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A stress-inducible protein associated with desiccation in lily pollen

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Abstract. This work characterizes a stress-inducible protein (LLA) associated with desiccation in lily (*Lilium longiflorum* Thunb. cv Snow Queen) pollen. Immunoblots of two-dimensional polyacrylamide gels of pollen protein indicated that the LLA-32 protein with a molecular mass of 32 kDa was heterogeneous and had an isoelectric point of 5.6. The protein was developmentally regulated at the later stage of pollen maturation during anther development. The accumulation of LLA-32 proteins remained steady in the mature pollen, but the protein disappeared during pollen germination. Subcellular fractionation of pollen proteins revealed that the protein was detected only in the cytoplasmic fraction, indicative of a gametophytic origin. Premature drying of developing pollen demonstrated that the LLA-32 doublet was associated with desiccation. The accumulation of the proteins can be experimentally manipulated by methyl jasmonate and salicylic acid as well as by mannitol and methyl viologen. Differential accumulation of the doublet polypeptides occurred with response to various stimuli. The upper band of LLA-32 doublet polypeptides markedly accumulated upon dehydration whereas the lower band of LLA-32 doublet significantly increased its level of accumulation when other stresses were applied.

Keywords: Desiccation; *Lilium longiflorum*; Pollen protein; Stress-inducible.

Introduction

Anthers are floral structures specialized for pollen production in higher plants. In young anthers, the sporogenous cells undergo meiosis and give rise to the formation of microspores at the tetrad stage. These microspores develop into microgametophytes after haploid mitosis and subsequently differentiate into mature pollen (Johri, 1984). In the final stage of maturation, pollen exhibits various degrees of desiccation and this natural drying process promotes pollen germinability to a great extent.

As an adaptive organ that facilitates the colonization of terrestrial habitats, pollen develops a number of unique features (Blackmore and Knox, 1990). First, the pollen grain is surrounded by a protective wall consisting of two layers, exine and intine. Second, sporopollenin in the exine is an insoluble polymer that is extremely resistant to drought and chemicals, including strong acids. Finally, the presence of soluble sugars such as sucrose in pollen may play an essential role in the acquired tolerance to desiccation (Hoekstra and van Roekel, 1988). Anhydrobiotic organisms are known to contain trehalose, a nonreducing disaccharide of glucose that is related to acquired tolerance to severe dehydration (Crowe et al., 1984). In addition to sugars, proteins are considered to be another component of desiccation tolerance. Various types of drought-inducible proteins have been identified (Skriver and Mundy, 1990; Shinozaki and Yamaguchi-Shinozaki, 1997). Among these, dehydrins are known to accumulate immediately before desiccation during seed development (Close et al., 1989; Dure, 1993). Dehydrins are also reported in mature pollen grains (Michel et al., 1994; Wang and Cutler, 1995). It has been suggested that these proteins play a role in protecting plant structures during water loss. Most genes that respond to drought are also abscisic acid (ABA)-responsive (Ingram and Bartels, 1996; Bray, 1997). We recently described two pollen-specific proteins that were immunologically unrelated to dehydrins in lily seeds (Wang et al., 1996, 1998). The two pollen proteins that accumulated shortly before anthesis were ABA and polyethylene glycol (PEG)-inducible (Wang et al., 1996). Herein, we continue our examination by using immunological and biochemical methods to characterize one of the two proteins during development and stress. The LLA-32 protein is a heterogeneous protein located in the cytoplasmic fraction of pollen grains. Premature drying of developing pollen demonstrates that the doublet polypeptides are associated with desiccation. The LLA-32 doublet polypeptides differentially increase their levels of accumulation upon dehydration and other stresses.

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Materials and Methods

Plant Materials

Plants of lily (*Lilium longiflorum* Thunb. cv Snow Queen) were grown in the field. Lily buds ranging from 7 to 175 mm were harvested at autumn and dissected to isolate anthers. Pollen was manually collected before anthesis on the basis of bud length or at anthesis (from 170 to 175-mm buds) or at indicated days after anthesis (DAA). Microspore mitosis was complete at a bud length around 65 mm, and pollen subsequently entered the maturation phase of development. To assure that the growing plants were healthy and free of pathogenic attack, the normal patterns of desiccation-associated proteins that accumulated in the developing anthers were monitored. Unless indicated elsewhere, pollen collected 1 day before flowering was used for germination experiments. Material was stored at -80°C until use.

