Programmed Cell Death of the Megagametophyte during Post-germinative Growth of White Spruce (Picea glauca) Seeds is Regulated by Reactive Oxygen Species and the Ubiquitin-mediated Proteolytic System

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The megagametophyte of white spruce (Picea glauca) seeds undergoes programmed cell death following seed germination. This process is characterized by distinct morphological and biochemical features, such as DNA fragmentation and the induction of proteases. Biphasic production of hydrogen peroxide was detected in the megagametophyte following seed germination. ROS scavengers or inhibitors of ROS production decreased caspase-like protease activity and slowed the progression of cell death. One catalase (CAT) of white spruce reacted with antibodies directed against cotton-seed CAT. The corresponding CAT gene was cloned and compared with the catalase genes of other plant species. The activity of the white spruce CAT enzyme was stimulated by tyrosine phosphorylation. The phosphorylated CAT was subjected to ubiquitination and degraded by the proteasome. Furthermore, the proteasome inhibitor MG132 inhibited the degradation of CAT and delayed cell death. These results suggest that the interplay of CAT and the ubiquitin-mediated proteolytic system is critical in the control of ROS production and subsequent cell death.

Introduction

The haploid megagametophyte of white spruce (Picea glauca) seeds is a living nutritive tissue that encloses the embryo at seed maturity. With the exception of the micropylar megagametophyte cells, most cells of this tissue undergo programmed cell death (PCD) after seed germination. This process is characterized by distinct morphological and biochemical features, such as DNA fragmentation, and induction of proteases including caspase-like protease (CLP) activities (He and Kermode 2003a,b).

Reactive oxygen species (ROS) such as H$_2$O$_2$ and hydroxyl radicals are produced during normal cellular metabolism. ROS and ROS signaling are involved in many aspects of seed physiology; ROS generation can cause oxidative stress and damage cellular components (e.g. proteins, lipids, DNA and polysaccharides) leading to seed deterioration. On the other hand, ROS and ROS signaling may also play a role in the termination of seed dormancy (Bailly 2004, Oracz et al. 2007, Müller et al. 2009, Oracz et al. 2009). So far two mechanisms have been put forth: one involving cell wall loosening to promote seed germination and elongation of the radicle (Müller et al. 2009, Oracz et al. 2009); the other implicating targeted changes in protein carbonylation patterns resulting in dormancy alleviation (Oracz et al. 2007). Progress made in recent years has further implicated ROS as mediators of PCD in plants (reviewed in Apel and Hirt 2004, Mittler et al. 2004), including cell death associated with the hypersensitive response (Lamb and Dixon 1997), barley aleurone cell death (Bethke and Jones 2001), leaf senescence (Pastori and del Río 1997) and camptothecin-induced death of tomato suspension cells (de Jong et al. 2002). Among the ROS, H$_2$O$_2$ is the most stable and is a key player in stress responses and PCD (Gechev et al. 2002, Gechev and Hille 2005). Sub-lethal doses of H$_2$O$_2$ can protect cells by activating antioxidant enzymes, whereas toxic concentrations can...

Tetrameric catalase (CAT; EC 1.11.1.6) converts H$_2$O$_2$ to H$_2$O and O$_2$. It is predominantly found in the peroxisome/glyoxysome, but the enzyme also resides in mitochondria, and in the cytoplasm (Bray et al. 2000). CAT has been viewed as a sink for H$_2$O$_2$, and has been studied extensively (Willekens et al. 1997). A barley CAT-deficient mutant exhibits accumulation of H$_2$O$_2$ under normal conditions, resulting in lipid peroxidation and disrupted membrane function in mesophyll cells (Kendall et al. 1983). Transgenic tobacco plants with a lowered capacity to metabolize H$_2$O$_2$ (as a result of antisense suppression of a gene encoding CAT) develop necrotic lesions, exhibit defense-related gene expression in the absence of elicitors and are hypersensitive to pathogen attack (Chamnongpol et al. 1996, Takahashi et al. 1997, Mittler et al. 1999).

There is some evidence to intimate that plant CATs may be regulated at the post-translational level. Calmodulin (CaM) can bind to and activate some plant CATs (Yang and Poovaiah 2002). Yeast two-hybrid analyses show interactions between three CATs of Arabidopsis and a nucleoside diphosphate kinase (NDK1) (Fukamatsu et al. 2003); however, NDK does not phosphorylate CAT (Yoshida et al. 2006), suggesting that CAT may be subject to other regulatory mechanisms.

In mammalian cells, an increase in intracellular protein tyrosine phosphorylation is one of the earliest events of oxidative stress responses (Yamamura 2002). Tyrosine phosphorylation is carried out by a protein tyrosine kinase; the kinase catalytic activity of this enzyme is regulated by a phosphatase (Yamamura 2002). Mammalian non-receptor tyrosine kinase c-Abl and the product of the c-abl-related gene (Arg) respond to oxidative stress (Kharbanda et al. 1995, Cao et al. 2003a). c-Abl is activated and targeted to mitochondria in H$_2$O$_2$-treated cells and induces loss of the mitochondrial transmembrane potential, resulting in cytochrome c release and induction of apoptosis (Sun et al. 2000, Kumar et al. 2001, Cao et al. 2003a). In addition, c-Abl and Arg can stimulate CAT and glutathione peroxidase activities by tyrosine phosphorylation (Cao et al. 2003b,c). The details of post-translational mechanisms that regulate the activities of ROS-scavenging enzymes have not been elucidated in plants.

