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ORIGINAL ARTICLE

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Expression and thermotolerance of calreticulin during pollen development in tobacco

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Abstract Glycoproteins 50, 55, 59 and 64 kDa with affinity to the lectin ConA occurring abundantly in mature tobacco pollen were shown to exhibit high tolerance against heating at 90°C for 30°min. The 59 kDa glycoprotein (GP59) was isolated by affinity chromatography on ConA-agarose followed by 2D-electrophoresis and identified by MS analysis as tobacco calreticulin with approximate pI 4.2. Identification of the protein was confirmed by immunoblotting with human anti-calreticulin and by labelling with a specific dye for Ca^{2+} -binding proteins (Stains All). Two acidic isoforms of 50 kDa glycoprotein in addition to GP59 displayed homology to calreticulin. RT-PCR revealed the presence of transcripts for calreticulin 59 kDa at all the stages of pollen development from microspore mitosis through the first 24 h of pollen tube growth. Immunodetection with anti-calreticulin and affinity to ConA on Western blots of total soluble proteins separated by 1D-SDS-PAGE showed that the protein first occurred at the mid-bicellular pollen stage, accumulated during pollen maturation and disappeared during 24 h of pollen tube growth. A thermotolerant form of GP59 was detected only in the terminal phase of pollen maturation and during 8 h of pollen tube growth. Results indicated that calreticulin 59 kDa is transcribed, translated and undergoes post-translational modification at a number of different stages of pollen development and that thermotolerance of the protein in mature pollen may be associated with high glycosylation. The thermotolerance of these glycoproteins could play a role in the protection of pollen against stress factors during dehydration and dispersal.

Keywords Calreticulin · Glycoproteins · Nicotiana tabacum L. · Pollen maturation

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Introduction

During the final stages of its maturation and after its release from the anthers, pollen is exposed to various stresses related to dehydration and temperature changes. The high sucrose and proline content play an important role in the dessication and heat tolerance of pollen. In addition to this osmoprotection, physiological mechanisms of plant tolerance to stress factors include synthesis of chaperones functioning in macromolecule alteration and membrane protection. Heat shock proteins (HSPs) belong to the best characterized chaperones. Activation of several HSP genes has been observed during pollen dehydration in tobacco (Žárský et al. 1995). Other plant proteins that accumulate in response to dehydration stress and that are proposed to function as chaperones are dehydrins. Dehydrins were initially identified in seeds, with expression that is stress-induced in vegetative tissues. Dehydrin promoter activity was also detected in the mature pollen of transgenic plants (Michel et al. 1994; Wang and Cutler 1995).

Calreticulin also plays the role of a molecular chaperone. Calreticulin is abundant in plant tissues such as roots and young leaves (Baldan et al. 1996; Denecke et al. 1995), germinating seeds (Denecke et al. 1995), and floral tissues (Chen et al. 1994; Denecke et al. 1995; Nelson et al. 1997). Plant calreticulins are up-regulated in response to a variety of stress stimuli (Denecke et al. 1995) and are highly expressed also during mitosis (Denecke et al. 1995) and embryogenesis (Borisjuk et al.

1998). Using immunobloting with anti-calreticulin antibody, calreticulin was found in the pollen of Liriodendron tulipifera (Navazio et al. 1998), in maize sperm cells (Williams et al. 1997), and was immunocytochemically detected in the pollen tubes and styles of Petunia hybrida (Chen et al. 1994; Denecke et al. 1995; Lenartowska et al. 2002).

Tobacco pollen maturation and dehydration have been shown to be characterized by an increase of many ConA-binding glycoproteins and the occurrence of some new ConA-binding glycoproteins (Říhová et al. 1996; Hrubá and Tupý 1999). In the present study, we describe one major heat-tolerant glycoprotein putatively identified as calreticulin. Its expression during pollen development extending from microspore and during the first 24 h of pollen tube growth was studied by RT-PCR, ConA-binding and immunodetection on Western blots and by examination of its occurrence in a thermotolerant form.

