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The nuclear localization signal of a pollen-specific, desiccation-associated protein of lily is necessary and sufficient for nuclear targeting

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Abstract. The lily LLA23 protein represents a novel member of water-deficit/ripening-induced protein family (Plant Cell Physiol. **41:** 477-485, 2000). Examination of the C-terminal half of LLA23 reveals the presence of basic regions that are reminiscent of a nuclear localization signal (NLS). To investigate the nuclear targeting property of NLS in LLA23, a green fluorescent protein (GFP) gene fused with the C-terminal half of LLA23 (GFP-LLA23) was constructed. In addition, the GFP alone and a mutGFP-ΔLLA23 that had the sequence for the putative NLS deleted were also constructed. All these three constructs were separately inserted into a bamboo mosaic potexvirus (BaMV) vector. Infection of BaMV in *Chenopodium quinoa* caused local lesions in leaves where the green fluorescence of fusion proteins could be visualized by fluorescence microscopy. The RNA blot and immunoblot analyses of BaMV-infected leaves indicated that the recombinant subgenomic RNA and the resulting protein were strongly detected. Fluorescence microscopic studies revealed that the NLS in LLA23 exhibited a property of nuclear targeting, showing highly condensed spots of green fluorescence in leaf cells, whereas GFP alone was apparently distributed throughout the cytoplasm. In contrast, a deletion of the NLS sequence resulted in exclusively cytoplasmic localization of the fusion protein. The nuclear location of the GFP-LLA23 protein in leaf cells was further confirmed by staining with 4´,6-diamidino-2-phenylindole. These results clearly demonstrate that the putative NLS in LLA23 is necessary and sufficient for import of the LLA23 protein into the nucleus.

Keywords: BaMV; Desiccation; *Lilium longiflorum*; NLS; Pollen-specific protein.

Abbreviations: BaMV, bamboo mosaic potexvirus; **DAPI,** 4´,6-diamidino-2-phenylindole; **GFP,** green fluorescent protein; **Lea,** late embryogenesis abundant; **NLS,** nuclear localization signal; **PAGE,** polyacrylamide gel electrophoresis; **PBS,** phosphate buffer saline; **PCR,** polymerase chain reaction.

Introduction

Pollen plays a crucial role in sexual reproduction of flowering plants. Within the anther, pollen mother cells undergo meiosis to produce a tetrad of haploid microspores that subsequently devide mitotically to form mature pollen grains. When pollen grows to full maturity, it exhibits various degrees of desiccation prior to anthesis and this desiccation process represents the last stage of pollen maturation.

The quantitative and qualitative changes in protein accumulation during pollen development have been documented in various plant species (Zarsky et al., 1985; Vergne and Dumas, 1988; Detchepare et al., 1989; Bedinger and Edgerton, 1990; Zarsky et al., 1995), but there have been few reports on pollen-specific proteins that accumulate in the maturing pollen. We have previously described the accumulation of a set of newly synthesized anther/pollen-specific proteins prior to anthesis during anther development in lily (Wang et al., 1992; Wang et al., 1996). Ueda and Tanaka (1995) have found two male gamete-specific histone variants in the generative nuclei of lily pollen. Recently, pollen-specific proteins were also described in pollen of tobacco and lily (Wittink et al., 2000; Ko et al., 2002; Mogami et al., 2002).

We have characterized a number of pollen-specific proteins related to dehydration in lily pollen (Wang et al., 1996, 1999; Ko et al., 2002). Of these, the LLA23 protein represents a novel member of the water-deficit/ripening-induced protein family (Wang et al., 1998; Huang et al., 2000). The water-deficit/ripening-induced proteins reported in various plant species possess a putative nuclear localization signal (NLS) at the C-terminus (Iusem et al., 1993; Canel et al., 1995; Silhavy et al., 1995; Chang et al., 1996; Schneider et al., 1997). Subcellular fractionation experiments performed by Iusem et al. (1993) indicated that the Asr protein is located primarily in the nucleus.