The ubiquitin–proteasome pathway is the main route for intracellular protein degradation in eukaryotes and the involvement of this pathway in the regulation of PCD is well established in animal systems. This pathway consists of the ubiquitin-conjugating system and the 26S proteasome. Ubiquitin is first covalently linked to cellular proteins; this targets the modified proteins for degradation by the proteasome (Hershko and Ciechanover 1998). Important regulators of apoptosis, including the B-cell lymphoma-2 (Bcl-2) family of proteins, the inhibitors of apoptosis (IAPs) and regulators of the inhibitor of nuclear factor-$\kappa$B kinase have been identified as substrates of the proteasome (Jesenberger and Jentsch 2002). In animals, CAT is targeted for ubiquitination and degraded by the proteasome during oxidative stress responses (Cao et al. 2003b). In contrast, the mechanisms of CAT degradation during post-germinative growth of seedlings, and at other stages of the plant life cycle are unknown.

We have reported previously that the majority of megagametophyte cells of white spruce (Picea glauca) seeds undergo PCD following seed germination (He and Kermode 2003a,b). Inhibition of CLP activity delays the onset of DNA fragmentation (He and Kermode 2003b). In this study, we further investigated the mechanisms involved in regulating megagametophyte cell death. Our data suggest that ROS are mechanistically connected to megagametophyte cell death. We also demonstrate that the activity of CAT is stimulated by phosphorylation of tyrosine residues when the H$_2$O$_2$ concentration increases. This modified CAT is subjected to ubiquitination, resulting in its degradation by the proteasome. These results suggest that the ubiquitin–proteasome pathway is involved in regulating megagametophyte cell death partly via degradation of CAT.

**Results**

**Generation of ROS is accompanied by cell death**

To investigate whether ROS are involved in the programmed death of white spruce megagametophyte cells, H$_2$O$_2$ and O$_2^-$ were determined by the luminal assay and nitro-blue tetrazolium (NBT) staining, respectively. Two peaks of H$_2$O$_2$ were detected in the megagametophyte following seed germination: the first peak occurred shortly after germination, when the radicle was ≤2 mm; the second peak occurred at a late post-germinative stage, when the seed’s radicle and hypocotyl were ~20 mm in length (stage 6, Fig. 1a). Unlike H$_2$O$_2$, generation of O$_2^-$ was detected only when the seed’s radicle and hypocotyl were ~20 mm in length (stage 6, Fig. 1b). A large proportion of megagametophyte cells died during the stages when the seed’s radicle and hypocotyl had increased from 15 to 25 mm (see Fig. 2, H$_2$O$_2$, from d 3 to d 5; refer also to Table 1). Thus, ROS production in the megagametophyte precedes or accompanies cell death. The first peak of H$_2$O$_2$ production (Fig. 1a) may be associated with the death of megagametophyte cells in the micropylar region of the white spruce seed, which appears to be associated with germination, or with very early post-germinative seedling growth (He and Kermode 2003a,b).

To further investigate the regulatory role of ROS in megagametophyte cell death, germinated seeds were treated with Tiron (an O$_2^-$ scavenger), dimethylthiourea (DMTU); a hydroxyl radical scavenger) or diphenylenediiodonium (DPI, an inhibitor of flavoenzymes, particularly NADPH oxidase; Doussie`re and Vignais 1992, Riganti et al. 2004). Treatment with Tiron (1 mM) and DPI (50 µM) significantly delayed megagametophyte cell death (P < 0.05; Fig. 2); cell death was not significantly changed in the presence of 1 mM DMTU (data not shown).

Because the targets of ROS are often intracellular macromolecules, we determined the stages associated with protein and
lipid oxidation/peroxidation. Protein carbonylation is an irreversible oxidative process resulting in loss of activity and degradation of the modified protein (Nyström 2005). Proteins modified in this manner can be detected by a 2,4-dinitrophenol (DNP) immunoassay. Since megagametophyte cells contain mainly storage proteins at early post-germinative stages, to detect carbonylated proteins, we loaded gels on the basis of an equal volume of protein extract (i.e. 10 µl out of 100 µl of soluble proteins extracted from 10 megagametophytes) at each stage rather than on an equal protein content basis. As shown in Fig. 3A, carbonylated proteins were detected at early post-germinative stages but rapidly increased at stages associated with extensive death of the megagametophyte (i.e. stages beyond stage 6). Lipid peroxidation, membrane damage caused by peroxidation of polyunsaturated fatty acids (Göbel et al. 2003), peaked at an earlier stage, when the seed’s radicle and hypocotyl is extensively damaged (Nyström 2005) or following germination. (A) Production of H$_2$O$_2$. Data are the means of three independent replicates ± SD. (B) Generation of O$_2^-$. Stages are defined by seedling length as noted in Table 1. Bar = 1 mm.

**Fig. 2** The effects of Tiron (1 mM) and DPI (50 μM) on the rate of megagametophyte cell death. Cell survival rate was estimated by TTC staining; 3–7 correspond to megagametophytes from germinated seeds (radicle ∼2 mm) after treatment with chemicals for 3–7 d, respectively. Data are the means of three independent replicates ± SD. Seedling lengths at d 3–7 of treatment are indicated in Table 1 (i.e., 15, 20, 25, 30 and 35 mm, respectively).

**Table 1** Definition of sampling stages for carrying out the experiments

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*Pre-germination and seedling (growth) stages are as follows: 0, seeds in germination conditions for 1 d (germination not completed); 2, seedling with radicle ∼2 mm; 5, seedling with ∼5 mm; 10, seedling with radicle and hypocotyl ∼10 mm; 15, seedling with radicle and hypocotyl ∼15 mm; 20, seedling with radicle, hypocotyl and cotyledons ∼20 mm; 25, seedling with radicle, hypocotyl and cotyledons ∼25 mm; 30, seedling with radicle, hypocotyl and cotyledons ∼30 mm; 35, seedling with radicle, hypocotyl and cotyledons ∼35 mm.