Materials and methods

Plant material

Tobacco plants (Nicotiana tabacum L. cv. Samsun) were grown in a greenhouse from April to October 2004, under daylight and temperatures ranging between 15 and 40°C. Microspores and pollen were isolated from anthers at seven developmental stages: (1) microspore mitosis, (2) early pollen, (3, 4) intermediate stages, (5) pollen maturation, (6) pre-anthesis pollen 1 day before shedding and (7) mature pollen, as described previously (Hrubá and Tupý 1999). Mature pollen was obtained from anthers collected just prior to anthesis and was allowed to dehisce in Petri dishes. Mature pollen was cultured for 4, 8, 16 and 24 h at 25°C as a shaken suspension culture at a density of 10 mg per 10 ml in 6% sucrose, 1.6 mM H_3BO_3. Pollen tubes were separated from the medium by filtration and stored in dry ice.

Protein extraction and electrophoretic analysis

Proteins were extracted and separated by electrophoresis as described previously (Hrubá et al. 2005). The homogenization buffer contained 50 mM Tris–HCl, pH 6.8, 10% sucrose, 1% mercaptoethanol and 0.02% sodium azide for 1D-SDS-PAGE according to Laemmli (1970) and 20 mM Tris–HCl, pH 8.0 for separations by 2D-SDS-PAGE. Proteins were detected by Coomassie Brilliant Blue R-250 (CBB) or by specific stains. The Ca^{2+} -binding proteins were visualized by the cationic carbocyanine dye (Stains All, Serva) according to the manufacturer's instructions. The GPs were blotted onto nitrocellulose (NC membrane 0.45 μm, Serva) at 300 mA for 2 h and detected with ConA and peroxidase binding followed by the reaction of peroxidase with 4-chloro-1-naphthol (Towbin et al. 1979).

Preparation of thermotolerant protein fraction

The extracts of soluble proteins in buffer for 1D-electrophoresis or 2D-electrophoresis were incubated at 90°C for 10 min, centrifuged at 20,000 g, 6°C for 15 min and the thermotolerant proteins were precipitated with 5 vol. acetone overnight at −20°C.

MS analysis of thermotolerant GPs

Thermotolerant proteins of mature pollen were fractionated by affinity chromatography on ConA-agarose (Hrubá and Tupý 1999) and the isolated GPs were separated by 2D-SDS-PAGE. The gel was stained with CBB and the spots for MS analysis were cut from the gel and stored in 5% acetic acid. The gel of each spot was chopped into small cubes (approximately 1 mm^3) and destained by several washings with 10 mM DTT, 0.1 M 4-ethylmorpholine acetate (pH 8.1) in 50% acetonitrile. The destained gel was washed with water, dehydrated with acetonitrile and reswollen in water. Next, the gel was partly dried using a SpeedVac concentrator and then reconstituted with a cleavage buffer containing 0.01% 2-mercaptoethanol, 0.1 M 4-ethylmorpholine acetate, 10% acetonitrile, 1 mM $CaCl_2$ and a sequencing grade trypsin (Promega, WI, USA, 50 ng/μl). Digestion was carried out overnight at 37°C. The resulting peptides were extracted with acetonitrile/trifluoracetic acid (40/1, v/v) and subjected to MS analysis.

Mass spectroscopic analysis was conducted using a matrix-assisted laser desorption/ionization reflectron time-of-flight MALDI-TOF mass spectrometer BIFLEX (Bruker-Franzen, Bremen, Germany) equipped with a nitrogen laser (337 nm) and a gridless delayed extraction ion source. Ion acceleration voltage was 19 kV and the reflectron voltage was set to 20 kV. Spectra were calibrated externally using the monoisotopic $[M+H]^+$ ion of peptide standards somatostatin (Sigma, MO, USA). A saturated solution of α-cyano-4-hydroxy-cinnamic acid in acetonitrile/trifluoracetic acid (50/0.2, v/v) was used as a MALDI matrix. An amount of 1 μl of matrix solution was mixed with 1 μl of the sample on the target and the droplet was allowed to dry at an ambient temperature. The MALDI-MS spectra were interpreted with the program ProFound (http://prowl.rockefeller.edu/profound_bin/WebProFound.exe) and MASCOT (http://www.matrixscience.com/search_form_select.html).

