulatory effect of ethanol on dormancy breaking in seeds specifically acts through alcohol dehydrogenase (ADH) activity (Cohn et al., 1989; Corbineau et al., 1991).

The research on dormancy in (potato) tubers has been reviewed by Claassens and Vreugdenhil (2000) and Suttle (2004). Although it has been shown that, as in seeds, GAs can break dormancy in tubers, it has not been established that the processes and signals that control dormancy in seeds and tubers are similar in all aspects. The potential of ethanol to break dormancy in tubers was demonstrated for Jerusalem artichoke (Petel et al., 1993). However, no details on the mode of action of ethanol in breaking dormancy in these tubers are known. Moreover, until now there has been no direct evidence of the potential of ethanol to break dormancy in potato tubers.

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Here a detailed analysis of the effects of ethanol on dormancy and sprouting of potato tubers is described. The effects of ethanol at the level of visible sprouting and at the level of gene activity were studied by monitoring *in vivo* expression of genes known to be involved in potato tuber formation (Verhees *et al.*, 2002). In this study, the mode of action of ethanol in the breaking of dormancy is investigated, whether the dormancy-breaking effect of ethanol acts through the GA pathway is determined, and a detailed view of the temporal and spatial regulation of gene expression in the tuber during ethanol action is given in relation to the renewed growth of the apical bud.

**Materials and methods**

**In vitro tuberization system**

* In vitro* potato plants (*Solanum tuberosum* cv. Binjte) were grown with a 16 h light period (50 W m<sup>−2</sup>, 20 °C). Single-node cuttings were taken and placed on a tuber-inducing medium consisting of modified Murashige and Skoog medium (Hendriks *et al.*, 1991). The explants were incubated in the dark at 20 °C. Tuber formation started on the 6th day and after 4 weeks the tubers were considered mature and dormant. The final diameter of the microtubers was ~3 mm and their fresh weight ~20 mg.

**Tuber treatments**

The apical buds of the microtubers exhibited a period of dormancy (~5 months) when stored untreated at ambient temperature. For the analysis of effects of various treatments on the rate of sprouting, 45 explants bearing sessile tubers were used, of which 30 tubers were 8-weeks-old and 15 tubers were 5-weeks-old. These explants were transferred to fresh tuber-inducing medium containing the various compounds to be tested and kept at 20 °C in darkness. Approximately 2 mm of the stem, bearing the tuber, was cut off to ensure uptake of the compounds to be tested through the stem cutting. In some cases the chemicals were applied as vapour; in these cases the compound was added as liquid to a piece of filter paper in a 3 cm Petri dish, placed on top of the medium in a 9 cm Petri dish. The explants were placed around the small Petri dish. The air volume in the Petri dish above the medium was 60 cm<sup>3</sup>. Concentrations of compounds applied: 1-propanol and 2-propanol, 87 mM; abscisic acid (ABA) and GA, 10 μM; acetaldehyde, 0.02% (5 mM); acetic acid, 0.004% (0.7 mM); chlororocholine chloride (CCC), 0.05% (3 mM); 4-methyl pyrazole (4-MP), 1 mM. Sprouting data presented in Figs 1, 3, and 5 (see Results) are based on the averages of 45 tubers. Rates of sprouting of control tubers in these figures are taken from the same experiment in which all treatments were run concurrently. Controls have been repeated independently at least three experiments and showed similar or lower rates of sprouting.

**Measurements of luciferase (LUC) activity in growing tubers**

To study expression of genes related to storage activity, two LUC reporter constructs were used, one under control of the potato *AGPaseS* promoter (Müller-Röber *et al.*, 1992) and the other under control of the potato *1PAT21* promoter (Bevan *et al.*, 1986) from *Solanum tuberosum*. L. Cell cycle-related gene expression was studied by monitoring promoter activity of the *Arabidopsis* genes *cycB1;1* (Ferreira *et al.*, 1994) and *CDC2a* (Hemerly *et al.*, 1993) fused to the LUC reporter (Sherf and Wood, 1994). As a control, the activity of a CaMV 35S promoter fused to the LUC reporter was used. The cloning of the reporter genes, transformation of potato, and the analysis of the different primary transgenic lines (*Solanum tuberosum* cv. Desiree) have been described before (Verhees *et al.*, 2002). For each reporter construct, two representative lines were selected for expression analysis. No major differences in the pattern of LUC expression were observed between the two independent transformed lines.

