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PCD and apical dominance release in potato tuber

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Release of apical dominance in potato tuber is accompanied by programmed cell death in the apical bud meristem

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Potato (*Solanum tuberosum* L.) tuber, a swollen underground stem, is used as a model system for the study of dormancy release and sprouting. Natural dormancy release, at room temperature, is initiated by tuber apical bud meristem (TAB-meristem) sprouting characterized by apical dominance (AD). Dormancy is shortened by treatments such as bromoethane (BE), which mimics the phenotype of dormancy release in cold storage by inducing early sprouting of several buds simultaneously. We studied the mechanisms governing TAB-meristem-dominance release. TAB-meristem decapitation resulted in the development of increasing numbers of axillary buds with time in cold storage, suggesting the need for autonomous dormancy release of each bud prior to control by the apical bud. Hallmarks of programmed cell death (PCD) were identified in the TAB-meristems during normal growth, and these were more extensive when AD was lost following either extended cold storage or BE treatment. Hallmarks included DNA fragmentation, induced gene expression of vacuolar processing enzyme 1 (*VPE1*) and elevated VPE activity. VPE1 protein was semi-purified from BE-treated apical buds and its endogenous activity was fully inhibited by a caspase-1-specific inhibitor (Ac-YVAD-CHO). Transmission electron microscopy further revealed PCD-related structural alterations in the TAB-meristem of BE-treated tubers: a knob-like body in the vacuole, development of cytoplasmic vesicles and budding-like nuclear segmentations. Treatment of tubers with BE and then VPE inhibitor induced faster growth and recovered AD in detached and nondetached apical buds, respectively. We hypothesize that PCD occurrence is associated with weakening of tuber AD, allowing early sprouting of mature lateral buds.
INTRODUCTION

Potato (*Solanum tuberosum* L.) is the world's largest food crop in terms of fresh produce after rice and wheat. Sprouting control during storage is one of the biggest challenges for the fresh market, prior to industrial processing, and in storage of seed-tubers (Coleman, 2000). After harvest, tuber buds are generally dormant and will not grow even if the tubers are placed under optimal conditions for sprouting (i.e. warm temperature, darkness, high humidity). Following a transition period of between 1 and 15 weeks depending on the storage conditions and variety, dormancy is broken and apical buds start to grow (Wiltshire and Cobb, 1996). The dormancy observed in postharvest potato tubers is defined as endodormancy (Lang et al., 1987), and is due to an unknown endogenous signal that mediates suppression of meristem growth (Suttle, 2004b). The duration of the endodormancy period is primarily dependent on the genotype, but other factors, such as growth conditions of the crop and storage conditions after tuber harvest, are also important (Turnbull and Hanke, 1985; Wiltshire and Cobb, 1996).

Endogenous plant hormones and their relative balance within the tuber are suggested to regulate endodormancy and sprouting (Turnbull and Hanke, 1985; Ji and Wang, 1988; Suttle, 2004a; Sorce et al., 2009; Suttle, 2009; Hartmann et al., 2011). Ethylene and abscisic acid (ABA) have been associated with the onset and maintenance of tuber dormancy (Suttle and Hultstrand, 1994), and molecular analysis has indicated that genes associated with the anabolic and catabolic metabolism of ABA correlate with dormancy in potato meristems and tubers (Simko et al., 1997; Ewing et al., 2004; Destefano-Beltran et al., 2006; Campbell et al., 2010). Gibberellins (GAs) are involved in sprout growth after dormancy cessation, but not with dormancy maintenance (Suttle, 2004a; Hartmann et al., 2011). Biologically active cytokinins increase over time in dormant potato tissues, suggesting a role for this class of hormones in loss of dormancy (Suttle, 1998, 2009; Hartmann et al., 2011). There is evidence that the most abundant naturally occurring auxin, IAA, is involved in the control of potato tuber dormancy (Sukhova et al., 1993; Sorce et al., 2000; 2009). Faivre-Rampant et al. (2004) demonstrated that an increase in transcript level of the auxin response factor gene (*ARF6*) is correlated with bud meristematic tissue development.

Although the postharvest potato tuber is used as a model system for the study of
metabolic processes associated with dormancy release, sprouting and aging, very few studies have been done on apical dominance (AD) during these processes. Krijthe (1962) described four stages of physiological development in storage after dormancy release: (i) AD where only one sprout develops, (ii) additional multiple buds sprouting as a result of reduced AD, (iii) branching of the sprouting stems, and (iv) in the aging mother tubers, sprout replacement by daughter tubers. Fauconnier et al. (2002) found that AD can last up to approximately 60 d of storage in cvs. Bintje and Désirée. Between 60 and 240 d of storage, sprout number per tuber increases regularly due to loss of AD. Low temperature (4°C as compared to 12°C) reduces sprouting capacity and AD, and increases the number of stems when the tubers eventually do sprout (Hartmans and Van Loon, 1987).

Previous studies have shown that immediately after harvest, during their dormant period, potatoes cannot be induced to sprout without some form of stress or exogenous hormone treatment (Struik and Wiersema, 1999; Suttle, 2009; Hartmann et al., 2011). In a previous study, we found that R-carvone application damages the meristem membrane, leading to local necrosis of the tuber apical bud meristem (TAB-meristem); a few weeks later, axillary bud growth is induced in the same sprouting eye (Teper-Bamnolker et al., 2010). Surprisingly, application of a very low dose of R-carvone induced sprouting of the tuber followed by minor necrosis of the TAB-meristem tip (Teper-Bamnolker et al., 2010).

On a large commercial scale, Rindite (a mixture of ethylene chlorhydrin, ethylene dichloride and carbon tetrachloride) (Rehman et al., 2001), bromoethane (BE) (Coleman, 1984), CS₂ (Meijers, 1972; Salimi et al., 2010) and GA₃ (Rappaport et al., 1957) have been used to break tuber seed dormancy. Both BE and Rindite cause rapid breakage of bud dormancy and the developing buds do not differ morphologically from buds of tubers undergoing natural dormancy release (Alexopoulos et al., 2009). The phytotoxic chemical BE shortens the natural dormancy period from 2 to 4 months to approximately 10 d (Destefano-Beltran et al., 2006; Campbell et al., 2008; Alexopoulos et al., 2009). Campbell et al. (2008) observed that transcript profiles in BE-induced cessation of dormancy are similar to those observed in natural dormancy release, suggesting that both follow a similar biological pattern. Nevertheless, in that study, a cDNA array was used which covered only part of the transcripts in potato. Thus, BE treatment can be used to compress and synchronize release from the dormant period, which is an advantage from an experimental standpoint (Campbell...
et al., 2008). In small-scale research experiments, dormancy can be released by the use of different catalase inhibitors (thiourea, aminotriazole and hydrogen cyanamide) or H$_2$O$_2$ (Bajji et al., 2007). However, the mode of action of phytotoxic chemicals in inducing dormancy release and altering apical bud dominance is poorly understood. We propose that the potato tuber exhibits stem-like behavior, with various strengths of apical bud dominance over other buds. We further suggest that programmed cell death (PCD) in the TAB-meristem is one of the mechanisms that regulates AD.

RESULTS

Altering Bud Dormancy and AD by Aging in Cold Storage and BE Treatment

We observed three main types of loss of AD in stored potato (Fig. 1): loss of dominance of the apical buds over those situated more basipetally on the tuber (type I); loss of dominance of the main bud in any given eye over the subtending axillary buds within the same eye (type II), and loss of dominance of the developing sprouts over their own branching, meaning that side stems emerge not from the base of the sprout as in type II (type III, Fig. 1).