Analysis of calreticulin during pollen development

Total or thermotolerant soluble proteins from different stages of pollen development were separated by 1D-SDS-PAGE, transblotted on nitrocellulose and assayed by ConA-peroxidase for GPs or with anti-calreticulin antibody. For Immunodetection, the blots were blocked in TBS containing 4% bovine serum albumine (BSA) for

1 h, washed with TTBS (TBS buffer containing 0.05% Tween-20) and incubated with polyclonal antibody raised against human calreticulin (Sigma) diluted 1:1000 in TBS with 1% BSA. Following extensive washing in TTBS, blots were incubated with secondary antibody goat anti-rat IgG conjugated to alkaline phosphatase (Stressgen), at dilution of 1:1000 in TBS with 1% BSA. Positive reactions were visualized by colour development using 4-nitroblue-tetrazolium chloride (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP).

RNA extraction and RT-PCR

RNA extraction from pollen at different stages of development was carried out using RNeasy Plant Mini Kit (Qiagen, Crawley, UK) according to the manufacturer's instructions. The RT-PCR was done according to Lalanne et al. (2004). The same starting amount of RNA was used for RT reaction and 1 µl of 50 times diluted RT product was used for PCR. The following gene-specific forward and reverse primers were designed using Primer3 software (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi): 5′-GGAAGGCTCCTTTGATTGACAAC-3′, 5′-ATCATCATCGGCATCATCATCTT-3′.

Results

Presence of thermotolerant GPs in mature pollen

ConA-peroxidase staining on Western blots of proteins from mature pollen separated by 1D-SDS-PAGE showed that heating of the extracts at 90°C resulted in degradation of most pollen GPs and that pollen contained abundant thermotolerant GPs of 50, 55, 59 and 64 kDa (Fig. 1). Even a 30-min incubation of protein samples at 90°C did not result in a significant decrease of the intensity of ConA-staining of these GPs on the blots. These results were confirmed on eight pollen batches collected at different periods during the growing season. In fractionation of thermotolerant proteins by affinity chromatography on ConA-agarose, GP 50 kDa (GP50) and GP 59 kDa (GP59) were easily eluted by 0.2 M α-D-methylmannopyranoside, while GP 64 kDa and GP 55 kDa remained bound on the column, presumably because of high glycosylation.

Identification of calreticulin by MS analysis of thermotolerant GPs

GP59 moved in 2D-SDS-PAGE as an acidic protein with an approximate pI of 4.2 (Fig. 2, spot 1) and was easily localized using ConA on a Western blot and with CBB on the gel. It stained blue with the carbocyanic dye Stains All (Fig. 2c), which stains blue acidic Ca^{2+}-binding proteins (Campbell et al. 1983). In addition to

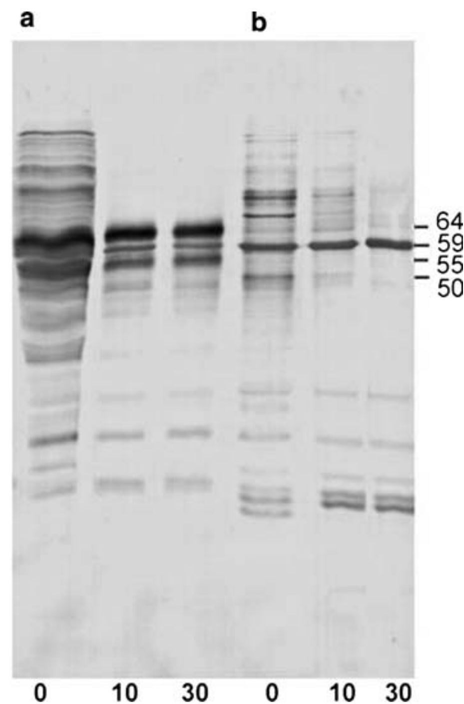

Fig. 1 Thermotolerance of pollen glycoproteins. A crude extract of soluble proteins from mature pollen (**a**) or its fraction of GPs separated by affinity chromatography on ConA-agarose (**b**) were incubated for 0, 10 or 30 min at 90°C and analysed by 1D-SDS-PAGE followed by protein transfer to nitrocellulose membrane and staining with ConA-peroxidase. The aliquots of extracts loaded on the gel corresponded to 1 mg (**a**) or 2.5 mg (**b**) of mature pollen

GP59, two acidic isoforms of GP50 (Fig. 2a, b, spots 2 and 3) and the protein 4 (Fig. 2b) were submitted to MS analysis. While spot 3 stained both with CBB and ConA, spots 2 and 4 were clearly detected only with CBB indicating low glycosylation. Based on the analysis using Profound (http://prowl.rockefeller.edu/profound_bin/WebProFound.exe) and MASCOT (http://www.matrixscience.com/search_form_select.html) websites and the MALDI-TOF MS data obtained, spots 1–3 showed identity with the protein calreticulin (T03691) and spot 4 could be identified as ankyrin-repeat protein HBP1 of tobacco.