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Proteins are translocated from the cytoplasm where they are synthesized into the nucleus either to perform basic cellular processes or in response to developmental or environmental signals (Harter et al., 1994). It is generally accepted that even for small proteins, nuclear import is mediated by nuclear localization signals characterized as short clusters of basic amino acids (Garcia-Bustos et al., 1991; Raikhel, 1992). Mechanisms to control nuclear targeting involve regulation of NLS binding by protein interaction and modulation of NLS activity by biochemical modification (Fobes, 1992; Goldfarb, 1994; Hunter and Karin, 1992). The LLA23 protein contains a segment of NLS at the C terminus (Huang et al., 2000). In a continuing effort to elucidate the function of the LLA23 protein, here we examined whether this putative NLS indeed functions to direct nuclear targeting of LLA23.

Materials and Methods

Plant Materials and Inoculation

Plants of lily (*Lilium longiflorum* Thunb. cv Snow Queen) were grown in the field. Plants of *Chenopodium quinoa* were grown in the green house. Leaves of *Chenopodium quinoa* were used for virus inoculation as previously described (Lin and Hsu, 1994). Each inoculum contained a mixture of 0.25 μg of bamboo mosaic potexvirus (BaMV) RNA per leaf.

RNA Blot

Total RNA was extracted from leaves of *C. quinoa* using the Ultraspec RNA™ isolation system (Biotecx Laboratories Inc., Houston, USA). RNA samples were electrophoresed in 1.0% formaldehyde-MOPS gels using standard procedures (Sambrook et al., 1989) and transferred onto nylon membranes. The membranes with immobilized RNA were prehybridized for 4 h at 42° C in medium containing 5 × SSC (1 × SSC is 0.15 M NaCl and 15 mM sodium citrate), 0.1% polyvinylpyrrolidone, 0.1% ficoll, 20 mM sodium phosphate, pH 6.5, 0.1% (w/v) SDS, 1% glycine, 50% formamide and 150 μg/ml of denatured salmon sperm DNA. For hybridization, the prehybridization solution was removed and replaced with hybridization buffer that contained the same components as the prehybridization buffer except for the addition of 1% glycine, denatured salmon sperm DNA (100 μg/ml) and random-primed ^{32}P-labeled green fluorescent protein (GFP) DNA (specification 8.0 × 10^8 cpm/μg). Hybridization was carried out at 42° C overnight with constant agitation. The membranes were washed at 42° C twice in 2 × SSC, 0.1% (w/v) SDS for 20 min followed by twice in 0.1 × SSC, 0.1% (w/v) SDS at 60° C for 20 min. The membrane was exposed to X-ray films (Konica AX) using 1 or 2 intensifying screens (DuPont).

Generation of GFP-LLA23 Fusion Constructs and in Vitro Transcription

To generate a chimeric fusion construct of GFP and LLA23 (GFP-LLA23), a DNA fragment that encoded the C-terminal half of LLA23 (amino acid residues 55 to 142) was first amplified by polymerase chain reaction (PCR) using LLA23 cDNA as a template using a 5′-primer 1 (5′-CGGCATGGACGAGCTGTACAAGGACTACGAGAAAG AGAAGAAGCAC-3′) and 3′-primer 3 (5′-TGCCTTAT CGCCGGCGTCGCTT AACCGAAGAAGTGG-3′) pairas shown in Figure 1A. In addition to containing a segment of LLA23 sequence at the N-terminus (underlined), the 5′-primer 1 also contains a C-terminal coding sequence of the GFP gene (without the terminator codon). The resulting PCR fragment of LLA23 (designated megaprimer 1) was fractionated on a 1% agarose gel and stained with ethidium bromide. The fragment was recovered by the NucleoTrap DNA Purification Kit (Clontech Laboratories Inc., Palo Alto, CA). Next, the GFP gene was used as a template and amplified by PCR with a 5′-primer of GFP (5′-AGATATCATGGTGAGCAAGGGCGAGGAGC-3′) and 3′-megaprimer 1 pair as described above. The resulting GFP-LLA23 fusion fragment was purified on a 1% agarose gel, stained with ethidium bromide. The fragment was again recovered by the NucleoTrap DNA Purification Kit and cloned into pGEM-T easy vector (Promega, Madison, WI). In the construction of mutGFP-ΔLLA23, a segment of 20 amino acids from residues 123 to 142 was deleted. This deletion resulted in a GFP-ΔLLA23 mutant lacking the NLS. The construction of mutGFP-ΔLLA23 was similar to that of GFP-LLA23 except that the 3′-primers 2 (5′-TGCCTTATCGCCGGCGTCGCTTACTCGTGGTGCTCA TGGAAGGTGT-3′) was used instead. The resulting 3′-megaprimer 2 wasthen paired with 5′-primer of GFP for PCR amplification using GFP as a template. The GFP alone was also amplified by PCR using a 5′-primer and 3′-primer (5′-CCCGGGCGGCGCGTTTACTTGTACAGCTCGTCC-3′)pair of GFP. After the nucleotide sequence of each construct was verified by DNA sequencing, the GFP, GFP-LLA23 and mutGFP-ΔLLA23 fragments were digested with *Eco* RV and *Not* I and cloned into the corresponding sites of pUC119 vector, which includes the T7 promoter and the whole genomic sequence of BaMV. Each plasmid construct was purified and its concentration was determined. Conditions for in vitro transcription of linearized plasmids were as described for brome mosaic virus (French and Ahlquist, 1987). The quantity and quality of the synthesized transcripts were verified by agarose gel electrophoresis before inoculation.