Freshly harvested tubers of cvs. Nicola and Désirée stored at 20°C for 45 and 60 d, respectively, maintained their AD (all three types) after sprouting, and in most tubers only the apical bud developed to a very long stem (Fig. 2, A and D, respectively). Tubers from the same batch were stored at 6°C for 60 d and then transferred to 20°C for an additional 30 d and 45 d of storage, respectively. This temperature/time treatment resulted in slower growth of the apical bud, loss of type I AD, and multiple bud sprouting in different areas of each tuber of both cultivars (Fig. 2, B and E). Application of 200 μl/L (container volume) of BE for 24 h induced sprouting in freshly harvested 'Nicola' and 'Désirée' tubers, which was evident within 10 to 20 d at 20°C (Fig. 2, C and F), whereas control tubers sprouted 30 and 45 d later, respectively (not shown). In both cultivars, sprouting induced by BE treatment resulted in the loss of AD of all types. Buds surrounding the apical buds tended to grow faster than those located in more distant segments of the tuber (Fig. 2, C and F). Loss of type I AD as a result of BE treatment was followed by loss of type III dominance, expressed
as excessive branching of the growing shoots (Fig. 2F).

**AD in Sprouting Tubers**

We checked whether the tuber exhibits classical stem-like behavior, and investigated the role of bud AD in determining lateral bud dormancy release and sprouting. Removing the apical bud after 30, 60 and 90 d in cold storage resulted in sprouting of an average of 1, 2 and 9 buds, respectively (Fig. 3, A–C). Aging in cold storage resulted in the sprouting of more buds due to removal of the apical bud, suggesting the need for each bud to reach maturity and autonomous dormancy release before it can be controlled by the TAB-meristem.

To characterize the role of the apical bud in AD (type I), we analyzed the effect of TAB-meristem removal on the sprouting pattern of nondormant 'Nicola' tubers stored in the cold for 90 d. Tubers were subjected to the following treatments, illustrated in Fig. 3D: (i) decapitation of their apical bud meristem; (ii) full removal of the apical complex; (iii) full removal of a lateral meristem complex; (iv) wounding between buds, similar to that created by full removal of the apical or lateral complex. The manipulated tubers were transferred from 6°C to 20°C and sprouting was followed for 24 d. Decapitation of the TAB-meristem induced accelerated loss of AD (type I); after 24 d, all buds were sprouting (Fig. 3E). Removing the apical complex (the TAB-meristem plus underlying cortex) induced loss of AD (type I) but axillary buds sprouted more moderately than when only the TAB-meristem was removed, and after 24 d, an average six buds were sprouting (Fig. 3E). Decapitation of the meristem of the lateral bud, or similar wounding of the skin between buds, did not impact AD or sprouting rate (Fig. 3E). Even excessive wounding of the tuber skin, without damaging the TAB-meristem, did not result in changing the timing or pattern of sprouting (not shown). Nontreated tubers reached an average of three sprouting buds after 24 d (Fig. 3E) with the apical bud much longer than the other two lateral buds (data not shown). We repeated this analysis in cv. Désirée and obtained similar results (not shown). These experiments emphasize the importance of TAB-meristem presence and viability in the control of lateral bud meristem growth, before sprouting is observed. We therefore investigated the relationship between cell viability specifically in the TAB-meristem and
Detection of PCD in TAB-Meristem Induces Loss of AD

Since both aging in cold storage and BE decrease AD, we suspected that viability of some of the meristem cells is altered, possibly as a result of induced PCD. We checked whether there is a correlation between incidence of PCD in the TAB-meristem and loss of AD (type I) during aging in cold storage. We hypothesized that the effect of excess PCD of the meristem cells would be similar to that of decapitation of the apical bud on AD.

A specific feature of PCD is the cleavage of genomic DNA at internucleosomal sites by endogenous nucleases. To detect fragmented nuclear DNA in situ, a terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) procedure was applied. TAB-meristem isolated from cv. Nicola tubers at harvest showed no symptoms of DNA fragmentation, either within the apical meristem or, initially, in the leaf primordium covering the TAB-meristem (Fig. 4, upper panel). After 30 and 45 d at 20°C, when AD is still in place, initial DNA fragmentation could be detected in the TAB-meristem (Fig. 4, AD). Incubating the tubers at 6°C for 60 d, which results in loss of AD, induced widespread and more extensive DNA fragmentation. After 90 d, the fragmentation had spread throughout the TAB-meristem (Fig. 4, ND), and the bud developed more slowly as compared to dominant apical bud.

Cells from the TAB-meristem tip, isolated from cv. Nicola tubers at harvest, had a meristematic characteristic (Ishida et al., 2009) and their nuclei were not stained by TUNEL (Fig. 5). TUNEL-positive cells that appeared 30 to 90 d after harvest tended to show a more non-circular nucleus and their staining became more condensed (Fig. 5). We concluded that DNA fragmentation develops and spreads in the TAB-meristem during tuber sprouting in parallel to AD loss.

Detection of PCD in TAB-Meristem Induced by BE Treatment

Exposure of freshly harvested tubers to a low dose of BE, which induces sprouting of all buds simultaneously, resulted in TUNEL-positive nuclei in the TAB-meristem cells 72
h after treatment (Fig. 6A). Non-bud meristem cell nuclei located under the tuber skin did not show any significant change (not shown). In contrast, nontreated tubers/buds showed only a few TUNEL-positive cells at the edge of the apical meristem and in the leaf primordium covering it (Fig. 6A). Vacuolar processing enzyme (VPE), which exhibits cysteine protease activity toward caspase-1 substrate, is involved in hypersensitive cell death, typical of PCD in plants (Hatsugai et al., 2004; Sanmartín et al., 2005; Zhang et al., 2010). In the BE-treated TAB-meristem, DNA fragmentation was followed by upregulation (400-fold) of \textit{vacuolar processing enzyme1} (\textit{VPE1}) gene expression measured immediately after exposure to 24 h BE treatment and 48 h after treatment initiation (Fig. 6B). No significant change in \textit{VPE1} expression was detected in nontreated tubers, up to 72 h after treatment initiation.

Six other genes which have been shown to be associated with stress in other plant species—\textit{Lesion Stimulating Disease1} (\textit{LSD1}) (Coupe et al., 2004), \textit{Peroxidase} (\textit{POX}) (Delaplace et al., 2008), \textit{Serine Palmitoyl Transferase} (\textit{SPT}) (Birch et al., 1999), \textit{Catalase 2} (\textit{CAT2}) (Mizuno et al., 2005), \textit{Zinnia Endonuclease-1} (\textit{ZEN1}) (Aoyagi et al., 1998; Ito and Fukuda, 2002), and \textit{Executer1} (\textit{EX1}) (Wagner et al., 2004)—were cloned from potato cv. Nicola, using the primers listed in Supplemental Material Table I (accession nos. JF773561–7, respectively, Supplemental Material Table II). Real-time RT-PCR of their cDNA fragments (using the specific primers listed in Supplemental Material Table II) showed no significant difference in their expression in the TAB-meristem of BE-treated tubers as compared to nontreated controls (data not shown).