**Fig. 3** Protein oxidation (carbonylation) (A) and lipid peroxidation (B) in megagametophytes of white spruce seeds following germination. Stages are defined by seedling length as noted in Table 1. Proteins were extracted from megagametophytes (100 µl/10 megagametophytes for each stage) and 10 µl of the protein extract was loaded in each lane. In (A), times beyond stage 6 were not shown as proteins were extensively carbonylated at these stages. In (B), the cell death percentage at each corresponding stage is indicated above the histogram bars. At the 15-mm seedling stage (stage 5), the majority of seed reserves have been mobilized. Data are the means of three independent replicates ± SD.

**Table 1** Definition of sampling stages for carrying out the experiments

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hypocotyl were ~15–20 mm in length (Fig. 3B). (At the 15-mm seedling stage, the majority of storage reserves have been mobilized; He and Kermode 2003a,b). These data suggest that ROS result in the oxidation of proteins and lipids; this process is initiated during early post-germinative stages and so it is possible that a select group of the carbonylated proteins and lipid hydroperoxides act as signals to promote cell death (reviewed in Maccarrone et al. 2001, Montillet et al. 2005, Nyström 2005), and are not simply generated as a consequence of the cell death process.

**Activation of CLPs is downstream of ROS signaling**

CLP activity in the megagametophyte increases following white spruce seed germination, coincident with megagametophyte cell death; when inhibited by a caspase-3 inhibitor, cell death and DNA fragmentation are delayed (He and Kermode 2003b). To investigate the relationship between ROS and CLP activity, germinated seeds (radicle ~2 mm) were treated with 100 µM carbenzoxy-valyl-alanyl-aspartyl[O-methyl]fluoromethylketone (zVAD-fmk) (a broad range inhibitor of caspases), or with 50 µM DPI (an inhibitor of NADPH oxidase and other flavoenzymes), and the CLP activity within the megagametophyte determined. (At the concentrations used, none of the inhibitors adversely affected seedling growth.) When treated with zVAD-fmk, the CLP activity of megagametophytes was maintained at a very low level while that in the megagametophytes of seeds imbibed on water (control) increased markedly after 3 d (Fig. 4A). Interestingly, the DPI treatment also suppressed CLP activity in the megagametophyte (Fig. 4A). DNA fragmentation is delayed in the megagametophyte by a caspase-3 inhibitor (He and Kermode 2003b); therefore, we also examined DNA fragmentation after DPI and zVAD-fmk treatments. Both chemicals also inhibited DNA fragmentation (Fig. 4B). Thus it is possible that ROS act upstream of CLP activation.

**CAT activity is stimulated by tyrosine phosphorylation.**

Taken together, the above results suggest that ROS play a role in mediating megagametophyte cell death. Since the cellular ROS level is partly controlled by ROS-scavenging enzymes, and CAT has been viewed as a sink for H₂O₂, we examined changes in the activity of the CATs in megagametophytes during and following germination. Proteins were extracted from megagametophytes at different stages and CAT activity was determined. By in-gel activity assays. As shown in Fig. 5A (upper panel), the CAT activity changed dynamically following seed germination: CAT activity decreased when radicles were ~10mm (stage 4), then increased to a maximum when seedlings were ~20 mm (stage 6). Activity was virtually absent after stage 7, when seedlings were >25 mm (stage 8). Western blot analysis using antibodies specific for CAT of cotton seeds indicated that the white spruce CAT protein increased at stages 4 and 6, but decreased thereafter (Fig. 5A, lower panel). Interestingly, the changes in overall CAT activity were not entirely consistent with the changes in the CAT protein level (Fig. 5A, upper and lower panels), particularly at stages 3–5. As an initial investigation of post-translational regulation of CAT activity, we added 1 mM Na₃VO₄ (a tyrosine phosphatase inhibitor; see later results) during protein extraction prior to the CAT in-gel activity assay and Western blot analysis. Compared with Fig. 5A, CAT activity increased at stages 4, 7 and 8 while the protein level remained at a similar level (Fig. 5B), suggesting that CAT activity at these stages may be subject to post-translational regulation.

In order to further investigate the complex regulation of CAT, we cloned the white spruce CAT gene. The steady-state level of transcripts corresponding to the CAT gene of white spruce seemed to vary only slightly following germination (Fig. 5C), with transcripts persisting even at later stages when CAT protein abundance and activity were virtually undetectable (e.g. stage 8). Multiple alignment of deduced amino acid sequences (Fig. 6) indicated that the CAT of white spruce shows 95%, 86%, 83%, 81% and 76% identity with the CATs of Scots pine, cotton, CAT-1 of tobacco, CAT-1 of maize and CAT 3 of Arabidopsis, respectively. The white spruce CAT also exhibits 76% identity with the CaM-binding region of Arabidopsis CAT 3 characterized by Yang and Poovaiah (2002). However, our in vitro CAT activity assays with CaM/Ca²⁺ or EDTA using immunoprecipitated CAT showed only a slight
increase in CAT activity in the presence of CaM (data not shown), suggesting that the activity of this particular CAT is likely regulated by other mechanisms.