Occurrence of calreticulin during pollen development

Immunoblotting using polyclonal antibody raised against human calreticulin showed affinity also to calreticulin of tobacco pollen. On Western blots of pollen proteins separated by 1D-SDS-PAGE, the calreticulin antibody recognized a single band corresponding to GP59 (Fig. 3). Similar changes of GP59 during pollen development could be observed using immunoblots with anti-calreticulin as on affinoblots with ConA. In extracts of total soluble proteins, labelling began in mid-bicellular pollen, increased during pollen maturation and disappeared during 24 h of pollen tube growth (Fig. 3a, b). In the thermotolerant protein fraction, the presence of

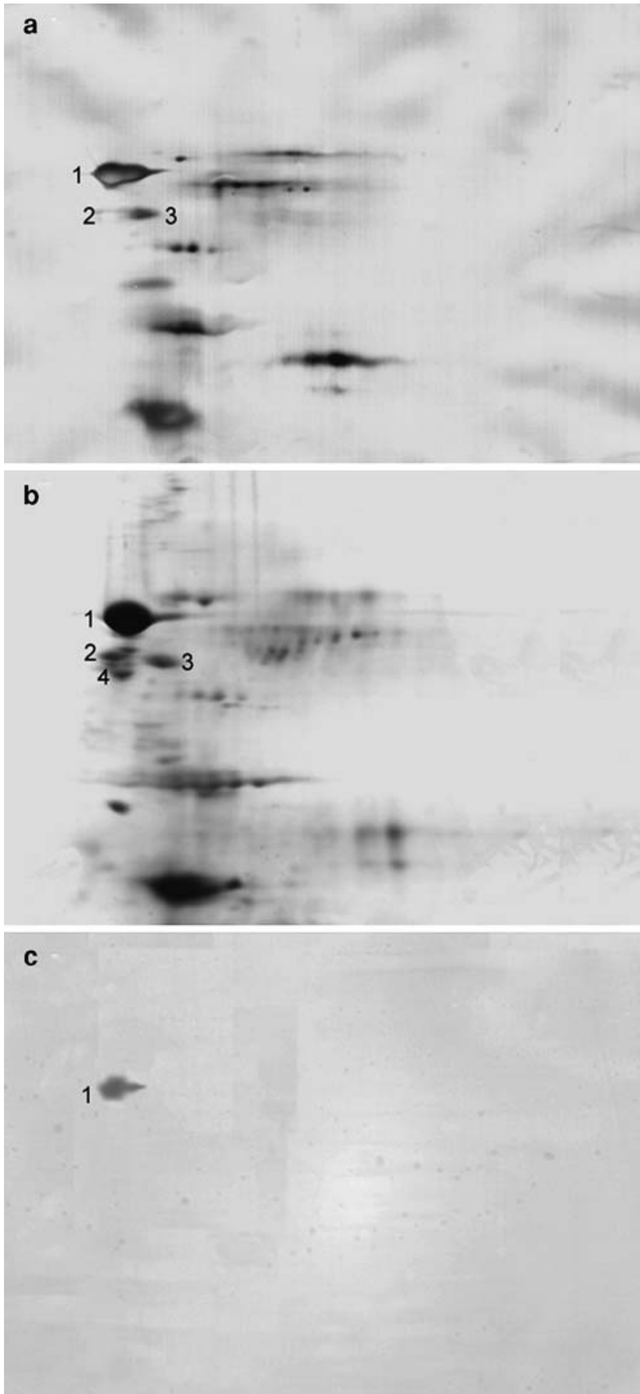

Fig. 2 2D-SDS-PAGE of thermotolerant GPs extracted from 300 mg of mature pollen and separated by affinity chromatography on ConA-agarose. GPs were detected on Western blots by ConA-peroxidase (**a**) or directly on the gel with CBB (**b**). **c** shows a blue stained spot on the gel with Stains All. MS analysis identified spots 1–3 as calreticulin and spot 4 as ankyrin-repeat protein HBP1

GP59 could be detected beginning on day 1 before anthesis (Fig. 3c, d). The earlier loss of GPs staining on ConA-affinoblot (Fig. 3c) than on immunoblot (Fig. 3d) during pollen tube growth might indicate possible changes in pollen tube GPs decreasing their affinity to ConA after heating.