We next measured the expression of a cysteine protease inhibitor gene (TC137660), reported to be downregulated during dormancy release in the TAB-meristem of potato (Campbell et al., 2008). The cysteine protease inhibitor gene was highly expressed after harvest (data not shown) followed by an additional increase in its expression after exposure to 20°C (Fig. 6C). In contrast, in BE-treated tubers, expression of the cysteine protease inhibitor gene was dramatically repressed, mainly during the first 48 h after treatment initiation (Fig. 6C). Within 72 h, its expression in the BE-treated tissue increased back to the level measured in the control tissue.

VPE activity was also found to be induced in the TAB-meristem following 24 h BE treatment, in parallel to the transcriptional activation of \textit{VPE1}. VPE activity was measured
using its specific c substrate, Ac-ESEN-MCA. A sevenfold increase in activity was found relative to the control 24 h after BE treatment initiation, and it remained high at 48 and 72 h of incubation (about tenfold that in the nontreated tubers) (Fig. 6D). A similar rise in VPE activity was observed in the lateral buds treated with BE (not shown). Application of Ac-YVAD-CHO, an inhibitor of caspase-1, reduced the measured VPE activity to the baseline, which was very similar to the level measured in meristems that were not treated by BE (Fig. 6D). We found the same pattern of VPE activity induction and specific inhibition by Ac-YVAD-CHO, using Z-AAN-MCA, another specific substrate of VPE purified from potato tuber (not shown).

To clarify whether BE induces VPE activity, impairing the tonoplast and leading to PCD, the fine intracellular structure of the TAB-meristem treated with BE was examined by transmission electron microscopy (TEM). BE treatment induced disruption of the tonoplast, and the formation of a knob-like body in the vacuole and small vesicles in the cytoplasm 48 h after treatment initiation (Fig. 7, C-D and G). These symptoms increased after 72 h (Fig. 7, E-F and H). The tonoplast of about 20 to 40% of the meristem cells was either not affected by the BE treatment, or symptoms were minor. Nontreated meristem showed no symptoms of tonoplast disruption (Fig. 7, A-B).

The overall morphology of the dying cells in the BE-treated TAB-meristem was analyzed at smaller magnification to further confirm the presence of hallmarks of PCD. Cells were analyzed by TEM 48 h and 72 h after initiation of BE treatment. In the early stages, numerous vacuoles were detected around the nucleus, as well as plastolysome-like structures containing electron-translucent cytoplasm (Fig. 8A). We detected budding-like nuclear segmentations resulting in the separation of nuclear fragments, inclusions of condensed chromatin within the provacuoles, clustering of nuclear pore complex during nuclear segmentation, and lobed nuclei (Filonova et al., 2000) (Fig. 8, B-C). At a moderately advanced stage of PCD (72 h after BE treatment initiation), the nucleus was pushed against the cell wall by the central vacuole which contained condensed chromatin and nuclear pore complex (Fig. 8D).

VPE was partially purified from TAB-meristem 48 h after BE treatment initiation, at the time when its higher activity was detected. Unexpectedly based on the theoretical pI of VPE1 (5.66), the protein did not bind to the cation-exchange column at pH 5.5 but did bind
well to the anion-exchange column (Fig. 9A), suggesting that its experimental pI is lower than the calculated one. The active fraction eluted from the size-exclusion column as a ca. 29-kDa protein (Fig. 9B), whereas its theoretical size is 53.7 kDa, suggesting that VPE1 undergoes processing to become active, as reported for other plant VPEs (Kuroyanagi et al., 2002; Kuroyanagi et al., 2005). During all three purification steps, only one activity peak was observed using both substrates, suggesting only one enzyme as the source (Fig. 9, A and B). Amino acid sequence analysis of the partially purified active fraction revealed nine peptides with sequences covering 33% of the predicted mature VPE1 (Fig. 9C).

To check whether VPE is involved in apical bud growth or dominance, we treated detached apical buds with the Ac-YVAD-CHO inhibitor, shown to inhibit TAB-meristem VPE activity (Fig. 6D). Ac-YVAD-CHO induced faster development of TAB-meristems at all concentrations tested (0, 50, 100 and 200 µM), with an optimum at 50 to 100 µM (Fig. 10A), while the 200 µM concentration was less effective (not shown).

Application of Ac-YVAD-CHO to the apical bud of cv. Ditta tubers, treated with BE, nullified BE's effect, induced early and faster growth of the apical buds and restored AD compared to controls (treated only with BE) after 4 d in 87, 67 and 60% of tubers treated with 50, 100 and 200 µM inhibitor, respectively (Fig. 10, B and C). Tubers that were not treated with Ac-YVAD-CHO showed 29% AD compared to 87% +/- 5% dominance for their non-BE-treated counterparts, that sprout 2 weeks later. Measurements performed after 5 to 7 d showed a reduction in the effect of Ac-YVAD-CHO application (data not shown).

DISCUSSION

Stress Induces Dormancy Release and Alters Apical Dominance

Several studies have shown that the length of the dormancy period depends on genetic background and is affected by pre- and postharvest conditions and physical or chemical stress (Sonnewald, 2001; Suttle, 2004b). Application of hydrogen cyanamide to grape buds results in the breakage of endodormancy and is used commercially to compensate for lack of natural chilling (Erez, 1987; Ophir et al., 2009). Teper-Bamnolker et al. (2010) showed that very low doses of the sprout inhibitor R-carvone induce early
sprouting and loss of AD. Whereas high doses of this inhibitor were shown to damage cellular membranes in the apical meristem, no such damage was detected at the sprout-inducing dose, suggesting a signaling effect of the latter (Teper-Bamnolker et al., 2010). The synthetic dormancy-terminating agent BE, a phytotoxic compound, has been used to induce rapid and highly synchronized sprouting of dormant tubers (Campbell et al., 2008; Alexopoulos et al., 2009). BE has been shown to affect the endogenous ABA content of tuber meristems, which plays a critical role in tuber dormancy control (Destefano-Beltran et al., 2006). In a potato microarray (POCI array) analysis, Campbell et al. (2008) observed similar transcript profiles for natural (cold-storage aging) vs. BE-induced dormancy release. We found a dramatic effect of BE application on dormancy and AD of the TAB-meristem, suggesting a connection between these two physiological conditions. Nonetheless, the two conditions are independent since apical bud of freshly harvested potato tubers can sprout earlier at room temperature than in the cold, while AD is preserved (Fig. 2).

The effect of mechanical damage to the apical bud on AD in the potato tuber shows the tuber's clear stem-like behavior. Growth of the apical bud, as expected, suppresses lateral bud development, and by removing it, axillary bud sprouting is induced, in increasing numbers as the tuber ages. This suggests the need for autonomous dormancy release of each bud before it can be controlled by the apical bud. Removing the apical complex had a milder effect on reducing AD than removing only the apical meristem, suggesting a specific role for the apical meristem base. It is logical to assume that the ability of BE to induce simultaneous growth of all buds is tied to its ability to reduce apical bud dominance, since the apical bud must sprout first in order to achieve dominance.