Protein Preparation, Electrophoresis and Immunoblotting

Phenol extraction of total protein was performed according to Wang et al. (1992). The infected leaves of *C. quinoa* were ground into a fine powder in liquid nitrogen with a motar and pestle. Protein concentration was determined by the dye binding Bio-Rad protein assay according to the supplier's instructions. Total protein was fractionated by SDS-polyacrylamide gel electrophoresis (PAGE) and either stained with Coomassie blue or electroblotted onto nitrocellulose (0.45 μm, Gelman Sciences, Ann Arbor, MI) (Wang et al., 1996). Blots were immunostained using a 1: 800 dilution of anti-GFP antiserum.

Fluorescence Microscopy

The infected leaves of *C. quinoa* were directly inspected using a fluorescence microscope. The infected leaves were treated with 0.5% Triton X-100 for 30 min before they were stained with 2.5 µg/ml 4´,6-diamidino-2-phenylindole (DAPI) in phosphate buffer saline (PBS) for 30 min at room temperature, and then washed with PBS. Fluorescence microscopy and photography were performed using BX51TRF and PM30 (Olympus, Tokyo, Japan), respectively.

Results

The protein encoded by LLA23 (accession number AF077629) belongs to a family of water-deficit/ripening-induced proteins (Huang et al., 2000). It contains a segment of the sequence KKTLKKENEEVEGKK near the C-terminus of the molecule (Figure 1A). It shows a putative bipartite motif of basic residues, K or R, characteristic of NLS. In both plants and animals, proteins are targeted to the nucleus by specific NLS characterized as short amino acid regions that are rich in basic residues (Garcia-Bustos et al., 1991).