Premature Drying and Germination Treatments

Pollen taken from anthers of different flower bud sizes was air-dried on the laboratory bench for 24 h at 25°C. For pollen germination, pollen (approximately 300 mg) was incubated in 4 ml of germination medium [0.29 M pentaerythritol, 300 μg ml^{-1} $Ca(NO_3)_2 \cdot 4H_2O$, 10 μg ml^{-1} H_3BO_3 and 100 μg ml^{-1} KNO_3] (Dickinson, 1978) at 30°C with shaking for 24 h. Where indicated, pollen was incubated in the medium containing various concentrations of salicylic acid (SA, sodium salt, Sigma), methyl jasmonate (MeJA, Bedoukian Research Inc., Danbury, CT, USA), mannitol or methyl viologen. To avoid imbibitional damage, frozen or dried pollen was routinely placed in humid air for 1 h prior to testing for germination (Hoekstra, 1984).

Preparation, Electrophoresis and Immunoblotting of Pollen Protein

The phenol extraction method was used to extract total protein from lily pollen (Wang et al., 1992). The total pollen protein was then subjected to two-dimensional polyacrylamide gel electrophoresis (2D-PAGE). The extraction of heat-stable proteins from pollen was performed according to Wang et al. (1996). Protein concentration was determined by the dye binding Bio-Rad protein assay according to the supplier's directions. The heat-stable protein fractionated by SDS-PAGE was overrun in order to have a better separation of the LLA-32 doublet polypeptides. The heat-stable protein on the gel was either stained with silver, or electroblotted onto nitrocellulose (0.45 μm, Gelman Sciences, Ann Arbor, MI, USA) (Wang et al., 1996). The 2D-PAGE-purified LLA-32 polypeptides (approximately 10 μg) in gel slices were ground in 0.5 ml of PBS (10 mM sodium phosphate, pH 7.4, 150 mM NaCl) and emulsified with 0.5 ml Freund's complete adjuvant (Sigma). The emulsion was intramuscularly injected into a local hen L2-BDS (kindly provided by Dr. Y. P. Lee, Dept. of Animal Science, National Chung Hsing Univ., Taichung, Taiwan). The second injection with approximately 10 μg protein in PBS/Freund's incomplete adjuvant was administered 4 weeks after the first one. A booster injection with the same amount of protein in PBS/Freund's incomplete adjuvant was then administered 1 week after the second injection. Eggs were collected a week after the booster injection. Immunoglobulins (Igs) were isolated from the egg yolks, aliquoted, and stored at -80°C in the presence of 0.1% sodium azide. Pre-immune IgGs used as controls were taken from eggs before injection. For each preparation of blot-affinity-purified antibodies, 750 μl of egg yolk IgG was used according to the method of Smith and Fisher (1984). Antibodies were eluted with elution buffer (5 mM glycine-HCl, pH 2.3, 500 mM NaCl, 0.5% [v/v] Tween 20, and 100 μg ml^{-1} BSA). The eluates were immediately neutralized by the addition of 1 M sodium phosphate (pH 7.7) to a final concentration of 50 mM. The eluates were aliquoted and stored at -80°C. Blots were immunostained using a 1:4 dilution of eluate of the affinity-purified LLA-32 antibodies.

Subcellular Fractionation of Pollen Proteins

Mature pollen (5 g) was suspended in 10 ml of 0.1 M Tris-HCl pH 8.0 containing 0.29 M pentaerythritol and 2.4 mM PMSF. Pollen was shaken for 1~2 min and centrifuged at 5,000 *g* for 3 min (water soluble fraction in the pollen wall). This process was repeated three times. Pollen remained intact under the above treatment when examined by microscopy. The final washed pollen was disrupted by passing it twice through a French Press (SLM-AMINCO FA-078, Rochester, NY, USA) at 10,000 p.s.i. in the Tris buffer as described above. The broken cells were centrifuged at 12,000 *g* to produce a crude wall fraction (pellet) and a cytoplasmic fraction (supernatant) (Chay et al., 1992). The pellet was successively washed in the following ways, each followed by centrifugation: four times with 100 ml of 0.05 M Tris-HCl pH 8.0, 12~15 times with 100 ml of deionized distilled water, once with 50 ml of 1% Triton X-100, once with 100 ml of deionized distilled water, once with 50 ml of 1% Triton X-100, and 8~11 times with deionized distilled water (Li et al., 1983). All washings were done at 4°C. The following extraction of protein from each fraction was according to the phenol method (Wang et al., 1992).