The shoot AD and branching regulatory system involves three long-range hormonal signals: auxin, which is synthesized mainly in young expanding leaves then moves down the plant in the polar transport stream, and strigolactone and cytokinins, synthesized in both the root and shoot, which move up the plant, most likely in the transpiration stream (Leyser, 2009; Müller and Leyser, 2011). Auxin clearly plays a role in AD, i.e. suppression of axillary buds by the apical meristem, but the mechanism by which the auxin signal is perceived in the axillary bud is subject to debate (Dun et al., 2006). Since the importance of TAB-meristem viability has been suggested, it follows that induction of local PCD in the meristem cells should result in altering bud AD.
PCD Is Enhanced in the Apical Meristem of Potato Tubers following BE Treatment

Loss of AD, induced by either aging in cold storage or BE treatment, was found to be strongly correlated with the occurrence of PCD in the TAB-meristem. This suggests a functional relationship between local PCD and AD control by affecting TAB-meristem cell viability. Even if most cells in the TAB-meristem lost viability, we might expect that an axillary meristem would grow in the same tuber eye, as shown in decapitation experiments or by adding sprout inhibitors (Teper-Bamnolker et al., 2010). While cold during prolonged storage cannot be separated from the aging effect, the short BE treatment's immediate effect on lateral bud growth suggests the important role of PCD in apical bud suppression of growth and dominance. Nevertheless, the decapitation experiments emphasize the sole apical bud's importance in AD.

PCD plays an important role in various stages of plant development, such as embryogenesis, self-incompatibility, xylogenesis and senescence, and in response to biotic or abiotic stress (Fukuda, 2004; Lam, 2004; Bozhkov et al., 2005; Van Breusegem and Dat, 2006; Della Mea et al., 2007; Turner et al., 2007; Bonneau et al., 2008; Lam, 2008). For example, PCD is induced in plants as a result of pathogen infection (Hatsugai et al., 2004; Zhang et al., 2010) or in the roots as a result of salt stress (Katsuhara and Shibasaka, 2000; Huh et al., 2002; Li and Dickman, 2004). The induction of PCD in plants by biotic/abiotic stresses suggests a role in adaptation to environmental stress (Williams and Dickman, 2008). We are aware of only one report regarding DNA fragmentation in senescing apical buds (long-day-grown G2 pea; Pisum sativum L.) following transition to reproductive growth (Li et al., 2004). DNA fragmentation has been detected in the apical meristems of wild-type plants, and is significantly inhibited in transgenic Arabidopsis overexpressing the PPF1 gene, altering calcium homeostasis. Here we detected DNA fragmentation in the developing TAB-meristem, suggesting developmental or environmentally induced PCD. BE treatment or aging of the tuber in cold storage induced the widespread appearance of TUNEL-positive cells in the TAB-meristem (Figs. 4–6). BE is characterized by its ability to induce early sprouting of all buds, as opposed to cold storage which delays sprouting. Nevertheless, both treatments resulted in reduced dominance of the tuber apical bud, suggesting a relationship
with their ability to induce PCD in the apical and lateral bud meristems. AD is known to be released in stored potato tubers during aging (Krijthe, 1962; Kumar and Knowles, 1993b), and tuber aging has been correlated with loss of membrane integrity due to peroxidative damage, the activities of scavenging enzymes and increased lipid oxidation (Kumar and Knowles, 1993a; Fauconnier et al., 2003; Delaplace et al., 2008). Although those studies measured oxidative changes in the tuber parenchyma, such changes might be associated with developmental or environmentally induced PCD as sprouting is part of tuber aging in storage.

It has been shown in a variety of plants that AD depends on polar auxin transport (reviewed by Müller and Leyser, 2011). Changes in TAB-meristem cell viability could affect auxin-signaling components. In a detailed study, Sorce et al. (2009) reported a progressive decrease in free IAA content in potato tubers during the dormancy period. In addition, immunolocalization studies showed accumulation of the hormone in the TAB-meristem and the vascular tissue beneath the dormant bud. In sprouting tubers, however, 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. Induction of PCD in the TAB-meristem may have a negative effect on auxin transport to lateral buds. Cytokinins and strigolactone, shown to be central players in AD (reviewed by Müller and Leyser, 2011), might be affected as well. The relationship between the observed PCD and transport of the relevant hormones will be examined in future studies.

Induction of both VPE1 expression and VPE activity was detected immediately after BE treatment, suggesting a rapid response of the meristem tissue. VPE, a cysteine protease, is the accepted plant counterpart of cysteinyl Asp-specific protease-1 (caspase-1) in animals (Hatsugai et al., 2006). This enzyme plays a crucial role in vacuolar collapse during plant PCD in both defense and development (Hara-Nishimura et al., 2005). Caspases belong to a class of specific cysteine proteases that show a high degree of specificity to an Asp residue in a recognition sequence comprised of at least four amino acids N-terminal to this cleavage site (Woltering, 2010). Plants do not have close orthologs of caspases but several caspase-like activities have been detected in plant extracts (Bonneau et al., 2008). Furthermore, use of mammalian caspase inhibitors has shown that plant caspase-like activities are required for PCD (Rotari et al., 2005; Bonneau et al., 2008). Biochemical and pharmacological studies
also support the involvement of caspase and serine proteases in plant PCD (Groover and Jones, 1999; Yano et al., 1999; Sasabe et al., 2000; Coffeen and Wolpert, 2004; Antao and Malcata, 2005). Plants do possess a phylogenetically distinct family of cysteine proteases, the VPEs (legumains) (Hatsugai et al., 2004; Kuroyanagi et al., 2005; Hatsugai et al., 2006), and serinyl Asp-specific proteases (saspases) (Coffeen and Wolpert, 2004; Piszczek and Gutman, 2007). In the process of PCD in plants, disruption of vacuoles or a change in the permeability of the tonoplast plays an essential role in speed of death and degree of recovery of the cell contents (Mino et al., 2007). VPEs, as well as other types of proteases (Bonneau et al., 2008; Lam, 2008; Reape and McCabe, 2008), are functionally but not structurally related to caspases (Vercammen et al., 2004; Watanabe and Lam, 2004; Bonneau et al., 2008). Control of PCD is essential for its containment to specific tissues; hence proteolysis by caspases and serine proteases is tightly regulated (Turk and Stoka, 2007).

We did not detect altered regulation of potato genes homologous to the antioxidant defense system genes usually activated in response to ROS generation, such as CAT2 (Mittler, 2002; Mizuno et al., 2005). The Arabidopsis thaliana stress-response gene, EX1, or gene homologs that lead to runaway cell death such as LSD1 (Coupe et al., 2004; Yao and Greenberg, 2006), were also not dramatically altered (not shown). We did not detect any effect of BE treatment on the potato SPT gene, which has been shown to be induced by Phytophthora infestans infection in the field-resistant potato cv. Stirling. The potato gene homologous to the S1-type nuclease, ZEN1, shown to function directly in nuclear DNA degradation during PCD of tracheary elements, was not regulated differently by BE treatment although we found TUNEL-positive cells in the vascular elements as well (Fig. 4). Introduction of ZEN1-antisense into Zinnia cell cultures specifically suppressed the degradation of nuclear DNA in tracheary elements undergoing PCD, but did not affect vacuolar collapse (Ito and Fukuda, 2002). This might explain the lack of coupling of the potato ZEN1 gene to VPE1 upregulation in potato. Assessing the potato ZEN1 gene expression levels during storage revealed minor changes in its expression (not shown).