In mouse cells, CAT is phosphorylated by a non-receptor tyrosine kinase (Cao et al. 2003a). Although tyrosine kinases have not been identified in plants, we detected high tyrosine kinase activity in the megagametophyte following germination of white spruce seeds (data not shown). To investigate whether the white spruce CAT was phosphorylated on tyrosine residues, the protein was extracted from megagametophytes when seedlings were ∼15–20 mm in length and CAT was immunoprecipitated with the anti-CAT antibody. The eluted protein was immunoblotted with anti-phosphotyrosine antibody. Fig. 7A (left) indicates that this CAT was phosphorylated on one or more tyrosine residues. Reciprocal immunoprecipitation with anti-phosphotyrosine antibody followed by immunoblotting with anti-CAT antibody provided further evidence that the CAT was phosphorylated on tyrosine residues (Fig. 7A, right). To verify the specificity of the anti-phosphotyrosine antibody, a membrane strip with anti-CAT-immunoprecipitated CAT was preincubated in 1 mM phosphoSer, phosphoThr or phosphoTyr, respectively, and then immunoblotted with anti-phosphotyrosine antibodies. As shown in Fig. 7B, only phosphoTyr blocked the antibody’s binding, indicating that the antibody is specific for phosphorylated tyrosine.

Since the CAT activity was low at some stages (e.g. stage 4), when the CAT protein was abundant, but increased at later stages (e.g. at stages 5 and 6) (Fig. 5), we speculated that tyrosine phosphorylation may act to stimulate CAT activity and that this post-translational modification may be responsive to the concentration of \( \text{H}_2\text{O}_2 \). Immunoprecipitation of CAT with anti-CAT was carried out with extracts of megagametophytes taken from seeds at different stages (when the endogenous \( \text{H}_2\text{O}_2 \) differed, i.e., at stages 4 and 6), or after young seedlings (∼2 mm) had been treated for 2 d with 1 mM \( \text{H}_2\text{O}_2 \). As shown in Fig. 8A, when the \( \text{H}_2\text{O}_2 \) concentration in megagametophytes was low, tyrosine phosphorylation of CAT was barely detected; when the endogenous \( \text{H}_2\text{O}_2 \) concentration increased, the amount of CAT subject to tyrosine phosphorylation increased. This indicates that the tyrosine phosphorylation of CAT is likely regulated by \( \text{H}_2\text{O}_2 \). To investigate whether tyrosine phosphorylation stimulates this CAT activity, we treated CAT immunoprecipitated protein (from extracts at stage 6) with alkaline phosphatase and then conducted the CAT activity assay. CAT activity was decreased ∼50% after phosphatase treatment (Fig. 8B), suggesting that CAT activity is stimulated by tyrosine phosphorylation.

**CAT is ubiquitinated and degraded by the proteasome**

The decreased level of CAT protein at stage 5 (Fig. 5) was very consistent and this occurred before the onset of extensive megagametophyte cell death, suggesting that the degradation of CAT may contribute to later cell death. Since in mammalian cells, CAT is degraded by the ubiquitin–proteasome pathway (Cao et al. 2003b), we investigated whether the white spruce CAT was ubiquitinated. Immunoprecipitation with an anti-CAT (anti-CAT) antibody or an anti-ubiquitin (anti-Ubi) antibody was carried out. The immunoprecipitates were then tested by Western blot analysis using anti-Ubi or anti-CAT antibodies. Anti-CAT immunoprecipitates (IPs) from megagametophytes at early pre-germinative stage did not react with anti-Ubi antibodies but IPs from megagametophytes at post-germination stages reacted with anti-Ubi antibodies over a range of electrophoretic mobilities (Fig. 9A, left). Furthermore, the...
density of immunoreactive CAT polypeptides increased as post-germinative seedling growth advanced (Fig. 9A). In the reciprocal experiment, immunoblotting of anti-Ubi IP with anti-CAT antibodies also showed an increase in ubiquitinated CAT at a later post-germination stage (Fig. 9A, right). These data indicate that the CAT was progressively ubiquitinated; this corresponded to stages when the $\text{H}_2\text{O}_2$ concentration was high (e.g. stage 6; Fig. 1). To investigate whether phosphorylation of CAT affects ubiquitination, Western blot analysis was conducted using CAT IP protein (at stages 1, 3 and 6) with anti-phosphoTyr antibodies. As shown in Fig. 9B, at an early germination stage, CAT was phosphorylated but polyubiquitinated CAT was barely detected; whereas at a later post-germinative stage, both phosphorylated CAT and phosphorylated polyubiquitinated CAT became more abundant.

To further investigate whether CAT is degraded by the proteasome, we treated germinated seeds (∼2 mm) for different durations with 50 µM N-benzyloxycarbonyl-leucyl-leucyl-leucinal (MG132; a proteasome inhibitor), and examined CAT protein levels in the megagametophyte. The CAT protein level...
in megagametophyte extracts from MG132-treated seedlings was maintained at a similar level throughout the 7-d period (Fig. 10A, B). In contrast, the lower molecular weight polypeptides that reacted with anti-CAT antibodies are possibly breakdown products of CAT (Fig. 10A left panel). These results suggest that this CAT was degraded by the ubiquitin–proteasome pathway. Significantly, inhibition of the proteasome by MG132 treatment also increased CAT activity at d 1 to d 4 (Fig. 10C). The same treatment delayed the peak of H$_2$O$_2$ level, reduced CLP activity and delayed cell death (Fig. 11). Although the CAT protein level was constant (Fig. 10A, right), the activity decreased greatly after 4 d of MG132 treatment (Fig. 10C).

This observation led us to test whether the CAT in the megagametophyte at later stages was still phosphorylated at tyrosine residues. Immunoprecipitation with anti-CAT was carried out using extracts of megagametophytes from germinated seeds (∼2 mm) that had been treated with MG132 for 1–7 d. The recovered CAT protein was immunoblotted with anti-phosphoTyr antibody (Fig. 12). As shown, the phosphorylation level of CAT became lower after the 5-d treatment, and was barely detected after the 7-d treatment (Fig. 12). This suggests that CAT was inactivated by dephosphorylation, possibly effected by a phosphatase.