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column used was 10 cm long (inner diameter of 1 cm). The collected fractions of the preparative HPLC step were not fractionated for the second time after methylation. Putative methyl-GA fractions were dried and trimethylsilylated by dissolving in a fresh mixture of BSTFA:TMCS:pyridine (20:1:79, by vol.) and heated for 20 min at 70 °C. The actual determination of GAs using a GC-MS system was performed as described by Kappers et al. (1997).

Abscisic acid

For the ABA determinations, tubers were grown on tuber-inducing medium and treated with or without ethanol as described for tuber samples for GA determinations. Each sample consisted of three tubers of different age (2-, 3-, and 4-months-old). Each sample was split into two, one consisting of the apical part + secondary structure (if any) and the other one consisting of the rest of the tuber tissue. The total number of tubers tested for sprouting in this experiment was about 25 per treatment.

The extraction procedure was modified after Berry and Bewley (1992) and Raikhel et al. (1987). The samples were freeze-dried and ground in liquid nitrogen. Approximately 10 mg of apical bud tissue and 50 mg of the rest of the tissue was mixed with, respectively, 400 and 800 μl of 80% (v/v) methanol, in which 0.1% (w/v) BHT (2,6-di-t-tert-butyl-4-methylphenol) was dissolved. After centrifugation (15 min, 14 000 g), the supernatant was stored at 4 °C and again the same amount of this extraction buffer was mixed with the pellet. After centrifugation, the supernatants were pooled, lyophilized, and resuspended in 200 μl of TBS buffer (25 mM TRIS–HCl, 100 mM NaCl, 1 mM MgCl2, pH 8.5). ABA was then quantified using the Phytodek monoclonal antibody ELISA method (Agdia Inc., USA).

Results

Ethanol breaks dormancy in tubers

The effect of ethanol on dormancy of tubers was tested by transferring in vitro tubers to medium with 0.5% ethanol and low or high levels of sucrose. Ethanol treatment, combined with a low level of sucrose resulted in the growth of sprouts, while ethanol in combination with a high level of sucrose resulted in development of secondary tubers. Thus, in both cases ethanol breaks dormancy, while the sucrose level determines the identity of the secondary structure. Control tubers, transferred to medium without ethanol, remained dormant, both on medium with 1% sucrose and with 8% sucrose. However, in some experiments sprouting was also observed a few weeks after transfer to fresh medium without ethanol (Fig. 1).

Figure 1 shows the effects of ethanol in the medium on the rate of sprouting of microtubers when they were transferred to medium containing 1% or 8% sucrose. Sprouting, i.e. visible growth of the apical bud, irrespective of the type of structure formed, was visible from 3 d after the start of the ethanol treatment. Close to 100% of sprouting was reached at day 5 or 6, both at high and low sucrose levels in the medium. In the control treatments (1% and 8% sucrose, without ethanol) sprouting started later, and at day 5 <5% of the tubers had sprouted.

Ethanol applied as vapour was also sufficient to induce tuber sprouting. In this case sprouting also started 3 d after onset of the treatment, but 100% of sprouting was reached later (Fig. 1).

Manipulation of endogenous ethanol levels and dormancy through anaerobiosis

To determine the relevance of the effect of exogenously applied ethanol on tuber dormancy, tests were carried out to see if manipulation of the storage conditions of the tubers can also result in accumulation of endogenous ethanol and in breaking of dormancy. For this purpose, in vitro-cultured tubers were stored under both aerobic and anaerobic conditions. The storage resulted in the accumulation of endogenous ethanol in the tubers under anaerobic conditions, while no such accumulation was observed in the tubers stored under aerobic conditions (data not shown). Moreover, after a few weeks, sprouting did occur in the anaerobic tubers, while no sprouting was observed in the control tubers. These results are in agreement with Rakitin and Suvorov (1935) who performed similar anaerobic-storage experiments with field-grown tubers.