Specific inhibitors of PCD in plant cells include cysteine protease inhibitors (Solomon et al., 1999), which presumably interact with the VPEs (Watanabe and Lam, 2004, 2006). Nevertheless, the role of cysteine protease inhibitors in potato dormancy remains unclear. Similar to our observation, the same cysteine protease inhibitor gene has
been reported to be downregulated during dormancy release induced by BE (Campbell et al., 2008). Specific protease inhibitors play crucial roles in the cellular regulation of proteases during the PCD process (Shi, 2002; Woltering et al., 2002; 2010). Remobilization of storage proteins accompanies sprouting, and an increase in cDNAs encoding cysteine protease in sprouting tubers has also been reported by Ronning et al., (2003). Interestingly, dormancy release in tuber meristems is accompanied by decreased expression of a number of protease inhibitors, including metallocarboxypeptidase, cysteine protease, aspartic protease and serine protease, as well as a number of other unspecified protease inhibitors (Campbell et al., 2008). Thus, termination of dormancy in potato tuber meristems results in decreased expression of inhibitors of all four major classes of plant proteases (Callis, 1995; Schaller, 2004). Although proteases have been shown to be involved in PCD (Solomon et al., 1999), there is evidence that they play a significant role in other developmental processes as well, such as epidermis, plastid, and shoot development (Tanaka et al., 2001; Kuriyama and Fukuda, 2002; Kuroda and Maliga, 2003).

Studies examining changes in gene expression associated with dormancy termination in raspberry buds also found that protease inhibitors are downregulated as dormancy terminates (Mazzitelli et al., 2007). Comparison of cold storage (“natural”) dormancy release to BE-induced dormancy release in postharvest potato revealed a commonality in the downregulation of transcripts of proteinase inhibitors related to PCD (Campbell et al., 2008).

It is possible that VPE1 and the cysteine protease inhibitor (TC137660) play a role in the PCD occurring during bud sprouting and AD release following BE treatment. Changes in the regulation of VPE1 and the protease inhibitor genes during cold-storage aging were minor as compared to those occurring with BE treatment (not shown), suggesting a long and moderate change during loss of AD. This raises the question of whether it is logical to look for gene regulation in gradual processes such as AD release in long storage. BE, as shown previously for potato dormancy release, serves as a research tool that compresses this biological process to a short and detectable period of time.

Treatment with BE clearly induced VPE activity in the apical and lateral bud meristem. Nevertheless, we suggest that only the activity in the TAB-meristem affects AD, similar to the decapitation experiments. The VPE1 that was partially purified from apical
buds 48 h after initiation of BE treatment had a different pI and MW than the theoretical
protein (Fig. 9), probably due to posttranslational processing. The *Arabidopsis* γVPE
preproprotein precursor has been shown to have a 22-aa signal peptide, and C-terminal and
N-terminal propeptides that are self-catalytically removed co-translationally, converting the
56-kDa preproprotein into a mature 40-kDa protein (Kuroyanagi et al., 2002). During all
three purification steps, only one activity peak was observed using both substrates,
suggesting only one enzyme as the source (Fig. 9, A and B). Analysis of the partially
purified active fraction revealed nine peptides covering 33% of the mature VPE1 protein
(Fig. 9C). The rise in VPE1 was correlated with vacuolization of meristematic cells. The
presence of VPE might be required for vacuolar disruption, probably followed by tonoplast
disruption and small vesicle formation (Fig. 7). We observed no change in the vacuole
tonoplast of some of the meristem cells following the BE treatment, which probably
remained viable. Later, DNA fragmentation, characteristic of PCD (van Doorn and
Woltering, 2005) is induced by BE in a sporadic way in the cells of the TAB-meristem (Fig.
6).

CONCLUSION

In summary, our data suggest that potato tubers exhibit AD behavior that is very
similar to that of other stems. Apical bud dominance affects only buds whose dormancy is
released. Developmental or environmentally induced PCD is involved in normal apical bud
meristem development. Accelerated and widespread PCD, as affected by aging and BE,
induces a reduction in apical bud dominance. The enzyme VPE1 could be a key factor in
TAB-meristem development and dominance: using a specific VPE inhibitor restored growth
and dominance of the apical bud, though this needs to be further proven in transgenic potato
tubers.

METHODS

**Plant Material and Potato Tuber Bud Disc Sprouting Assay**
Three potato (*Solanum tuberosum* L.) cultivars that are commonly grown in Israel were used: Nicola, Désirée and Ditta. Tubers were grown in two main areas of the country, the Sharon and the northern Negev, under standard field conditions. Harvested tubers were cured for 2 weeks, during which time the temperature was gradually reduced from 25 to 6°C; they were then stored in the dark at 6°C in 95% humidity generated by an ultrasonic humidifier (SMD Technology, Rehovot, Israel).

**Decapitation Experiments**

Apical meristem was excised with a sterile 0.5 x 16 mm needle under an MZFLIII stereomicroscope (Leica, Wetzlar, Germany). Decapitation of apical complex, and excision of lateral meristem located in the fifth position and nonmeristematic tissue were performed with a borer (Ø 0.5 cm, 3 mm penetration). Treated tubers were stored at 20°C, 95% RH. Sprouting buds were counted every 3 d, 3 to 24 d after treatment. Buds were defined as sprouting when they reached 3 mm in length.

**BE Treatments**

BE treatments were performed as described by Law and Suttle (2002). In the BE experiments, only tubers that were verified to be dormant, and had not sprouted after 2 weeks at 20°C (Campbell et al., 2008), were used. Each treatment contained three replicates of four glass chambers containing 10 tubers each, for a total of 120 tubers, which were sealed and exposed to filter paper soaked with 0.2 mL BE (Sigma, Rehovot, Israel) per L of container volume. The sealed glass chambers were incubated for 24 h at room temperature in the dark. After treatment, tubers were placed in a chemical hood for 6 h at room temperature (to allow the release of absorbed BE vapors) and subsequently stored at 20°C in the dark in 95% humidity. Using a cork borer (Ø 0.5 cm, 3 mm penetration), the apical complex was excised at several time points: immediately after treatment (24 h), and at 48 and 72 h after treatment. Excised meristems were immediately frozen in liquid nitrogen and stored at -80°C for later quantitative real-time RT-PCR analysis and measurements of VPE activity.
Tubers were washed with tap water and incubated for 24 h at 20°C. Treatment of whole tubers with Ac-YVAD-CHO (Peptide Institute, Osaka, Japan) was performed as described by Suttle (2009) for hormones, with some modifications. Briefly, a small (about 3-mm deep) cavity was made below the apical bud of 30 tubers per treatment, using a 200-μL pipette tip. Test solutions were prepared in 2% (v/v) DMSO and 2 μL Ac-YVAD-CHO suspension was introduced daily, for 5 d, into each cavity by pipetting (controls received 2% DMSO only). Treated tubers were incubated in the dark at 20°C and 97% RH.

For the detached bud sprouting assay, we used a modification of the method described by Rentzsch et al. (2011). Briefly, the tuber was surface-sterilized for 5 min in 1% (v/v) NaOCl and then washed for 5 min in tap water. Tuber bud (‘eye’) discs of 6-mm diameter were excised using a cork borer and cut to 5-mm height. Ca. 16 bud discs were washed three times with MES buffer and incubated for 5 min under shaking submerged in 5 mL of 2% DMSO without (0) or with 50, 100 or 200 μM Ac-YVAD-CHO.