**Discussion**

We previously showed that the programmed death of megagametophyte cells is an active process that is temporally and spatially controlled (He and Kermode 2003a, b). In this study, we examined the involvement of ROS and the ubiquitin–proteasome pathway in regulating PCD of the white spruce
CAT is ubiquitinated and degraded by the 26S proteasome. (A) Western blot analyses of immunoprecipitated complexes (IP). Crude proteins were extracted from megagametophytes of seeds (at stages 1, 3 and 6 as defined in Table 1) and normalized by CAT protein level (lower panel). Immunoprecipitation was carried out with anti-CAT (left) or anti-Ubi antibodies (right), and the resultant IP complexes were immunoblotted (IB) with anti-Ubi antibodies or anti-CAT antibodies, respectively, as indicated. As a control, rabbit IgG (for rabbit anti-CAT) or mouse IgG (for mouse anti-Ubi) was also used to immunoprecipitate protein extracts from megagametophyte at stage 6. * Indicates non-specific binding to mouse anti-Ubi. (B) Western blot analysis of CAT-IP with anti-phosphoTyr antibodies. M, pre-stained protein ladder.

Megagametophyte cell death is ROS mediated

ROS are key players in the regulation of PCD (Lamb and Dixon 1997, Jabs 1999, Overmyer et al. 2003); H$_2$O$_2$, potentially acts as a regulatory signal in plants (reviewed in Gechev and Hille 2005). ROS generation is associated with several forms of plant PCD, including that of cereal aleurone layer cells, leaf senescence and the hypersensitive response (Alvarez et al. 1998, Bethke and Jones 2001, del Rio et al. 2003, Palma and Kermode 2003, Zapata et al. 2005). In the white spruce megagametophyte, cell death occurs when storage reserve mobilization is completed; in addition, cell death follows a temporal and spatial pattern in which the megagametophyte cells within the micropylar region of the seed die first (He and Kermode 2003a,b). H$_2$O$_2$ production was biphasic in the megagametophyte following germination of white spruce seeds; O$_2$ generation was detected only at a time associated with extensive death of the megagametophyte. The early phase of H$_2$O$_2$ production may be necessary for initiating some signal transduction pathways; e.g. the activation of a Ca$^{2+}$-dependent protein kinase appears to be involved (data not shown). The second phase of ROS production (including O$_2$ and H$_2$O$_2$), may be involved in initiating and/or executing cell death, as increases in CLP activity, protein carbonylation and lipid peroxidation coincided with the second phase of ROS generation. Biphasic production of ROS has also been observed in other forms of plant PCD, such as the hypersensitive response and ozone-induced cell death (Schraudner et al. 1998, Dorey et al. 1999). ROS produced in the megagametophyte are likely from the plasma membrane (e.g. the plasma membrane NADPH oxidase inhibitor DPI delays cell death), glyoxysomes (particularly during storage lipid mobilization) and mitochondria. In cells metabolizing fatty acids such as the megagametophyte, glyoxysomes, which contain $\beta$-oxidation cycle enzymes, generate ROS via fatty acyl CoA oxidase, a flavoprotein that reduces O$_2$ directly to H$_2$O$_2$. Because plant membranes are permeable to H$_2$O$_2$, the H$_2$O$_2$ produced by both the glyoxysome and the mitochondrion (during electron transport) will influence the cytosolic ROS concentrations (Pastori and del Rio 1997). A determination of the subcellular localization of ROS generation may help to elucidate the regulatory roles of ROS in megagametophyte cell death.

Treatment of germinated seeds with DPI (an inhibitor of flavoenzymes, particularly NADPH oxidase) or with Tiron (a scavenger of O$_2$) delayed the rate of megagametophyte cell death, suggesting that there is a causal relationship between ROS and cell death. DPI also suppressed CLP activity and delayed DNA fragmentation; therefore, the mechanism by which ROS mediate cell death is at least partially via the activation of CLPs. Interestingly, the onset and rate of cell death was not significantly changed in the presence of the hydroxyl radical scavenger, 1mM DMTU, suggesting that this specific ROS may not be involved in megagametophyte cell death.

Protein carbonylation is widely used as a marker for oxidative stress and aging (Chevion et al. 2000, Stadtman and Levine 2000, Levine 2002). Carbonylated proteins were detected before the stages associated with extensive megagametophyte cell death. Protein carbonylation during the induction of apoptosis may result in the modification of a restricted number of proteins (England et al. 2004). A reduction in glycolysis due to...
the carbonylation of glycolytic enzymes occurs in human leukemia (HL) 60 cells, in which apoptosis is induced by treatment with etoposide (VP-16, a topoisomerase II inhibitor that acts as a DNA-damaging agent) (England et al. 2004). Exogenous salicylic acid treatment leads to a marked increase in the carbonylation of the α-subunits of 12S cruciferin storage proteins in Arabidopsis seeds, a process that may facilitate storage protein mobilization (Rajjou et al. 2006). It is possible that ROS-induced protein carbonylation in megagametophyte cells has dual roles: facilitating storage protein mobilization and signaling cell death.

Lipid peroxidation can be generated by ROS directly attacking membrane lipids, or by lipoxygenases activated after oxidative stress (Maccarrone et al. 2001, Göbel et al. 2003). The role of lipid peroxidation in megagametophyte cell death awaits further investigation. This process has been implicated in the PCD of animal as well as plant cells (Sandstrom et al. 1995, Aoshima et al. 1997, Maccarrone et al. 2001, Gechev et al. 2002).