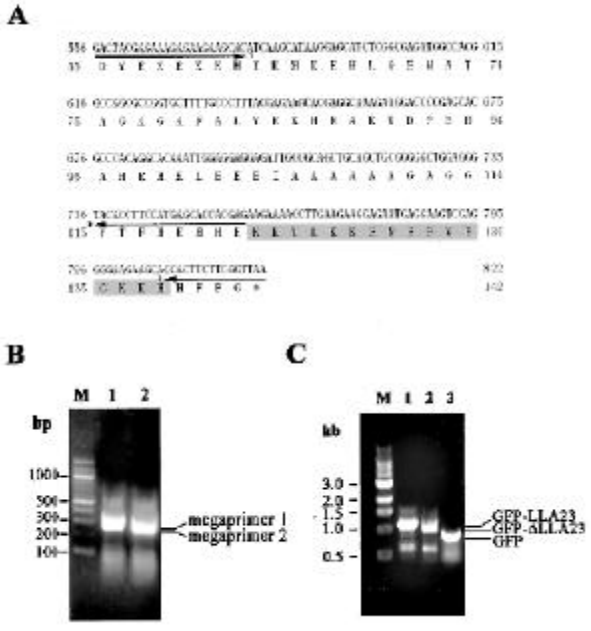

Figure 1. Generation of GFP-LLA23 and mutGFP-ΔLLA23 constructs. A, A partial coding sequence of LLA23 cDNA and its predicted amino acid sequence. The shaded region indicates a segment of the putative NLS sequence. Primers 1-3 are indicated by long arrows: 1 represents the LLA23 portion of the 3´-GFP-5´-LLA23 primer and 2 and 3 represent 3´-ΔLLA23 (without an NLS) and 3´-LLA23 primers, respectively; B, Synthesis of megaprimers. DNA fragments that encode the C-terminal half of LLA23 and ΔLLA23 were separately amplified by PCR. The resulting PCR fragments, designated megaprimer 1 and 2, were fractionated on a 1% agarose gel and stained with ethidium bromide; C, Generation of fusion constructs. The GFP was used as a template and amplified by PCR using a 5´-primer of GFP and either 3´-megaprimer 1 or 3´-megaprimer 2. The resulting GFP-LLA23 and GFP-ΔLLA23 fusion fragments were purified on a 1% agarose gel and stained with ethidium bromide. In B and C, M indicates the lane containing marker DNA fragment of various sizes.

To investigate the function of the putative NLS in LLA23, a GFP gene was used as a reporter. Using the LLA23 cDNA as a template, megaprimers were amplified by PCR using a 5´-primer 1 and either 3´-primer 2 or 3´-primer 3 (Figure 1, A and B). Subsequently, the GFP was used as a template and amplified by PCR using a 5´-primer of GFP and either purified 3´-megaprimer 1 or 3´-megaprimer 2. The resulting constructs contained the GFP sequence fused with the C-terminal sequence of *LLA23* (GFP-LLA23) and the GFP sequence with the C-terminal sequence of *LLA23* without the NLS sequence (mutGFP-ΔLLA23), respectively (Figure 1C). The fusion constructs were inserted into a bamboo mosaic potexvirus (BaMV) vector and BaMV RNAs from each construct was prepared in vitro. Infection of BaMV in *C. quinoa* caused local lesions in leaves (Figure 2A), where the green fluorescence of fusion proteins could be visualized by fluorescence microscopy (Figure 3). RNA blot analysis of BaMV-infected leaves indicated that the virus subgenomic RNA (sgRNAx), which contained the RNA transcribed from the fusion construct, strongly hybridized to the ^{32}P-labeled GFP DNA probe. The other two RNA bands, virus genomic RNA (gRNA) and subgenomic RNA1 (sgRNA1), also hybridized to the GFP probe (Figure 2B). Total protein was extracted from BaMV-infected leaves of *C. quinoa* and fractionated by SDS-PAGE. Immunoblot analysis using GFP-specific antiserum suggested that expression of these constructs in infected leaves resulted in the synthesis of fusion proteins of predicted sizes (Figure 2C). The mock contains only BaMV itself, and thus no GFP protein could be possibly detected.

Fluorescence microscopic studies indicated that the NLS in LLA23 exhibited a property of nuclear localization signals, showing highly condensed spots of green fluorescence in leaf cells (Figure 3B), whereas GFP alone was apparently distributed in the cytoplasm of whole leaf cells (Figure 3A). In contrast, deletion of the NLS sequence (mutGFP-ΔLLA23) resulted in exclusively cytoplasmic localization of the fusion protein (Figure 3C). Further, the nuclear location of GFP-LLA23 in leaf cells was confirmed by DAPI staining (Figure 3, D and E). These results clearly demonstrate that the NLS in LLA23 is necessary and sufficient for nuclear localization.

Discussion

The LLA23 protein of lily is a novel member of the water-deficit/ripening-induced protein family. The protein is unique in that it is pollen-specific (Wang et al., 1996), whereas the other members of the family are found in rip-

ening fruits and vegetative organs of various plant species (Iusem et al., 1993; Silhavy et al., 1995; Chang et al., 1996; Schneider et al., 1997). The LLA23 protein is developmentally regulated and accumulates immediately before anthesis (Wang et al., 1998). Changes in protein accumulation at the stage prior to anthesis during anther development have been described in various plant species (Mandaron et al., 1990; Michel et al., 1994; Wang and Cutler, 1995). However, no protein has been shown to be related to desiccation. We have demonstrated that the accumulation of both LLA23 RNA and protein responds to desiccation and many other environmental stresses (Wang et al., 1998; Huang et al., 2000).