Histological Analysis—TUNEL and DAPI Staining

Histological analyses were performed on 10-μm-thick bud meristem sections cut by microtome. Samples were stained according to the method described by Teper-Bamnolker (2010). For TUNEL staining, fixed tissues were rehydrated with Histoclear and decreasing concentrations of ethanol (100, 70 and 30%). Tissue permeabilization was performed with 20 μg/mL Proteinase K (Gibco BRL) in 10 mM Tris 7.5 and 5 mM EDTA pH 8 at 37°C for 30 min. After washing the tissue twice with PBS, lysing enzyme (4 mg/mL) in 5 mM EDTA pH 8 was added for 20 min with incubation at 37°C. TUNEL reaction was performed on slides using the In Situ Cell Death Detection kit with fluorescein (Roche Applied Science) according to the manufacturer’s instructions. To visualize nuclei in meristem cells, samples were stained with 4’-6-diamidino-2-phenylindole (DAPI, Sigma) at 1 μg/mL in PBS buffer for 10 min. DAPI and TUNEL-positive staining were observed with an IX81/FV500
confocal laser-scanning microscope (CLSM; Olympus) equipped with a 488 nm argon ion laser and 405 nm diode laser. DAPI was excited with the 405 nm diode laser and the emission was collected through a BA 430–460 nm filter. TUNEL was excited with 488 nm light and the emission was collected through a BA505IF filter. The transmitted light images were obtained using Nomarski differential interference contrast (DIC), and 3D images were obtained using the FluoView 500 software supplied with the CLSM.

Histological Analysis—TEM

TAB-meristem tissues were fixed in 3.5% (v/v) glutaraldehyde in PBS, and then rinsed and postfixed in 1% (v/v) OsO₄ in PBS. Following several washes in PBS, the tissues were stained with uranyl acetate. The samples were dehydrated by passing them through an ethanol series and acetone and were then embedded in Agar100 epoxy resin (Agar Scientific, Cambridge, UK). Thin sections were cut, treated with uranyl acetate/lead citrate, and examined with a Tecnai G2 Spirit transmission electron microscope (TEM) (FEI, Phillips, Netherlands). Representative photos are presented.

Cloning of Cell Death Regulatory Genes from Potato cv. Nicola

Gene sequences in *S. tuberosum* were found or predicted based on translated BLAST searches from the most closely related available proteins (usually tomato and *Arabidopsis*). Primers were designed according to potato expressed sequence tags (ESTs) and mRNA (in GenBank or Solgenomics, [http://solgenomics.net/](http://solgenomics.net/)) and were derived from individual ESTs or clustered/aligned ESTs (Supplemental Material Table I). PCR products obtained from DNA and cDNA of cv. Nicola were cloned in pGEMT vector (Promega) and transformed into competent JM109 cells (Promega). Plasmids were extracted with GenElute™ Plasmid Miniprep kit (Sigma) and sequenced using vector primers SP6 and T7 (Cheng et al., 2007) by Hylabs Ltd. (Rehovot, Israel). PCR was performed with TaKaRa Ex Taq™ (Takara Bio, Shiga, Japan) for 35 cycles of denaturation at 95°C for 40 s, annealing under a temperature gradient of 55 to 62°C for 40 s, and extension at 72°C for 1 min, followed by a final
extension step of 10 min at 72°C. Sequences were BLAST-analyzed to verify their identity
and used for real-time PCR primer design.

**DNA Isolation**

DNA was extracted from potato cv. Nicola leaves. Samples were crushed and mixed
thoroughly with DNA extraction buffer (200 mM Tris-HCl pH 7.5, 250 mM NaCl, 25 mM
EDTA pH 8.0, 0.5% w/v SDS dissolved in double-distilled water). After centrifugation
(20,000g for 5 min), the supernatant was mixed gently with isopropanol. After a second
centrifugation, the supernatant was decanted and the residue was dissolved in 50 μL double-
distilled water.

**RNA Isolation**

Potato cv. Nicola bud meristems were isolated using a cork borer (Ø 0.5 cm, 3 mm
penetration). Tissue samples were collected from 120 tubers (divided into three independent
replicates) after BE treatment or during 6°C postharvest storage and immediately frozen in
liquid N₂ and stored at -80°C until use. Total RNA was isolated according to Logemann et
al. (1987). After extraction, RNA samples were treated with Turbo DNase (Ambion) to
remove contaminating DNA according to the manufacturer’s protocol. Concentrations of
RNA samples were measured with a ND-1000 spectrophotometer (Nanodrop Technologies)
and purity was verified by optical density absorption ratio at 260 nm and 280 nm
(OD_{260}/OD_{280} between 1.80 and 2.05), and OD_{260}/OD_{230} (between 2.00 and 2.30). Sample
integrity was evaluated by electrophoresis on 1% agarose gels (Lonza, Rockland, ME)
containing 0.5 μg/mL Safe-View Nucleic Acid Stain (NBS Biologicals). Observation of
intact 18S and 28S rRNA subunits and absence of smears in the gel indicated minimal RNA
degradation.

**cDNA Synthesis**
cDNA was synthesized from 1.5 μg of total potato RNA using the Verso cDNA kit (ABgene) according to the manufacturer’s specifications. Cloned sequences with the longest perfect repetitions and flanking regions that permitted primer design were selected for real-time PCR primer design (Supplemental Material Table II) using Primer Express 2.0 (Applied Biosystems) and synthesized by Sigma. If possible, for each gene tested, at least one of the primers used spanned an exon-exon border so that only cDNA could be amplified. Two transcripts of VPE are known in potato (http://www.ncbi.nlm.nih.gov/): VPE1 (EU605871.1) and VPE2 (EU605872). The VPE1 nucleotide sequence exhibits 98% identity with that of VPE2. Specific primers for VPE1 and VPE2 revealed that only VPE1 is expressed in TAB-meristems of 'Nicola' potato tubers treated with BE (data not shown). As housekeeping genes, we used ubiquitin (ubi3, L22576) or actin (TC133139) as described in previous studies (Kloosterman et al., 2005; Campbell et al., 2010).

**mRNA Regulation Analysis**

Quantitative real-time RT-PCR analysis of potato cDNA was performed using the ABsolute QPCR SYBR Green Mix kit (ABgene). Reactions were run on a Corbett Research Rotor-Gene 3000 cycler. To check for DNA contamination, we ran a reaction with RNA only. A standard curve was obtained for each gene using a cDNA mix of analyzed samples. Reactions for each gene in each cDNA sample were repeated independently at least four times. Quantification of each gene was performed using Corbett Research Rotor-Gene software. The expression of each gene was an average of at least three replicates. We only used replicates in which the standard deviation of a population was <1.5% of the average. Relative expression of a gene in a certain sample was initially obtained by dividing the gene level (in arbitrary units) by that of the housekeeping gene (in arbitrary units). The sample with the lowest expression level was given a relative value of 1.

**VPE Activity**

VPE activity was measured using the method reported by Mino et al. (2007) with some modifications. The frozen apical meristems were homogenized in extraction buffer
(50 mM sodium acetate pH 5.5, 50 mM NaCl, 1 mM EDTA and 100 mM DTT) under ice-cold conditions. The homogenate was centrifuged at 15,000g for 15 min at 4°C and the supernatant was used for the enzyme assay. Ac-ESEN-MCA and Z-AAN-MCA (100 µM; Peptide Institute) were used as the substrates for the reactions, and the amount of 7-amino-4-methyl-coumarin (AMC) released was determined spectrophotometrically at an excitation wavelength of 380 nm and an emission wavelength of 460 nm (Enspire, 2003 Multi Label Reader, Perkin Elmer) after 2 h incubation at room temperature. A known amount of AMC was used for calibration. To confirm that the hydrolyzing activity was from VPE, 200 µM Ac-YVAD-CHO, which specifically inhibits VPE (Hatsugai et al., 2004; Mino et al., 2007) was added to the reaction mixture. Protein content was determined with a BCATM Protein Assay kit (Pierce) using bovine serum albumin as the standard.