Results

LLA-32 Protein is Heterogeneous and Developmentally Regulated

Total protein extracted from mature pollen was fractionated by 2D-PAGE. A 2D-PAGE immunoblot was incubated with affinity-purified anti-LLA-32 antibodies to show that the LLA-32 protein was heterogeneous and acidic, having an isoelectric point (pI) of 5.6 (Figure 1A). Affinity-purified antibodies to LLA-32 recognized three polypeptides, one with a slightly higher molecular weight (Figure 1B). 2D-PAGE of total pollen protein revealed that

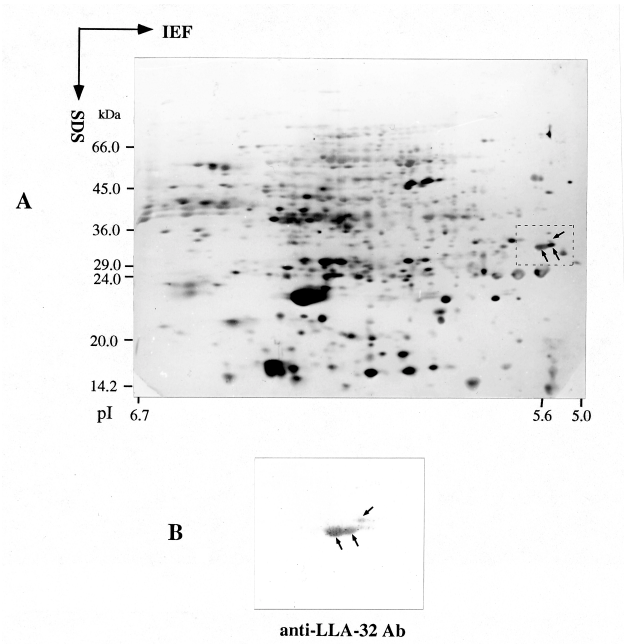

Figure 1. The heterogeneity of pollen LLA-32 proteins in *L. longiflorum*. Total protein (120 µg) extracted from pollen was electrophoresed by 2D-PAGE and either stained with silver (A) or electroblotted onto nitrocellulose, and immunologically detected using affinity-purified LLA-32 antibodies (B). Instead of using 120 µg, 2.4 mg of total protein was fractionated and electroblotted for the immunodetection of LLA-32 proteins. The LLA-32 proteins are indicated by arrows. Positions of molecular mass marker proteins are indicated.

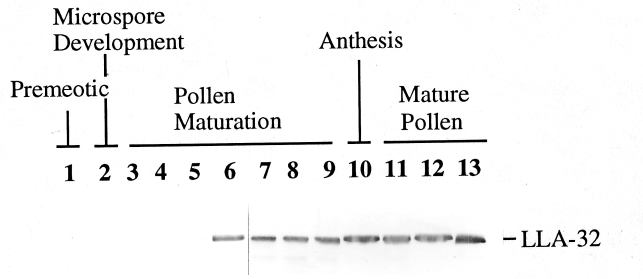

Figure 2. Accumulation of LLA-32 proteins in developing anthers of *L. longiflorum*. Heat-stable proteins were extracted from various size classes of anthers: 1 = 9 mm (16-mm buds); 2 = 17 mm (35-mm buds); 3 = 21 mm (70-mm buds); 4 = 22 mm (90-mm buds); 5 = 22 mm (110-mm buds); 6 = 22 mm (130-mm buds); 7 = 23 mm (140-mm buds); 8 = 23 mm (150-mm buds); 9 = 23 mm (160-mm buds); 10 = 23 mm (from 170 to 175-mm buds, at anthesis) and from mature pollen at indicated days after anthesis (DAA): 11 = 1 DAA; 12 = 3 DAA; 13 = 5 DAA. Total protein was extracted from each anther size classes and from mature pollen and heat-treated at 90°C for 10 min. Heat-stable proteins (5 µg) were fractionated by SDS-PAGE, electroblotted onto nitrocellulose, and immunochemically detected using affinity-purified LLA-32 antibodies.