Effects of ethanol on gene expression

The effect of ethanol on in vivo gene expression throughout the entire process of dormancy breaking was studied, using potato LUC reporter lines (Verhees et al., 2002). To study changes in cell cycle and storage-related gene expression during ethanol-induced sprouting, reporter plants were used containing the promoter of cell cycle- (cycB1;1 and CDC2a) or storage-related genes (AGPaseS and λPat21) fused to the coding sequence of the LUC reporter genes. For each reporter line the LUC activities were quantified in the area covering the whole tuber, after transfer to medium with ethanol (0.5%) combined with 1% or 8% sucrose. Figure 2 shows the average LUC activities expressed as a percentage of the initial LUC activity in the whole tuber at t=0. In the control tubers (35S-LUC) the transfer to the different types of fresh medium (1% or 8% sucrose, with or without ethanol) did not result in significant differences in LUC activity; the activity declined within 10 h to about 50% of the initial value (Fig. 2A). For the other reporter lines, the change in LUC activity was strongly affected by the type of fresh medium and the presence of ethanol.
lines, the transfer to medium with 8% sucrose (no ethanol) led to a similar gradual decline in LUC activity. By contrast, transfer to medium with 1% sucrose resulted in a transient increase in LUC activity between 5 h and 35 h for cycB1;1, CDC2a, and kPat21. The AGPaseS activity continuously declined after transfer to medium with 1% sucrose, indicating that the reduction in sucrose supply affects the maintenance of AGPaseS gene expression in the tuber. When tubers were transferred to medium with ethanol, a strong decline was observed in the activity of both cell cycle- (Fig. 2B, C) and storage-related promoters (Fig. 2D, E). In the presence of ethanol, the activity profiles in these reporter tubers did not differ between tubers transferred to medium with 1% and 8% of sucrose. In some cases, the strong decline in the whole tuber area masked the local changes in the apical bud region, which was therefore also measured separately (see below). On medium with ethanol, sprouting only occurred 50 h after transfer (cf. Fig. 1), indicating that the decline in cell cycle and storage-related gene expression in the whole tubers preceded visible growth of the buds.

Mode of action of ethanol in breaking tuber dormancy

For seeds it has been suggested that conversion of ethanol via alcohol dehydrogenase (ADH) is required for the effect of ethanol on breaking of dormancy (Cohn et al., 1989). The validity of this hypothesis for breaking of dormancy by ethanol in potato tubers was tested in three ways: (i) by blocking ADH activity; (ii) by comparing the effects of primary and secondary alcohols, since only primary alcohols are substrates for ADH (Corbineau et al., 1991); (iii) by testing the effects of the products resulting from
conversion of ethanol by ADH, namely, acetaldehyde and acetic acid. Effects of these treatments were analysed both by scoring visible sprouting and by quantifying the effects of the treatments on $\lambda$Pat21 gene expression.

(i) Blocking ADH activity by 4-MP resulted in the abolition of the effect of ethanol on sprouting (Fig. 3A). In the absence of ethanol, 4-MP also diminished the rate of sprouting (eventually up to 60%). 4-MP also completely prevented the ethanol-induced lowering of $\lambda$Pat21 expression (Fig. 4A).

(ii) 1-Propanol induced sprouting of tubers at a similar rate as ethanol, although with a 1 d delay (Fig. 3B). 2-Propanol had no effect on sprouting. Similarly, only 1-propanol significantly affected expression of $\lambda$Pat21, while 2-propanol was ineffective (Fig. 4B). Similar results were found for expression of AGPase, cycB1;1, and CDC2a (data not shown).

(iii) Figure 3B shows that neither acetaldehyde (0.02%) nor acetic acid (0.004%) was able to induce sprouting. Rather, both treatments resulted in lower rates of sprouting as compared with the control. Other concentrations tested also failed to induce sprouting (data not shown). Application of acetaldehyde did not result in reduction of $\lambda$Pat21 expression (Fig. 4C).

Are GAs or ABA involved in dormancy breaking by ethanol?

The hormone ABA has been implicated in the control of dormancy and has been shown to prevent second growth of tubers (Van den Berg et al., 1991; Ewing et al., 2004). Whether the effects of ethanol could be blocked by applications of ABA was tested. Figure 5 shows that ABA at 10 $\mu$M nearly completely prevented sprouting in control tubers. Moreover, this concentration of ABA also partly counteracted the effect of 0.5% ethanol on tuber sprouting. Higher concentrations of ABA were even more effective (data not shown). Despite this clear interaction between ethanol and ABA on the rate of sprouting, no effect of ABA was found on the ethanol-induced decrease of storage-related reporter activities (Fig. 6).