Partial Purification of VPE1

TAB-meristems of cv. Nicola potato tubers were isolated using a cork borer (ø 0.5 cm, 3 mm penetration). Tissue samples were collected 48 h after the beginning of BE treatment and immediately frozen in liquid N$_2$ and stored at -80°C until use. Ground tissue (6 g) was homogenized in 25 mL extraction buffer [50 mM sodium acetate pH 5.5, 10 mM thiourea, 10 mM CaCl$_2$, 1 mM PMSF, 5 mM DTT and 0.6 g poly(vinylpolypyrrolidone) (PVPP, Sigma)] under ice-cold conditions. The homogenate was centrifuged at 12,000g for 30 min at 4°C and the supernatant was agitated with 0.6 g PVPP for 2 min, then a second centrifugation was performed and the supernatant was adjusted to 10 mM EDTA by adding 0.5 M EDTA pH 8.5 and used for purification. VPE1 was purified in three steps using a fast-performance liquid chromatography (FPLC) system (ÄKTAexplorer™, GE Healthcare) at 4°C. Fractions were checked for VPE activity, as previously described, using 100 µM Ac-ESEN-MCA and 100 µM Z-AAN-MCA. In the first step, supernatant was loaded on a 1-mL cation-exchange column (SP sepharose HP, GE Healthcare), previously equilibrated with 15 column volume (CV) of 50 mM sodium acetate pH 5.5. The unbound fraction was loaded (2 mL/min) on the anion-exchange column (Mono Q 10/100 GL, GE Healthcare) previously equilibrated
with 15 CV of 50 mM sodium acetate pH 5.5. The sample was eluted with 20 CV of a linear salt gradient (0–1 M NaCl). The absorbance was monitored at 280 nm, and 1.5-mL fractions were collected. Active fractions were pooled, dialyzed against double-distilled water at 4°C for 15 h (>3500 MW cut-off) and lyophilized. The lyophilized protein was dissolved in 2.5 mL of 50 mM sodium acetate pH 5.5 and loaded (1 mL/min) on Superdex-75 (GE Healthcare) previously equilibrated with 2 CV of 50 mM sodium acetate pH 5.5. Purified samples were collected in 1.5-mL tubes. Column calibration was carried out using blue dextran (2000 kDa), conalbumin (75 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), ribonuclease A (13.7 kDa), aprotinin (6.5 kDa) in a protein standard kit (GE Healthcare) using the same running conditions.

Fractions corresponding to peaks of activity were pooled, lyophilized, reduced with 2.8 mM DTT (60°C for 30 min), modified with 8.8 mM iodoacetamide in 100 mM ammonium bicarbonate (in the dark, at room temperature for 30 min) and digested in 10% ACN and 10 mM ammonium bicarbonate with modified trypsin (Promega) overnight at 37°C. The resulting tryptic peptides were resolved by reverse-phase chromatography on 0.075 x 200-mm fused silica capillaries (J&W) packed with Reprosil reverse-phase material (Dr. Maisch, GmbH, Germany). The peptides were eluted with linear 65-min gradients of 5 to 45% and 15 min at 95% acetonitrile with 0.1% formic acid in water at flow rates of 0.25 μL/min. Mass spectrometry was performed by an ion-trap mass spectrometer (Orbitrap, Thermo) in positive mode using repetitively full MS scan followed by collision-induced dissociation (CID) of the seven most dominant ions selected from the first MS scan. The MS data were clustered and analyzed using Discoverer software (Thermo-Finnigan), and searched against the Solanum tuberosum and Solanum lycopersicum sequences in the NCBI-nr database. MS was conducted at the Smoler Proteomics Center, Technion, Israel.
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Figure 1. Typical types of loss of apical dominance in stored potato tubers as demonstrated in cv. Désirée. Type I is loss of dominance of the apical buds over those situated more basipetally on the tuber. Type II is loss of dominance of the main bud in any given eye over the subtending axillary buds within the same eye. Type III is loss of dominance of the developing sprout over its own branching, meaning that the side stems do not come out from the base of the sprout as in type II.

Figure 2. Effect of storage conditions and bromoethane treatment on apical dominance in cv. Nicola (A, B and C) and Désirée (D, E and F) postharvest tubers. (A) and (D) Sprouting in apical dominance form after 45 and 60 d of dark storage at 20°C, respectively. (B) and (E) Sprouting in nondominance form after 60 d at 6°C plus 30 and 45 d of dark storage at 20°C, respectively. (C) and (F) Loss of apical dominance after forced sprouting, 10 and 20 d after BE treatment, respectively.

Figure 3. Dominance of apical meristem in nondormant postharvest cv. Nicola potatoes. (A), (B) and (C) Removal of apical bud following 30, 60 and 90 d in cold storage results in sprouting of an average of 1, 2 and 9 buds, respectively. (D) Schematic presentation of decapitation in potato tubers. (i) AP, apical meristem; (ii) AP Com, apical complex representing apical meristem with its underlying cortex; (iii) LT, lateral complex positioned 5 buds from the apical meristem. The control was unwounded (Control) or wounded between buds (iv; Wound). In each treatment, 40 tubers were decapitated. (E) Number of sprouting buds after decapitation. Error bars represent the standard deviation of four replicates.

Figure 4. Storage conditions that induce loss of apical dominance in postharvest 'Nicola' potatoes induce DNA fragmentation in the apical meristem cells. Histological analyses were performed on 10-µm-thick bud meristem sections. The cells were counterstained in situ with DAPI (blue color represents nuclei) followed by TUNEL reagents (green color represents DNA fragmentation). Corresponding phase contrast image (PhC) of meristem tissue is also shown. AD, conditions that induce apical dominance and include storage at 20°C for 30 and 45 d. ND, apical meristem cells isolated from tubers stored for 60 and 90 d at 6°C in the
dark. Tubers lost their apical dominance after 45–60 d in cold storage plus 30 d at 20°C. Scale bars = 100 µm.

**Figure 5.** Changes in nucleus morphology and DNA fragmentation in apical bud cells during dormancy release and sprouting. Histological analyses were performed on 10-µm-thick bud tip meristem sections. The cells were counterstained *in situ* with DAPI (blue color) followed by TUNEL reagent (green color represents DNA fragmentation). Corresponding phase contrast image (PhC) of meristem tissue is also shown. Apical meristem cells were isolated from tubers stored at 6°C in the dark. Each row (from top to bottom) represents a typical stage in the nuclear morphology of TUNEL-positive cells, 0–90 d postharvest. Scale bars = 10 µm.

**Figure 6.** Programmed cell death in potato apical meristem cells following bromoethane (BE) treatment. (A) Tuber apical meristem 48 h after exposure to BE treatment as compared to untreated (Control) tubers. Histological analyses were performed on 10-µm-thick TAB-meristem sections. The cells were counterstained in situ with DAPI followed by TUNEL reagents. Corresponding phase contrast image (PhC) of meristem tissue is also shown. Scale bars = 200 µm. (B) and (C) VPE1 and cysteine protease inhibitor TC137660 gene expression following BE treatment as analyzed by real-time RT-PCR. Data present the average of three experiments. (D) VPE activity in the apical bud after BE treatment and with the VPE inhibitor Ac-YVAD-CHO. Error bars represent standard error of three replicates.