**CAT activity is regulated by tyrosine phosphorylation**

There are at least three CAT genes in white spruce megagametophyte (data not shown) and one full-length cDNA was cloned. The steady-state level of transcripts encoding this CAT in the megagametophyte remained similar following seed germination and throughout the study period, while the protein level and activity changed dramatically (Fig. 5). Ca/CaM regulates some plant CAT activities (Yang and Poovaiah 2002). The alignment of the deduced amino sequences of the white spruce CAT and the CATs of several species showed that they all shared a high degree of homology in the CaM binding site. Three CATs of *Arabidopsis* interact with NDP kinase (NDPK) (Fukamatsu et al. 2003). However, NDPK does not phosphorylate CAT (Yoshida et al. 2006). In animal cells, CAT interacts with non-receptor tyrosine kinases: c-Abl and Arg (Cao et al. 2003a). When the H$_2$O$_2$ concentration reaches 0.25–1.0 mM, CAT is phosphorylated by c-Abl and Arg, resulting in an increase in its activity. In post-germinative white spruce megagametophyte cells, one CAT (that reacted with antibodies specific for cotton seed CAT) was phosphorylated on tyrosine residue(s), a modification that stimulates its activity. Furthermore, tyrosine phosphorylation was enhanced when the H$_2$O$_2$ level was elevated. Thus at least in white spruce megagametophyte cells, tyrosine phosphorylation of CAT appears to be one aspect of ROS signaling. At present, we do not know the site(s) of tyrosine phosphorylation on the white spruce CAT.

Signal transduction by protein tyrosine phosphorylation has been identified (e.g. it appears to be involved in ABA signaling; Ghelis et al. 2008), but this process is not well understood in plants. Mitogen-activated protein kinases (MAPKs) are subject to regulation by tyrosine phosphorylation (Gupta and Luan 2003). Protein tyrosine kinases have not been identified in plant cells; therefore it is hypothesized that tyrosine phosphorylation is...
carried out by dual specificity kinases (Rudrabhatla et al. 2006). A genome-wide analysis in Arabidopsis indicates that there are 57 different protein kinases that have tyrosine kinase motifs, and animal non-receptor tyrosine kinases are structurally related to putative plant tyrosine kinases (Rudrabhatla et al. 2006). In white spruce megagametophyte protein extracts, immunoprecipitation conducted with the anti-CAT antibody, co-precipitates a kinase with tyrosine phosphorylation activity along with the CAT (data not shown). Whether this kinase is a dual specificity kinase or a tyrosine-specific kinase awaits further investigation.

The ubiquitin–proteasome pathway is involved in proteolysis of CAT and potentially regulates megagametophyte cell death

The involvement of the ubiquitin–proteasome pathway in apoptosis of animal cells has been well characterized. The Bcl-2 family of proteins, the IAPs, p53 and the inhibitors of nuclear factor-B and of nuclear factor-B kinase, are all ubiquitin–proteasome substrates (Jesenberger and Jentsch 2002). However, the roles of the ubiquitin–proteasome pathway in plant PCD are only beginning to be elucidated; nevertheless, the limited data available suggest its involvement. In transgenic tobacco plants, virus-induced gene silencing of two different subunits of the 26S proteasome (i.e. the α6 subunit of the 20S proteasome and the RPN9 subunit of the 19S regulatory complex) leads to PCD (Kim et al. 2003). In heat-shocked tobacco BY-2 suspension cells, the proteasome inhibitor MG132 decreases the rate of cell death. Coincidentally, this inhibitor reduces ROS production, inhibits cytochrome c release from mitochondria to the cytosol and inhibits caspase-3-like protease activity (Vacca et al. 2007).

ROS-scavenging enzymes are key players in regulating levels of ROS in cells; these enzymes appear to be down-regulated before or during cell death in plants (Mittler et al. 1999, Fath et al. 2001). In this study we demonstrated that one CAT in the megagametophyte was ubiquitinated and degraded before extensive cell death occurred. The tyrosine-phosphorylated CAT appeared to be a major target of ubiquitination and the degree of ubiquitination was progressively increased at stages associated with high endogenous H₂O₂ levels. In addition, treatment of germinated white spruce seeds with the proteasome inhibitor MG132 was associated with an increase in CAT activity and a decrease in cell death.

Fig. 11 The effect of the proteasome inhibitor MG132 on production of H₂O₂ in megagametophytes (A), CLP activity (B) and cell death (C). 1-7 correspond to megagametophytes from germinated seeds (radicle ~2 mm) treated with MG132 or H₂O for 1-7 d, respectively. Data are the means of three independent replicates ± SD.

Fig. 12 CAT is not tyrosine-phosphorylated at a later stage after MG132 treatment. Immunoprecipitation with anti-CAT antibodies was carried out using protein extracts from megagametophytes derived from seedlings that had been treated with MG132 for 1-7 d. See text for details. An equal amount of CAT IP was loaded in each lane for duplicate Western blots (upper and lower panels). Top panel shows blotting with anti-phosphoTyr antibodies; lower panel shows duplicate blotted with anti-CAT antibodies, confirming that similar amounts of CAT were loaded.
with increased CAT activity from d 1 to d 4 of the treatment. MG132 treatment also delayed cell death, suggesting that degradation of CAT by the ubiquitin–proteasome pathway is an integral mechanism facilitating H₂O₂ accumulation and thus promoting PCD.