There is no single and strict consensus NLS, but there are some general rules for NLSs (Garcia-Bustos et al., 1991). These include: (1) there are typically short sequences; (2) they contain a high proportion of positively charged amino acids; (3) they are not removed following nuclear localization. Previous analysis of LLA23 identified a putative NLS near the C-terminus of the protein (Figure 1A), and this finding prompts us to test whether this NLS indeed functions in nuclear targeting.

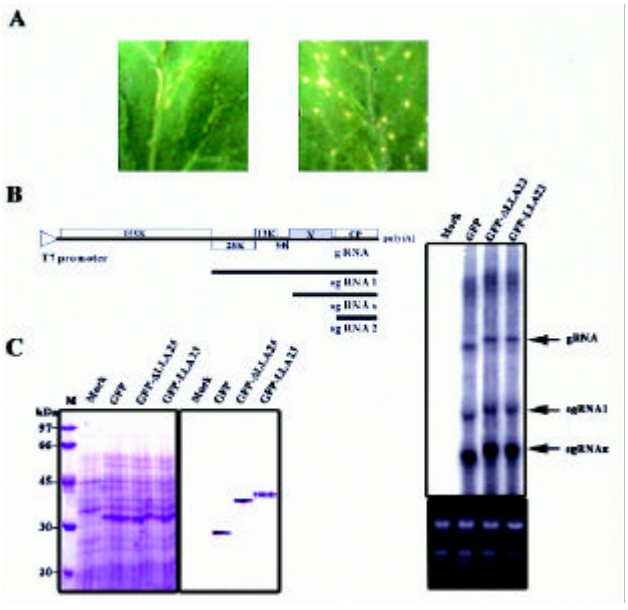

Figure 2. Biochemical analyses of leaves of *C. quinoa* infected by various BaMV constructs. A, The healthy leaves (left) of *C. quinoa* of 4-week-old plants and symptoms shown on 8-day BaMV-infected leaves (right); B, Detection of BaMV fusion RNA in infected leaves. The left-hand side shows a map of BaMV genomic RNA (gRNA) and subgenomic (sg) RNA1, RNA2 and RNAx. X indicates the GFP or fusion constructs. The BaMV gRNA encodes a number of viral proteins with molecular masses of 155, 28, 13 and 6 kDa and coat protein (CP). Total RNA was isolated from infected leaves by BaMV. RNA samples (3 µg) were denatured, fractionated on a formaldehyde-agarose gel, transferred to a nylon membrane (top), and hybridized to a ^{32}P-labeled GFP DNA. Nearly equal amounts of total RNA were loaded in each lane, as determined by ethidium bromide staining of the gel (bottom); C, Immunoblot detection of various GFP-LLA23 fusion proteins in BaMV-infected leaves. Equal amounts (3 µg) of leaf extracts prepared from infected leaves were electrophoresed by SDS-PAGE and either stained with Coomassie blue (left) or electroblotted onto nitrocellulose and immunologically detected using anti-GFP antiserum (right). M indicates molecular mass marker proteins (97, 66, 45, 30, and 20 kDa).

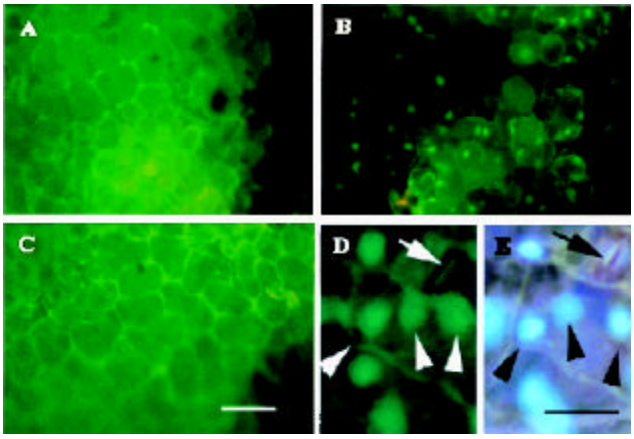

Figure 3. Requirement of the NLS in LLA23 for nuclear localization. Eight days after infection, leaves of *C. quinoa* were inspected by fluorescence microscopy. A-C, Images of subcellular localization of GFP, GFP-LLA23 and mutGFP-ΔLLA23 fusions, respectively; D-E, An infected leaf was first detergent-treated and then stained with DAPI. The locations of GFP-LLA23 (D) were exactly the same as those stained with DAPI (E). Arrows indicate the corresponding guard cells. Arrowheads indicate GFP in the nuclei. Scale bar represents 20 µm.