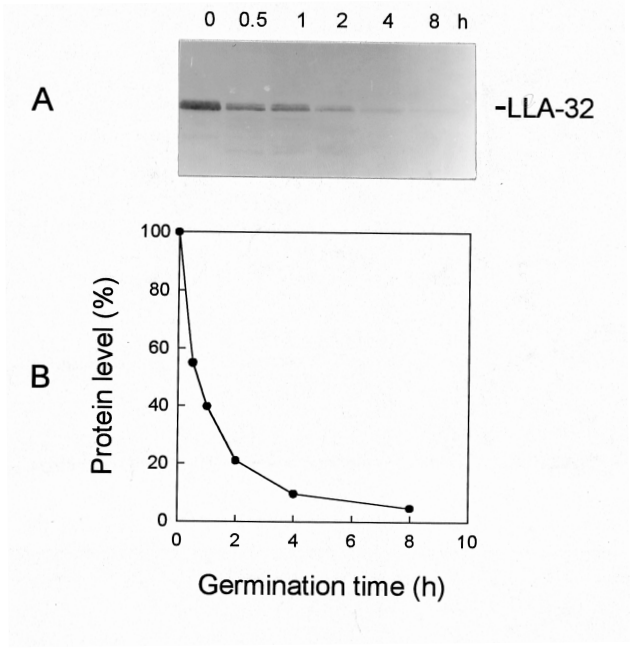

Figure 3. Time course of the disappearance of LLA-32 proteins in germinating pollen of *L. longiflorum*. Mature pollen (0.2 g) was incubated at 30°C in the germination medium at indicated hours after germination. Total protein was extracted from ungerminated and germinated pollen and heat-treated at 90°C for 10 min. Heat-stable proteins (5 µg) were fractionated by SDS-PAGE, electroblotted onto nitrocellulose, and immunochemically detected using affinity-purified LLA-32 antibodies (A). The LLA-32 protein levels (B), estimated by scanning the optical density of immunostained signals shown in (A), are expressed as percentages of the maximal levels found in ungerminated pollen. Each data point is the average of signals from three independent immunoblots.

antibodies to LLA-32 only specifically recognized LLA-32 proteins. No other protein was detected by affinity-purified LLA-32 antibodies. Therefore, instead of using total protein, heat-stable proteins were extracted and used in the following experiments to simplify the protein patterns in a gel.

To determine whether LLA-32 protein could be detected in the phases of premeiotic and microspore development, heat-stable proteins from various size classes of anthers were fractionated by SDS-PAGE. An immunoblot revealed that LLA-32 was detected only at the later stage of pollen maturation (Figure 2). The temporal distribution of LLA-32 during anther development was consistent with the earlier report of its detection in the phase of pollen maturation by silver staining (Wang et al., 1992). In addition, the accumulation of LLA-32 proteins in the mature pollen remained steady even after five DAA. However, the proteins disappeared when pollen was incubated in the germinating buffer for 4 h or longer (Figure 3). The germinating buffer contains 0.29 M pentaerythritol instead of 0.3 M sucrose.

Subcellular Fractionation of LLA-32 Proteins

To investigate the subcellular location of LLA-32 protein, pollen was initially suspended in Tris buffer pH 8.0 and shaken to release water-soluble proteins that were

embedded in the pollen wall. The final washed pollen was disrupted through a French Press, and then the broken cells were centrifuged to produce a crude wall/membrane fraction (pellet) and a cytoplasmic fraction (supernatant). Next, the pellet was successively washed with buffers (in Materials and Methods) and no protein in the supernatant was detected by Bio-Rad protein assay after the final washing with deionized distilled water (data not shown). Pollen protein extracted from each fraction was resolved by SDS-PAGE (Figure 4A). Immunoblots of total protein from each fraction revealed that the LLA-32 proteins were undetected in the pollen wall (lanes B1 and B3). The proteins were detected only in the cytoplasmic fraction, indicative of a gametophytic origin (lanes B2).

Influence of Premature Drying on the Accumulation of LLA-32 Proteins

To determine drying effect on the accumulation of LLA-32 proteins, pollen was removed from developing anthers of indicated bud lengths and air-dried for 24 h. Heat-stable proteins were extracted from various developmental stages of pollen from 140 to 170-mm buds and from mature pollen at anthesis (from 170 to 175-mm buds). SDS-PAGE analysis indicated that a number of heat-stable polypeptides, including the LLA-32 proteins, increased their levels of accumulation upon premature drying (Figure 5A). Immunoblot analyses also revealed that the upper band of LLA-32 doublet significantly increased its level of accumulation whereas no appreciable induction of the lower band of LLA-32 doublet occurred upon drying (Figure 5B).