In summary, ROS mediate the post-germinative death of white spruce megagametophyte cells at least in part via the regulation of CLP activity. The ubiquitin–proteasome pathway appears to modulate both the ROS-scavenging enzymes and CLPs. One of the white spruce megagametophyte CATs is phosphorylated on tyrosine residue(s) and ubiquitinated, and is thereafter degraded by the 26S proteasome; this modulation appears to be responsive to the H₂O₂ concentration. We propose a model of the regulation of CAT in post-germinative megagametophyte PCD: at stage 4 before extensive cell death occurs, CAT activity is down-regulated, possibly by its dephosphorylation by a tyrosine phosphatase (tyrosine phosphatase and suppression of phosphatase activity favoring cell death. Activation of a specific protein tyrosine kinase or a dual specificity kinase leads to the phosphorylation of CAT and results in an increase in CAT activity. Increased CAT activity decreases the H₂O₂ level. However, the phosphorylated CAT becomes a substrate for the ubiquitin–proteasome pathway. The degradation of CAT facilitates the accumulation of H₂O₂, and this in part results in cell death.

Materials and Methods

Plant materials
White spruce (P. glauca) seeds of seed lot 32776 were obtained from the Tree Seed Centre in Surrey (B.C. Canada). Seeds were surface-sterilized in 1.5% H₂O₂ for 20 min, rinsed several times in sterile distilled water and imbibed in sterile distilled water for 24 h. Seeds were then placed in seed boxes (Hoffman Manufacturing Co., Albany, OR, USA) containing one Kimpak and one Whatman No. 1 filter paper and 45 ml of sterile distilled water and moist chilled for 3 weeks at 4°C in darkness. Seeds were then transferred to germination conditions (30°C days, 20°C nights, 8-h photoperiod; light intensity at 25 µmol m⁻² s⁻¹, PAR 400–700 nm). At different time points following germination (see Table 1), seeds were dissected under a dissecting microscope into megagametophytes or embryo parts (radicle, hypocotyl and cotyledons) and megagametophytes were used fresh or were immediately frozen in liquid nitrogen or dry ice and stored at −80°C until use.

Chemical treatments of germinated seeds
Germinated seeds (radicle ~2 mm) were treated with 50 µM DPI (Sigma Chemical Co., St Louis, MO, USA), 1 mM Tiron (Sigma), 100 µM zVAD-fmk (a cell-permeable, irreversible, pan-caspase inhibitor; Calbiochem, San Diego, CA, USA) or 50 µM MG132 (Calbiochem); in all cases except DPI, the solution was replaced every 2 d because of the chemical’s instability. Megagametophytes were sampled at different time points following the treatments (Table 1).

CLP activity assay
Proteins were extracted from megagametophytes at different times after germinated seeds were treated with various chemicals (Table 1) and CLP activity assays were performed according to He and Kermode (2003b) using caspase-3 substrate II (Ac-DEVD-AMC) (Calbiochem). Protein concentration was determined using Bio-Rad Dc Protein Assay kit (Bio-Rad Laboratories Ltd, Mississauga, ON, Canada).

H₂O₂ assay
H₂O₂ in the megagametophyte of seeds at different post-germinative stages was determined by a chemiluminescence reaction with luminol (Warm and Laties 1982, Palma and Kermode 2003). Ten megagametophytes were extracted in 200 µl of 10% trichloroacetic acid (TCA) and centrifuged at 14, 950×g for 10 min. The supernatant was neutralized with 0.1 M KOH. A 5-µl aliquot of the extract was added to 50 µl of 5-amino-2, 3-dihydro-1,4-phthalazinedione (luminol; 1.1 mM in 50 mM potassium phosphate pH 7.9) and 800 µl of potassium phosphate buffer (50 mM, pH 7.9) in a clear 1.5-ml Eppendorf tube. The reaction was started with 100 µl of potassium ferri-cyanide (14 mM in double-distilled deionized water, freshly prepared). Chemiluminescence was determined using a TD-20/20 luminometer (Turner Design, Sunnyvale, CA, USA), integrated for 15 s immediately following the start of the reaction. The concentration of H₂O₂ was determined using a standard curve of known concentrations reacted with luminol.

In-gel activity assay for CATs
Protein extracts were fractionated on 10% (w/v) non-denaturing polyacrylamide gels for 6 h at 4°C. For the CAT activity assay, gels were soaked in double-distilled deionized water for 15 min and then incubated in 0.03% H₂O₂ for 10 min. After rinsing gels with double-distilled deionized water, gels were stained in a solution of 1% (w/v) ferric chloride and 1% potassium ferricyanide. The reaction was stopped by rinsing gels with deionized water. Clear bands indicate CAT activity (Palma and Kermode 2003).

Detection of superoxide anion production
Longitudinal sections of megagametophytes at different stages were stained with NBT as described by Labs et al. (1996). Megagametophyte sections were immersed in 0.1% NBT (10 mM potassium phosphate buffer, pH 7.5) for 15 min and rinsed with double-distilled deionized water.

Determination of viability
Viability assays were performed according to the method described in Palma and Kermode (2003) with modifications.
Megagametophytes (10 megagametophytes/time point) were stained in 1% (w/v) 2,3,5-triphenyltetrazolium chloride (TTC) (pH 5.8, 50 mM phosphate buffer) solution for 2 h at 25°C and rinsed with double-distilled deionized water. TTC is reduced in living cells by dehydrogenases to produce insoluble red TTC formazan crystals (Towill and Mazur 1979). The megagametophytes were then extracted with 100% ethanol at 70°C for 2 h. The optical density at 485 nm of the supernatant was determined and the cell survival rate was calibrated by a standard curve (the living cells were killed in liquid N₂ by freezing and thawing the cells four times).

**Lipid peroxidation assay**

Lipid peroxidation was measured by the method described in de Pinto et al. (2002). Megagametophytes (10 per stage) were homogenized in 250 µl of 0.1% (w/v) TCA. The homogenate was centrifuged at 10,000 × g for 10 min. Supernatant (200 µl) was diluted 1:5 (v/v) with 20% (w/v) trichloroacetic acid containing 0.5% (w/v) thiobarbituric acid. The mixture was heated at 95°C for 30 min and cooled in an ice bath, after which it was centrifuged at 10,000 × g for 10 min, and the absorbance of the supernatant was determined at 532 nm. The value for non-specific absorption at 600 nm was subtracted from the 532 nm reading. The concentration of malondialdehyde was calculated using an extinction coefficient of 155 µM cm⁻¹⁻¹.