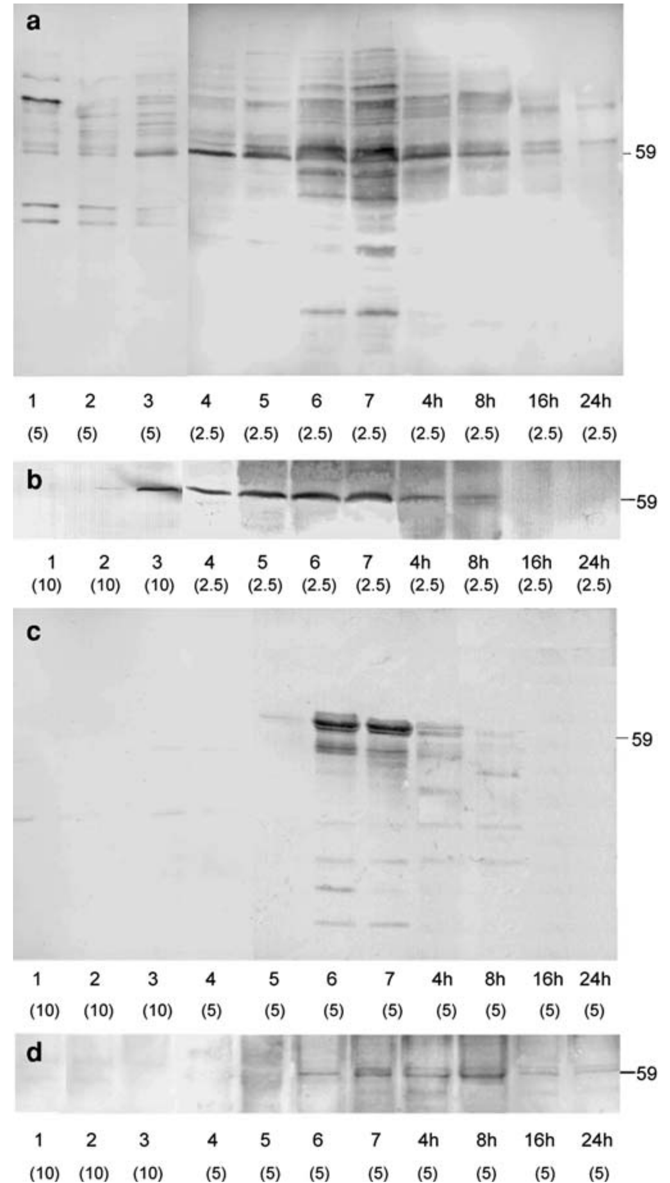

Fig. 3 Developmental changes of pollen calreticulin. Proteins were extracted from mitotic microspores (1) and from early (2), mid- (3, 4), maturing (5), almost mature (6) and mature (7) pollen and from pollen tubes grown in vitro for 4, 8, 16 and 24 h. Total soluble (**a**, **b**) or thermotolerant (**c**, **d**) proteins were separated on 1D-SDS-PAGE, electroblotted onto nitrocellulose and detected using ConA-peroxidase labelling (**a**, **c**) or by anti-calreticulin antibody (**b**, **d**). Lanes were loaded with proteins corresponding to pollen from the numbers of anthers given in parentheses

Transcription of calreticulin gene during pollen development

The expression profile of calreticulin during pollen development was examined by RT-PCR analysis of RNA isolated from stages 1–7 of pollen development and during 4 and 24 h of pollen tube growth. The RT-PCR primers were designed according to the tobacco calreticulin gene T03691 (Denecke et al. 1995). The results showed that the calreticulin gene transcript is

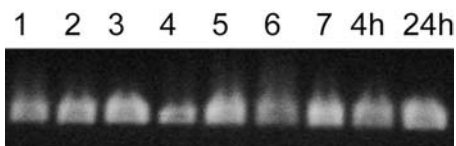

Fig. 4 RT-PCR analysis of calreticulin gene (TO3691) expression during pollen development in anthers (stages 1–7) and in vitro pollen germination for 4 and 24 h. For description of the developmental stages (see Fig. 3)

present at all the developmental stages examined, from microspore mitosis to pollen maturation and in the first 24 h of pollen tube growth (Fig. 4).