Influence of Various Stresses on the Accumulation of LLA-32 Proteins

As reported elsewhere, some dehydration-responsive genes are also induced by osmotic stress in various plant tissues (Leonardi et al., 1995). The LLA-32 proteins are both PEG-8000 and ABA-inducible (Wang et al., 1996). To further investigate the inductive effect of various environmental stresses on the LLA-32 proteins, we took advantage of the disappearance of the proteins during pollen germination (Figure 6, lane 2). After incubation with various stimuli in germinating buffer for 24 h, pollen protein was extracted, heat-treated, and fractionated by SDS-PAGE. Immunoblot analyses revealed that a maximum accumulation of LLA-32 proteins was observed when 180 μM MeJA, 1% mannitol, 30 μM SA, or 1 mM methyl viologen was applied (Figure 6). Contradictory to a marked induction of the upper band of LLA-32 proteins upon dehydration, the lower band of LLA-32 proteins significantly increased its level of accumulation when other stresses were applied. However, the protein levels dropped dramatically when the concentration reached 10% mannitol or 100 μM SA whereas the protein levels remained steady when the concentration of MeJA or methyl viologen increased to 540 μM or 10 mM, respectively (data not shown).

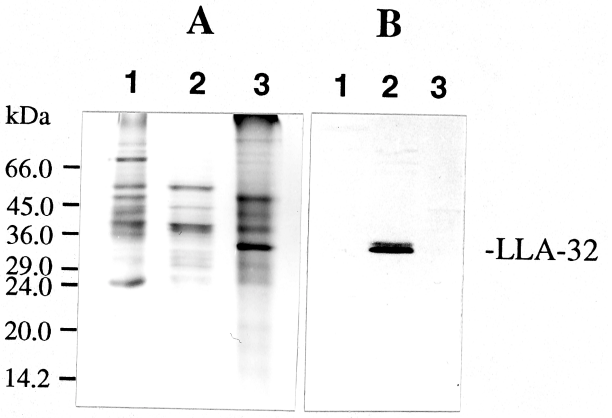

Figure 4. Subcellular immunodetection of LLA-32 proteins in the mature pollen of *L. longiflorum*. Total protein (2 μg) from each fraction was electrophoresed by SDS-PAGE and either stained with silver (A) or electroblotted onto nitrocellulose, and immunochemically detected using affinity-purified LLA-32 antibodies (B). Instead of using 2 μg, 6 μg of heat-stable proteins was fractionated and electroblotted for immunochemical detection. Water-soluble fraction of pollen wall (lane 1), cytoplasmic fraction of pollen grains (lane 2), and pollen wall/membrane fraction (lane 3). Positions of molecular mass marker proteins are indicated.

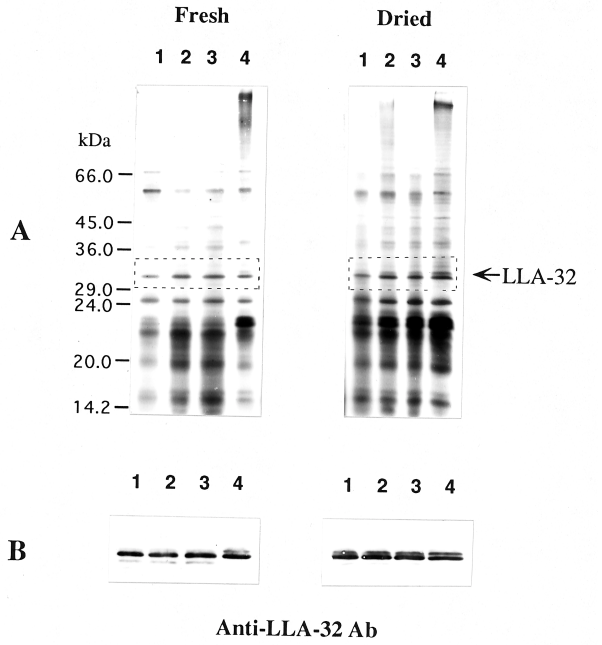

Figure 5. Effect of premature drying on the accumulation of LLA-32 in developing pollen of *L. longiflorum*. Pollen was removed from anthers of three consecutive flower bud sizes: 140~150 mm (lane 1), 150~160 mm (lane 2), 160~170 mm (lane 3) or at anthesis (lane 4). Pollen protein was extracted and heat-treated at 90°C for 10 min. Heat-stable protein (2 μg) was fractionated by SDS-PAGE and either silver-stained (A) or electroblotted onto nitrocellulose, and immunochemically detected using affinity-purified LLA-32 antibodies (B). Instead of using 2 μg, 6 μg of heat-stable proteins was fractionated and electroblotted for immunochemical detection. Positions of molecular mass marker proteins are indicated.