**Detection of carbonylated proteins**

Proteins were extracted from megagametophytes of seeds at different stages following germination [extraction buffer: 25 mM Tris–HCl pH 7.0, 0.05% Triton X-100, 1 mM dithiothreitol (DTT), 20 µM leupeptin and 0.5 mM phenylmethylsulfonyl fluoride (PMSF)]. Carbonylated proteins were detected using an OxyBlot™ Protein Oxidation Detection Kit (Chemicon International, Temecula, CA, USA) according to the manufacturer’s instructions.

**DNA isolation and electrophoresis**

Genomic DNA was extracted from megagametophytes of seeds at different time points following the chemical treatments noted above using methods described in He and Kermode (2003a). DNA samples (10 µg) were loaded on a 1.8% agarose gel for analysis of DNA fragmentation. After electrophoresis, the gel was stained with ethidium bromide.

**Immunoprecipitation and Western blot analysis**

Proteins were extracted with a buffer consisting of 50 mM Tris–HCl pH 7.5, 150 mM NaCl, 0.1% (v/v) Triton X-100, 5 mM EDTA, 1 mM PMSF and 10 µM MG132. Immunoprecipitation was performed using protein A–Sepharose beads (for rabbit polyclonal anti-CAT antibodies) or protein G–Sepharose beads (for mouse monoclonal anti-Ubi antibodies) according to the manufacturer’s instructions (BioVision, Mountain View, CA, USA).

For Western blot analyses, immunoprecipitated proteins or proteins extracted from megagametophytes were fractionated on 10% SDS–PAGE gels and then electroblotted onto nitrocellulose membranes. Membranes were blocked with 5% skimmed milk powder in phosphate-buffered saline (PBS) containing 0.05% Tween-20 (1 h at room temperature) and then incubated in the primary antibodies (anti-CAT, 1:1000 dilution; Kurence and Trelease 1986; anti-Ubi, 1:500 dilution) for 2 h at room temperature. The blot was then washed three times in PBS containing 0.05% Tween-20 (each 20 min) and incubated in goat anti-rabbit or anti-mouse secondary antibody conjugated to alkaline phosphatase (Bio-Rad Laboratories Ltd, Mississauga, ON, Canada). Following three washes (20 min each) in PBS containing 0.05% Tween-20, immunodetection was achieved using NBT and 5-bromo-4-chloro-3-indolyl phosphate as substrates. Densitometry analysis of the immunoblots was carried out using TotalLab 100 software following the manufacturer’s instructions (Nonlinear USA Inc., Durham, NC, USA).

**Cloning the CAT gene of white spruce**

Immunoprecipitated CAT was analyzed by liquid chromatography–tandem mass spectrometry (LC-MS/MS) and degenerate primers were designed based on the deduced amino acid sequence. The forward primer was: 5'-NGGCNCGNTTGGAGAY-3', and the reverse primer was: 5'-NGCCTCNACCANCK-3'. Total RNA was isolated from germinated white spruce megagametophyte as described by He and Kermode (2003b). RT–PCR was performed using Reverse Aid H (Fermentas International Inc., Burlington, ON, Canada) and Phusion (Finnzymes Inc., Woburn, MA, USA) kits. However, no specific PCR products were obtained probably due to high degeneracy. Therefore, the forward primer was combined with a universal oligo(dT) primer [5'-AAGCAGTGTTACACCGAGATAC(T)₃VN-3'] (BD SMART™ RACE cDNA Amplification Kit; BD Biosciences Clontech, Mississauga, ON, Canada). PCR conditions were: 94°C, 3 min; followed by 35 cycles: 94°C, 30 s; 48°C, 30 s and 72°C, 2 min. The final extension was at 72°C for 10 min. After gel electrophoresis, a 1.7-kb fragment of the reverse transcriptase (RT)–PCR products was recovered. The fragment was purified using the QiAprep Spin Miniprep Kit (Qiagen Sciences, Germantown, MD, USA) and the insert was sequenced. Three different cDNA sequences were obtained from nine cDNA clones. The deduced amino acid sequence of one cDNA was identical to the deduced amino acid sequence from the LC-MS/MS data. To obtain the full length of the CAT cDNA, the universal primer mix from the BD SMART RACE cDNA Amplification Kit was combined with two CAT gene-specific reverse primers: 5'-TGTCTAGGGTCACCTCACAATAA-3' (RCAT1) and 5'-ATCGA TGAACCAGTCTACAGGA-3' (RCAT2). Three PCR fragments were obtained but none of their sequences matched the CAT cDNA sequence. A BLAST with the CAT partial cDNA sequence was carried out and an unknown mRNA sequence (GenBank access no. EF085146) from Sitka spruce (Picea sitchensis) was found to
be 99% identical to the white spruce cDNA. Therefore, a forward primer was designed: 5'-ATGGATCTTACAAACACC-3'. This primer and RCAT1 were used to amplify the 5' end of the CAT cDNA. The PCR conditions were: 94°C, 2 min; followed by 30 cycles: 94°C, 30 s; 55°C, 30 s and 72°C, 1 min. Final extension was at 72°C for 10 min. A second PCR product of 220bp was obtained, sub-cloned and sequenced. Alignment of the two cDNA sequences showed an identical overlapping region of 120bp indicating that they originated from the same gene.

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**References**