GAs are able to break the dormancy of seeds (Koornneef and Van der Veen, 1980; Groot and Karssen, 1987) and potato tubers (Boo, 1961). It was, therefore, of interest to test whether effects of ethanol on sprouting were mediated through changes in endogenous GA levels. A possible role of endogenous GA in the mode of action of ethanol was analysed by studying the effect of the GA-synthesis inhibitor CCC, in the presence of ethanol. Figure 5 shows that CCC did not abolish ethanol-induced sprouting, but delayed it by 1 d. Application of GA without ethanol resulted in rapid and complete sprouting 1 d earlier than that obtained with ethanol (data not shown). Application of ethanol with CCC resulted in rapid sprouting, regardless of the presence or absence of additional GA (Fig. 5). Moreover, CCC did not affect ethanol-induced reduction of the $\lambda$Pat21-LUC reporter gene activity (Fig. 6) or ethanol-induced reduction of AGPase, cycB1;1, and CDC2a reporter gene activity (data not shown).

Viability of non-sprouting tubers

Because not all treatments showed 100% sprouting at the time the observations in Figs 1, 3, and 5 were made (after approximately 1 month), the viability of the non-sprouting tubers was checked. When, for a given treatment, the rate of sprouting after 3 months was below 80%, the tubers were transferred to fresh medium, containing 1% sucrose and various additions. Effects of the ADH inhibitor 4-MP are presented in (A); effects of products and substrates of ADH are given in (B).

Identity of the secondary growth structure is determined by sucrose and GA levels

As shown above, the level of sucrose in the medium influenced the type of new structure formed from the apical bud of the tuber under the influence of ethanol: at low sucrose (1%) mainly shoots were formed, whereas high sucrose (8%) resulted in the formation of secondary tubers.
This effect of sucrose might be osmotic. Therefore, the effect of sorbitol in the presence of 1% sucrose, resulting in the same osmotic potential as an 8% sucrose medium, was tested. No significant increase in the percentage of secondary tubers was found as compared with medium with 1% sucrose only (data not shown).

Since GA and ABA have been shown to influence tuber formation in potatoes (Xu et al., 1998; Vreugdenhil and Sergeeva, 1999), effects of these regulators on the formation of secondary structures were also tested. ABA when applied simultaneously with ethanol, in the presence of 1% sucrose, partly blocked sprouting (Fig. 5), and only shoots were formed (Table 1). Inhibiting the synthesis of
GA by CCC resulted in 100% formation of secondary tubers, even at low sucrose levels in the medium. The opposite effect, i.e. 100% formation of sprouts, was caused by exogenous application of GA (Table 1).

Levels of endogenous GAs and ABA in tubers
Xu et al. (1998) presented evidence that the level of sucrose in the medium of single-node cuttings growing in vitro affected endogenous GA levels in the developing bud, i.e. low sucrose in the medium would result in high GA levels, inducing shoot growth, and high sucrose levels would lead to low GA levels, allowing tuber formation. Therefore, endogenous GA (and ABA) levels were determined in order to test whether the effect of sucrose on the structures formed after ethanol application could be explained by parallel changes in hormone levels. The level of GA in the tubers before transfer was 4.8 ng g⁻¹ FW. When transferred to medium with high sucrose, the level of GA remained relatively high, namely, 4.0 and 2.0 ng g⁻¹ FW in the absence and presence of ethanol, respectively. However, when transferred to low-sucrose medium, the GA level did not increase, but dropped by over 15-fold. Again, in the presence of ethanol, the level of GA was lower than in its absence.

Initially, the level of ABA was 250 ng g⁻¹ FW, and no major changes were observed in any of the treatments. No major differences were observed between ABA levels in buds as compared with the rest of the tuber (data not shown).