We generated a construct of GFP-LLA23 along with a mutGFP-ΔLLA23 which did not contain the NLS sequence of LLA23. GFP-LLA23 was localized predominantly to the nuclei of leaf cells (Figure 3B) whereas the mutGFP-ΔLLA23 resulted in exclusively cytoplasmic distribution of the fusion protein in leaf cells (Figure 3C). These results clearly demonstrate that the NLS in LLA23 is necessary and sufficient for nuclear localization. Similar results were also observed by confocal scanning laser microscopy (data not shown). To identify the critical basic amino acids for nuclear localization of LLA23, we have generated several mutant constructs by replacing alanine residues with lysines at the NLS in LLA23. The work of mutagenesis on NLS is in progress.

The presence of more than one NLS in nuclear proteins is apparently very frequent (Garcia-Bustos et al., 1991; Raikhel, 1992). However, a single NLS in the LLA23 protein is sufficient to direct GFP to the nucleus. It is striking that the overall bipartite NLS structure is conserved between monocots and dicots (Huang et al., 2000). Together with the reported presence of putative bipartite NLSs in a high proportion of plant b-ZIP proteins (Raikhel, 1992; Varagona et al., 1992), these observations suggest that the bipartite structure may be the most prevalent NLS configuration in plants, across a spectrum of divergent nuclear proteins.

The physiological function of LLA23 is yet unknown. Because it is abundant and hydrophilic, two characteristics similar to dehydrins (Close et al., 1993), we suggest that the LLA23 protein in pollen grains may play a role similar to late embryogenesis abundant (lea) proteins in seeds. The lea gene products are supposed to protect cellular structures from the deleterious effect of water loss. While lea proteins protect the cytoplasm from desiccation, LLA23 might do the same for the DNA in the nucleus. The Asr protein was reported to be in the nucleus (Iusem et al., 1993). Silhavy et al. (1995) also suggested that the function of the DS2 may be the protection of the nuclear DNA from desiccation (Silhavy et al., 1995). Using BaMV as a viral vector, we have clearly demonstrated that the protein contains a functional NLS and this NLS is necessary and sufficient for the import of the protein into the nucleus.

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百合與乾燥有關的花粉專一性蛋白質的入核序列是必需且充分的標入核內訊號

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百合 LLA23 蛋白質代表 water-deficit/ripening-induced 蛋白質家族中新的一員 (Plant Cell Physiol. 41: 477-485, 2000)。LLA23 蛋白質的 C 端有一鹼性區和置核序列 (NLS) 相類似。為了研究 LLA23 蛋白質的置核序列是否具有標的核的特性，將綠色螢光蛋白質基因和 LLA23 蛋白質的 C 半端基因銜接建構成 GFP-LLA23。同樣地，也建構單獨的 GFP 及刪除 LLA23 蛋白質基因置核序列而成的 GFP-ΔLLA23，分別插入竹嵌紋病毒載體中。此竹嵌紋病毒會感染白藜葉片而造成區域性壞疽的形成，其病毒產生的融合蛋白質可藉由螢光顯微鏡觀察綠色螢光。利用 RNA 和免疫轉漬法分析，顯示出感染的白藜葉片中重組的次基因組 RNA 和其所轉譯的蛋白質均能明顯的偵測到。螢光顯微鏡觀察的結果顯示，含有置核序列的 GFP-LLA23 融合蛋白質具有標的核的性質，在葉細胞內形成高度集中的綠色光點；而接種只有 GFP 的葉片，其綠色螢光顯然遍布在細胞質中。相反的，刪除置核序列後的 GFP-ΔLLA23，其融合蛋白質完全留在細胞質中。GFP-LLA23 融合蛋白質位於細胞核內可以染核的 4’,6-diamidino-2-phenylindole 加以證實。這些結果清楚的證明 LLA23 蛋白質的置核序列是必需的且能充分地將 LLA23 蛋白質標的到細胞核內。

關鍵詞：竹嵌紋病毒；乾燥；百合；置核序列；花粉專一性蛋白質。