**Figure 7.** Transmission electron micrographs of tissue from apical meristems of potato tubers after BE treatment. Note that the treatment induced the formation of a knob-like body in the vacuole and small vesicles in the cytoplasm, and disrupted the tonoplast. (A) and (B) Apical meristem cells of nontreated tubers. (C–H) Apical meristem cells 48 h (C, D, G) and 72 h (E, F, H) after the beginning of BE treatment. Typical and representative abnormalities found in the cells are indicated by arrows and arrowheads. Inset in E shows a more highly magnified view of the boxed area. D and F show a magnified view of knob-like bodies marked by black brackets in C and E, respectively. Arrows (in D and F) indicate knob-like bodies formed on the tonoplast. Arrowheads in E indicate disruption of the tonoplast.
Arrows in H indicate small vesicles formed in the cytoplasm. Bars, 5 \(\mu\)m in A, C, E, 2 \(\mu\)m in G, 1 \(\mu\)m in H, 700 nm in B, D and F. Abbreviations: cw, cell wall; sg, starch granule; v, vacuole; t, tonoplast.

**Figure 8.** Changes in nucleus and cytoplasm in the apical meristem cells of potato tubers after BE treatment. Cells were analyzed by transmission electron microscopy 48 h (A-C) and 72 h (D) after BE treatment. (A) Cell with early markers of PCD. Formation of numerous vacuoles around the nucleus and plastolysome-like structures containing more electron-translucent cytoplasm (asterisks; inset shows a higher magnification). (B) Budding-like nuclear segmentations resulted in separation of nuclear fragments (brackets; inset shows a higher magnification) into the adjacent cytoplasm. (C) Nuclear degradation detected at early stages of cytoplasm lysis, before formation of central vacuole. Note the inclusions of condensed chromatin (arrowheads) within provacuoles, the clustering of nuclear pore complex (arrows) during nuclear segmentation, and the lobed nucleus (stars). (D) Cell at moderately advanced stage of PCD. The nucleus is held against the cell wall by a central vacuole and condensed chromatin and nuclear pore complex can be detected in the central vacuole (arrowheads). Bars, 1 \(\mu\)m in A, inset in B and C, 2 \(\mu\)m in C and D, 5 \(\mu\)m in B. Abbreviations: cw, cell wall; cv, central vacuolar; n, nucleus; NPC, nuclear pore complex; pv, provacuoles; sg, starch granule; t, tonoplast.

**Figure 9.** Partial purification and identification of endogenous VPE1 from apical bud meristem of BE-treated tuber. (A) Anion-exchange chromatography; VPE activity (columns) was measured using the substrate Ac-ESEN-MCA. (B) Samples eluted after 50–65 mL were loaded onto a size-exclusion column, and VPE activity was measured using substrate Z-AAN-MCA. Samples eluted at 61–72 mL were collected. Fluorescence was measured at excitation 380 nm, emission 460 nm. (C) Peptides identified by LC-MS/MS as VPE1 are underlined.

**Figure 10.** Effect of VPE inhibitor (Ac-YVAD-CHO) application on detached apical bud growth (A) and apical dominance restoration in BE-treated tubers (B and C). Bars (placed at the bud base) = 500 \(\mu\)m. Arrows in B indicate apical buds. Error bars represent standard
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Figure 6. Programmed cell death in potato apical meristem cells following bromoethane (BE) treatment. (A) Tuber apical meristem 48 h after exposure to BE treatment as compared to untreated (Control) tubers. Histological analyses were performed on 10-μm-thick apical bud meristem sections. The cells were counterstained in situ with DAPI followed by TUNEL reagents. Corresponding phase contrast image (PhC) of meristem tissue is also shown. Scale bars = 200 μm. (B) and (C) VPE1 and cysteine protease inhibitor TC137660 gene expression following BE treatment as analyzed by real-time RT-PCR. Data present the average of three experiments. (D) VPE activity in the apical meristem after BE treatment. Error bars represent standard error of the mean.
Figure 7. Transmission electron micrographs of tissue from apical meristems of potato tubers after BE treatment. Note that the treatment induced the formation of a knob-like body in the vacuole and small vesicles in the cytoplasm, and disrupted the tonoplast. (A) and (B) Apical meristem cells of nontreated tubers. (C–H) Apical meristem cells 48 h (C, D, G) and 72 h (E, F, H) after the beginning of BE treatment. Typical and representative abnormalities found in the cells are indicated by arrows and arrowheads. Inset in E shows a more highly magnified view of the boxed area. D and F show a magnified view of knob-like bodies marked by black brackets in C and E, respectively. Arrows (in D and F) indicate knob-like bodies formed on the tonoplast. Arrowheads in E indicate disruption of the tonoplast. Arrows in H indicate small vesicles formed in the cytoplasm. Bars, 5 μm in A, C, E, 2 μm in G, 1 μm in H, 700 nm in B, D and F. Abbreviations: cw, cell wall; sg, starch granule; v, vacuole; t, tonoplast.
**Figure 8.** Changes in nucleus and cytoplasm in the apical meristem cells of potato tubers after BE treatment. Cells were analyzed by transmission electron microscopy 48 h (A-C) and 72 h (D) after BE treatment. (A) Cell with early markers of PCD. Formation of numerous vacuoles around the nucleus and plastolysome-like structures containing more electron-translucent cytoplasm (asterisks; inset shows a higher magnification). (B) Budding-like nuclear segmentations resulted in separation of nuclear fragments (brackets; inset shows a higher magnification) into the adjacent cytoplasm. (C) Nuclear degradation detected at early stages of cytoplasm lysis, before formation of central vacuole. Note the inclusions of condensed chromatin (arrowheads) within provacuoles, the clustering of nuclear pore complex (arrows) during nuclear segmentation, and the lobed nucleus (stars). (D) Cell at moderately advanced stage of PCD. The nucleus is held against the cell wall by a central vacuole and condensed chromatin and nuclear pore complex can be detected in the central vacuole (arrowheads). Bars, 1 μm in A, inset in B and C, 2 μm in C and D, 5 μm in B. Abbreviations: cw, cell wall; cv, central vacuolar; n, nucleus; NPC, nuclear pore complex; pv, provacuoles; sg, starch granule; t, tonoplast.
Figure 9. Partial purification and identification of endogenous VPE1 from apical bud meristem of BE-treated tuber. (A) Anion-exchange chromatography; VPE activity (columns) was measured using the substrate Ac-ESEN-MCA. (B) Samples eluted after 50–65 mL were loaded onto a size-exclusion column, and VPE activity was measured using substrate Z-AAN-MCA. Samples eluted at 65–70 mL were detected. Fluorescence was measured at excitation 380 nm, emission 460 nm. (C) Peptides identified by LC-MS/MS as VPE1 are underlined.
Figure 10. Effect of VPE inhibitor (Ac-YVAD-CHO) application on detached apical bud growth (A) and apical dominance restoration in BE-treated tubers (B and C). Arrows in A indicate buds, arrowheads in B indicate apical buds. Error bars represent standard error of three replicates.