Effects of ethanol on gene expression in the apical bud
The quantification of LUC reporter activity in the whole tuber area (region I, Fig. 7A) clearly shows the effect of ethanol treatment on gene expression (Figs 2, 6). However, different, more local, changes in reporter-gene activity may be masked in these average values. Changes in LUC reporter activity were therefore also quantified separately in the area of the apical bud (region II) and a central sub-area of the tuber (region III), as specified in Fig. 7A. As an example, Fig. 7B shows the quantified LUC activity in an individual tuber expressing the cycB1;1-luc reporter gene. The graph shows that during the initial 20 h after transfer to medium with ethanol, the cell cycle-related LUC activity declined, both in the tuber area and in the bud. However, 40 h after transfer, the average cycB1;1-luc activity started to increase in the bud region, whereas the average LUC activity in the tuber sub-region remained low (Fig. 7B). A similar differential response between bud and tuber region during ethanol-induced dormancy breaking was observed for the other reporter genes. Therefore, for further analysis, only cycB1;1 and AGPaseS expression in the bud region were used to test the effect of different treatments. For the analysis of each treatment, LUC activity in the bud region of 25 individual tubers at different time points was normalized for activity at t=15 h after onset of the treatment, and the average (normalized) LUC activity was calculated. On medium with ethanol and 8% sucrose, the average cycB1;1-luc activity in the tuber region declined, while in the apical bud region the reporter gene activity showed a strong up-regulation at ~20 h after the start of the treatment (Fig. 8A). This effect of ethanol clearly preceded the visible effect on growth of the bud (occurring on average at t=72 h; Fig. 1). By contrast, in tubers transferred to medium with ethanol and 1% sucrose, cycB1;1 promoter activity in tuber and apical bud region remained very similar (Fig. 8B). Similar results were obtained for the tubers expressing the AGPaseS-luc reporter; tubers on 8% sucrose showed an enhanced activity for AGPaseS in the bud region relative to the tuber region, whereas no differential AGPaseS expression between tuber and bud was observed on 1% sucrose medium (data not shown).

ABA does not influence effects of ethanol on gene expression in the apical bud
The possible effects of ABA on the dynamics in cycB1;1 and AGPaseS expression in the bud region of tubers transferred to medium with ethanol and 8% sucrose were studied. Under these conditions bud sprouting is inhibited by ABA. Figure 9 shows the average (normalized for activity at t=15 h) LUC activities for the cycB1;1 and AGPaseS reporters, as measured in bud and tuber regions at 70 h after transfer. For both the cycB1;1 and the AGPaseS reporter tubers, the control treatment (transfer to the same medium) resulted in similar activity in bud and tuber region. The treatment with ethanol resulted, for both reporters, in a differential response of activity between bud and tuber region (decrease in tuber region, increase for cycB1;1, and no change for AGPaseS in the apical bud area; see also Fig. 8A). This differential effect of ethanol on promoter activity was not affected by 100 μM ABA, even though ABA blocked ethanol-induced outgrowth of the bud (Fig. 9).

Table 1. Identities of secondary growth structures on potato tubers
Effects of various treatments on the identity of new organs formed from the apical bud of microtubers after 1 month when dormancy is broken by treatment with 0.5% ethanol. The numbers are percentages of the total number of tubers that were scored per treatment (n=45).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Sprouts (%)</th>
<th>Tubers and thickened sprouts (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1% Sucrose+ethanol</td>
<td>62</td>
<td>38</td>
</tr>
<tr>
<td>1% Sucrose+ethanol+ABA&lt;b&gt;</td>
<td>73</td>
<td>0</td>
</tr>
<tr>
<td>1% Sucrose+ethanol+CCC</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>1% Sucrose+ethanol+GA</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>8% Sucrose+ethanol</td>
<td>0</td>
<td>100</td>
</tr>
</tbody>
</table>

<b>After 1 month 27% of these tubers had not sprouted, but sprouting was observed in these tubers after 6 months.
4-MP blocks the effects of ethanol on gene expression in the apical bud

To test whether the effect of ethanol on differential cycB1;1 and AGPaseS promoter activity in bud and tuber region was mediated through ADH activity, tubers were transferred to medium with 8% sucrose, ethanol (0.5%), and 4-MP (1 mM). For both reporter lines the average LUC activity at \( t = 70 \) h, in the tuber sub-region and apical bud region was calculated. The LUC activity was again normalized to that at \( t = 15 \) h. The results show that, by contrast with ABA, the inhibitor of ADH activity completely abolished the ethanol-induced down-regulation of gene activity in the tuber region and the up-regulation of cycB1;1-luc activity in the bud region (Fig. 9). In this experiment the growth of the apical bud was completely prevented by 4-MP, an even stronger suppression than noted earlier on medium containing 4-MP and 1% sucrose (Fig. 3A).