Discussion

In our previous report on the proteins of lily anthers, we suggested that the LLA-32 protein is pollen-specific (Wang et al., 1996). In this study, affinity-purified antibodies were used to determine the heterogeneity and distribution of the protein during pollen development and germination. 2D-PAGE immunoblot resolved the doublet of LLA-32 into a number of polypeptides. Subcellular fractionation of pollen proteins indicated that LLA-32 proteins were not in the pollen wall. Thus, the protein does not belong to the family of many sporophytic proteins in the pollen wall involved in cell-cell recognition (Heslop-Harrison and Heslop-Harrison, 1973). The protein was observed in the cytoplasmic fraction of pollen grains (Figure 4), implying a gametophytic nature. Because proteins in the intine layer of the pollen wall may be released to the cytoplasmic fraction upon the breakage of pollen grains, the location of LLA-32 proteins in the intine layer can not be excluded.

The accumulation of LLA-32 polypeptides in anther development correlates with desiccation that occurs naturally in the pollen (Wang et al., 1996). Premature drying treatment of developing pollen further demonstrates that the accumulation of the proteins is associated with desiccation (Figure 5). This finding corresponds to the observation that proteins in dry pollen are resistant to drying and freeze-drying (Wolkers and Hoekstra, 1995). As widely suggested, dehydrins act as an essential component against water stress; however, they may need to work in concert with oligosaccharides to develop desiccation tolerance (Blackman et al., 1992). The LLA-32 proteins, which are not dehydrins (Wang et al., 1996), may belong to a novel class of proteins related to dehydration. It is interesting that one of the doublet polypeptides responds to dehydration more significantly than the other (Figure 5).

Kermode (1990) contended that dehydration in developing seeds is a critical switch, from a developmental to a germination program. The developmental switch is acquired at seed ages closer to full maturity, during which desiccation occurs and specific proteins accumulate. Developing pollen exhibits various degrees of desiccation immediately before maturation. Therefore, a similar switch from a developmental to a germination mode may exist in pollen. Premature drying treatment of developing pollen possibly results in a suppressive developmental program and a "switching-on" of the germination program. In that sense, the appearance of LLA-32 proteins may serve as an indicator for such a switch.

In addition to being induced by dehydration, the LLA-32 proteins are MeJA-inducible (Figure 6A). Similar to the LLA-32 proteins, several jasmonate-induced proteins (JIPs), which accumulate under water stress conditions, have been reported elsewhere (Reinbothe et al., 1992, 1994). The LLA-32 protein is also ABA-inducible (Wang et al., 1996). Plant cells generally react to various environmental stresses in different ways, but the production of ABA-responsive (Rab) proteins is shared by most all