Discussion

This study demonstrates some ConA-binding GPs of mature tobacco pollen that exhibit high thermotolerance, suggesting their possible involvement in pollen protection against stress conditions. The MS analysis of thermotolerant GPs separated from non-glycosylated proteins by affinity chromatography on ConA-agarose revealed the presence of one abundant and two minor acidic isoforms of calreticulin. The identification of the major isoform 59 kDa was confirmed as a Ca^{2+}-binding protein by immunoblotting with human anti-calreticulin and blue staining with Stains All.

Calreticulin belongs to resident proteins of the ER carrying a C-terminal targeting signal responsible for their retention within the ER. Calreticulin, which is highly conserved between plants and animals, possesses three characteristic domains: negatively charged N-terminal domain with HDEL retention signal, the N-terminal domain and the central proline rich P-domain. In plants two or more isoforms of calreticulin exist. Three calreticulin isoforms were described in Arabidopsis (Nelson et al. 1997) that form two distinct groups (CRT1 and CRT2/CRT3). These two distinct groups of genes differ in the number of negatively charged amino acids in the C-terminal region, in the number of potential N-glycosylation sites and in their expression pattern (Persson et al. 2001). Calreticulin is proposed to be a multifunctional protein. It acts as a molecular chaperone by promoting proper protein folding and it has been also implicated in Ca^{2+} storage and intracellular signalling. It might also have a role in the modulation of gene expression (Burns et al. 1994) and regulation of cell adhesiveness (Coppolino et al. 1997). Calreticulin has been shown to be induced by stress (Sharma et al. 2004) and to protect cells by suppressing protein aggregation and inhibiting thermal denaturation (Saito et al. 1999).

Studies on immunodetection of calreticulin in mature pollen of L. tulipifera L. (Navazio et al. 1998), maize sperm cells (Williams et al. 1997) and petunia pollen tubes (Lenartowska et al. 2002) are concerned with the role of calreticulin as a Ca^{2+} -binding protein but there is no report on the regulation of calreticulin expression during pollen development and on its heat tolerance. The developmental study presented here revealed the presence of similar levels of the calreticulin transcript at all the stages of pollen development, beginning with microspore mitosis through the first 24 h of pollen tube growth, whereas its translation product could be detected by immunoblotting and ConA-staining from the bicellular stage of pollen development through the first 8 h of pollen tube growth; maximum labelling occurred in mature ungerminated pollen. Calreticulin mRNA may thus be present in pollen in an inactive form ready for translation under certain developmental conditions. The presence of inactive mRNA transcripts in tobacco pollen has been reported encoding 69 kDa glycoprotein (Štorchová et al. 1994; Honys et al. 2000). It is of interest that calreticulin as a thermotolerant protein could be detected only in almost mature and mature pollen and during 8 h of pollen tube growth. It seems probable that thermotolerance of calreticulin may depend on the extent and type of glycosylation. The glycosylation pattern of plant calreticulin seems to be heterogeneous and does not appear to be a conserved property of the protein. The number of potential N-glycosylation sites differs not only among particular species, but also among particular isoforms of the calreticulin gene in Arabidopsis (Persson et al. 2001) and L. tulipifera (Navazio et al. 1995). The glycosylation status of calreticulin can also respond to environmental stress conditions, as has been described in Chinese hamster ovary cells (Jethmalani et al. 1994). In these cells, calreticulin is not glycosylated under non-stress conditions; however, it is immediately glycosylated during acute heat stress. Heat shock also up-regulates calreticulin expression in different kinds of cultured human lung cells (Conway et al. 1995). Dependence of heat-tolerance on glycosylation has been demonstrated in Escherichia β-glucanase (Meldgaard and Svendsen 1994) and in barley α-glucosidase (Clark et al. 2004). Here we show that glycosylated calreticulin exhibits high heat-tolerance in mature pollen. To the best of our knowledge, there are no other reports concerning the thermotolerance of calreticulin. The heat-tolerant calreticulin with attached N-glycans may play a role in the protection of pollen against dessication and temperature stresses—an effect that may be exerted during the last stages of its maturation and after its release from the anthers.

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