Discussion

Ethanol breaks dormancy of potato tubers

In agronomic practices, early and temporary release of dormancy resulting in the formation of secondary and tertiary tubers is commonly known as second growth (Van den Berg et al., 1990; Ewing and Struik, 1992). This undesirable process may result in substantial reductions in tuber yield and poor tuber quality due to decreased starch levels and poor tuber shape. Second growth may occur in the field when environmental conditions during tuber growth become adverse for tuber growth, for example, high temperature or excess nitrogen supply (Bodlaender et al., 1964; Van den Berg et al., 1990). Assuming that dormancy gradually develops in the tuber (Claassens and Vreugdenhil, 2000), this second growth could also be seen as (early) breaking of dormancy.

Here it has been shown that application of low amounts of ethanol, applied as vapour or dissolved in the medium, can result in similar activation of bud growth of tubers grown in vitro. The dormancy-breaking effect of ethanol was visible either as the growth of a sprout, or as the formation of secondary and tertiary tubers. The novel observation of the direct action of ethanol fits circumstantial indications that ethanol may be involved in dormancy breaking under field conditions. For instance, Van Loon and Bouma (1978) found that soil compaction results in severe induction of second growth. This may now be attributed to poor aeration of soil resulting in anaerobic conditions and accumulation of ethanol, produced either by the tuber itself or by the soil micro-organisms. Rakitin and Suvorov (1935) already reported that anaerobic conditions resulted in ethanol accumulation in tubers and caused premature sprouting.

Ethanol suppresses tuber growth-related gene expression

Within 10 h after transfer to medium supplemented with ethanol, cell cycle and storage-related gene expression (as quantified by LUC activity in the whole tuber) declined relative to that in tubers on medium without ethanol (Fig. 2B–E). This indicates that processes within the tuber, namely cell division and synthesis of reserves, normally associated with tuber development (Verhees et al., 2002), are rapidly blocked by ethanol. As a control, expression of the CaMV 35S promoter in tubers under similar conditions was monitored (Fig. 2A). In these tubers no effect of ethanol on LUC activity was observed, suggesting that the changes in cell cycle- and storage-related LUC activity are most likely due to changed promoter activity of these reporter genes and do not result from other more general changes which may affect in vivo LUC activity, for example, ATP, oxygen, or luciferin uptake.

The early effects of ethanol on cell cycle and storage-related gene expression in the tuber tissue are not directly related to detectable growth activity of the apical bud: the down-regulation of gene expression in tuber tissue was visible within \( \sim 10 \) h, while the actual growth of the apical bud was only visible at 2–3 d after transfer to medium with...
ethanol (Fig. 1). Moreover, in older tubers the effect of ethanol on cell cycle-related gene expression was as rapid as in young tubers (within ∼10 h), but here sprouting was only visible after 5 d (data not shown).

Cell cycle-related gene expression in the apical bud does not always relate to growth of the apical bud

The LUC activity images allowed separate analysis of gene activity in tuber tissue and in the apical bud region. These analyses showed an up-regulation of cell cycle- and storage-related genes in the apical bud when tubers were placed on medium with ethanol and 8% sucrose, but not on medium with ethanol and 1% sucrose. Growth activity of the apical bud is therefore not necessarily related to additional up-regulation of cell cycle promoter activity. But also up-regulation of cell cycle-related gene expression in the apical bud is not always related to apical bud growth, as in the presence of ethanol and ABA, growth of the bud was completely prevented but cell cycle gene activity was still up-regulated (Fig. 9). Combined, these results show that cell cycle-related gene activity and bud sprouting are at least partly independently regulated and that ABA acts downstream from ethanol-induced gene expression in the signal transduction network that leads to secondary growth.