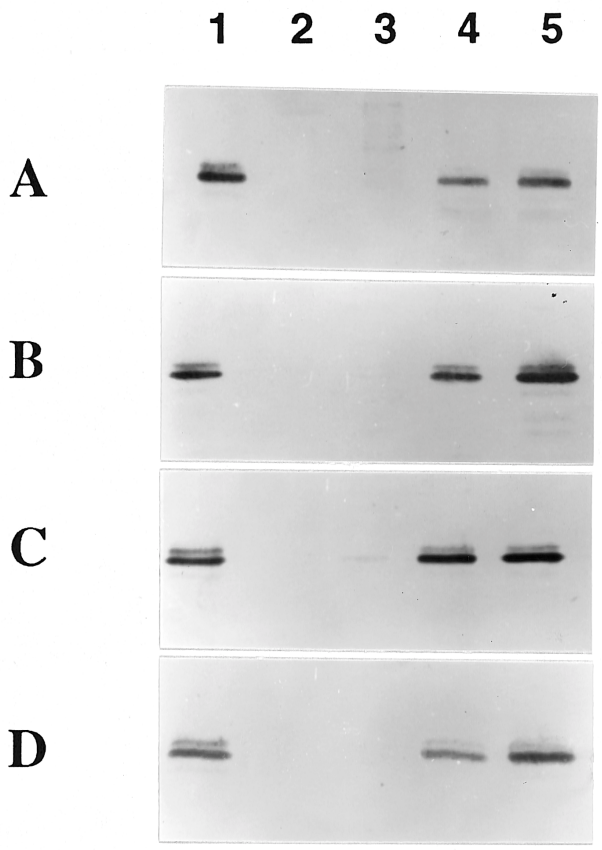

Figure 6. Induction of LLA-32 proteins in pollen of *L. longiflorum* by various stimuli during germination. Pollen harvested 1 day before anthesis (lane 1) was incubated in a germination buffer at 30°C for 24 h without any stimulus (lane 2), or with the application of 45, 90 and 180 µM MeJA (lanes A3-5); of 0.1, 0.5 and 1% mannitol (lanes B3-5); of 1, 10, and 30 µM SA (lanes C3-5) and of 10, 100 and 1000 µM methyl viologen (lanes D3-5). Pollen protein was extracted and heat-treated at 90°C for 10 min. Heat-stable proteins (6 µg) were fractionated by SDS-PAGE, electroblotted onto nitrocellulose, and immunochemically detected using affinity-purified LLA-32 antibodies, respectively.

of them (Leonardi et al., 1995). It has been proposed that water loss induces an increase in endogenous ABA level that subsequently activates JA biosynthesis, resulting in an increase of endogenous JA. Correspondingly, an increase in JA level activates the expression of wound-inducible genes (Wasternack and Parthier, 1997). This model might account for why many of those JIPs are also ABA-inducible (Reinbothe et al., 1994). Nevertheless, some identified JIPs do not exhibit an induction by ABA (Moons et al., 1997; Wasternack and Parthier, 1997).

The LLA-32 proteins are also induced by PEG (Wang et al., 1996) and mannitol (Figure 6B). In addition, the LLA-32 proteins are methyl viologen-inducible (Figure 6D). Methyl viologen, a potent oxidant, is known to enhance O_2^- production that may be involved in the initiation of oxidative damage (Iturbe-Ormaetxe et al., 1998). Moreover, the LLA-32 proteins are SA-inducible (Figure 6C). SA is

another endogenous signal that apparently plays a prominent role in defense against pathogenic attack, particularly in a localized tissue death where pathogen penetrates (Yang et al., 1997). Besides, SA is a signal in the pathway leading to systemic acquired resistance (SAR) (Ryals et al., 1994). The fact that the LLA-32 proteins can be induced by SA implies that these desiccation-related proteins may also be pathogen-induced. We have detected significant increases of LLA-32 proteins even at the earlier stages of pollen maturation during which the protein is normally undetectable; in this case, the batch of growing lily plants from which anthers were dissected to isolate proteins has been attacked by unknown pathogens (data not shown).

Herein, we report on a heterogeneous, desiccation-related protein that developmentally accumulates immediately before anthesis during anther/pollen development. The differential accumulation of the proteins can be experimentally manipulated by premature drying, plant growth regulators, or other stresses.

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百合花粉一與乾燥有關之逆境誘發蛋白

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本實驗分析百合 (*Lilium longiflorum* Thunb. cv. 雪后) 花粉中一與乾燥失水有關的逆境誘發蛋白 (LLA)。花粉蛋白之雙向電泳及免疫轉漬分析顯示 LLA-32 是具有多型，等電點爲 5.6，分子量爲 32 kDa的多胜太。此花粉蛋白在花藥發育的過程中，只在花粉成熟的晚期才累積表現。花粉成熟以後 LLA-32蛋白的量仍維持一定，但是當花粉萌發四小時後， LLA-32 蛋白即消失。花粉蛋白經次細胞分離的實驗顯示 LLA-32蛋白是位於花粉的細胞質內。將發育中的花粉先行乾燥處理可証明此蛋白確與乾燥有關。LLA-32 doublet 蛋白的累積可經由外加 methyl jasmonate、salicyilic acid、mannitol 和 methyl viologen 等方式來加以操控。LLA-32 doublet受乾燥和其他逆境而有不同程度的累積。LLA-32 doublet的一個多胜太受乾燥影響而明顯增加；LLA-32 doublet的另一個多胜太則受其他逆境的影響明顯增加 。

關鍵詞：乾燥；百合；花粉蛋白；逆境誘發。