Mode of action of ethanol in breaking tuber dormancy

ADH action is required for ethanol effects on dormancy and gene expression: Treatment with 4-MP inhibited sprouting and prevented ethanol-induced down-regulation of gene expression (Figs 3A, 4A). Since 4-MP is an inhibitor of plant ADH activity (Perata and Alpi, 1991), this suggests a role for ADH in the effect of ethanol in breaking of dormancy. A role for ADH enzyme activity in dormancy breaking by alcohols is further substantiated by the fact that only ADH substrates (primary alcohols) and not secondary alcohols affect dormancy and gene expression (Figs 3B, 4B). The fact that 4-MP alone also inhibits sprouting suggests either that the compound itself inhibits dormancy breaking, or that ADH activity plays a role in breaking dormancy, even in the absence of exogenous ethanol (Fig. 3A).

Products of ADH do not affect sprouting or gene expression: Cohn et al. (1989) suggested that for an alcohol to affect dormancy of true seeds it has to be converted by ADH to the corresponding aldehyde or carboxylic acid. Therefore, it was expected that acetaldehyde or acetic acid would also break dormancy in potato microtubers. Treatment with acetaldehyde or acetic acid did not induce sprouting nor did these compounds affect gene expression in the tubers.
in the same way as ethanol (Figs 3B, 4C). From these observations it is concluded that conversion of ethanol to acetaldehyde and/or acetic acid by ADH is not required for breaking of dormancy in potato tubers. Alternatively, the difference in effect of ethanol and the products of the ADH enzyme reaction may reflect differences in uptake from the medium between ethanol and acetaldehyde or acetic acid by the tubers. The latter option is not very likely, however, since these compounds appeared to penetrate into the tubers as judged from the delaying effect on tuber sprouting (Fig. 3). Moreover, application of acetaldehyde or acetic acid as vapour yielded similar results (data not shown).

The experimental data indicate that the action of ADH activity is important for dormancy breaking, but the products have no effect. The conversion of alcohols to their aldehydes by ADH is accompanied by the reduction of NAD$^+$ to NADH. Thus, ADH action may increase the NADH/NAD$^+$ ratio in the cell. Gallais et al. (1998) studied pyridine nucleotide levels and redox charges of non-dormant and dormant caryopses of oat (Avena sativa L.). They noticed an increase in NADH content and the ratio NADH/(NADH+NAD$^+$) [catabolic redox charge (CRC)] during early germination of non-dormant caryopses, which differed significantly from the NADH content and CRC of non-germinating dormant caryopses. They also found that ethanol provokes increases in NADH and consequently in CRC, which suggests that germination is enhanced or accelerated when the NADH content increases (Gallais et al., 1998). The present data are consistent with the hypothesis that the effect of ethanol on tuber sprouting may be mediated through an increase in NADH levels or CRC, due to the enzymatic activity of ADH.

**GA does not act downstream of ethanol signalling:** Plant hormones, especially GAs and ABA, have been suggested to be involved in the regulation of potato tuberization and sprouting (Vreugdenhil and Struik, 1989; Ewing and Struik, 1992). Application of GA to tubers can induce bud activation which results in sprouting (Fig. 5; Claassens and Vreugdenhil, 2000). However, CCC, an inhibitor of GA biosynthesis, did not prevent ethanol-induced bud activation, nor did it prevent down-regulation of the reporter gene activities in tuber tissue, or up-regulation of reporter gene activity in the apical bud region (Figs 5, 6). The application of CCC resulted in a change in organ identity of the new structures, indicating that the compound did penetrate the tuber cells. This suggests that GA does not act downstream of the ethanol signalling pathway leading to sprouting.

**ABA acts downstream of ethanol signalling:** ABA, when applied alone, nearly completely prevented sprouting. This is consistent with the finding that a decrease in ABA levels in tubers occurs under second-growth-inducing conditions (Van den Berg et al., 1991). ABA partly counteracted the effect of ethanol on sprouting (Figs 5, 6). This suggests that ethanol might act by lowering endogenous ABA levels. However, this hypothesis is not supported by the ABA measurements in tubers under various conditions. Although the present data indicate that ethanol does not act via changes in the levels of GA or ABA, it remains possible that ethanol affects tissue sensitivity towards either of the two hormones.

Figure 10 summarizes the steps that it is concluded are likely to be involved in ethanol-induced breaking of tuber dormancy.

**Acknowledgement**

The work presented here was partly funded by the European Union’s Biotech Framework IV as part of the project ‘Biology of tuber dormancy and sprouting’ (BIO4-CT96-0529).

**References**


