Mutational analysis of the signal for a nuclear localization of proteins which accumulate specifically during meiosis in lily microsporocytes

Abstract LIM5 and LIM13 are novel meiosis-associated genes isolated from *Lilium longiflorum*. The presence of a hydrophobic N-terminal region predicted from the amino acid sequence has suggested that they function as extracellular structural components. However, both proteins also contain clusters of basic amino acids which may function as nuclear localization signals. To investigate the cellular localization of the protein, we tagged the C-termini of LIM5 and LIM13 with a green fluorescent protein. Transient expression of fusion proteins in onion epidermal cells revealed nuclear localization activity of both proteins. Mutational analysis indicated that amino acid sequences that constitute bipartite-type nuclear localization signals are necessary and sufficient for the intracellular localization of both proteins.

Key words Green fluorescent protein · Lily · Meiosis · Microprojectile bombardment · Nuclear localization

Abbreviations GFP: Green fluorescent protein · NLS: Nuclear localization signal

Introduction

One of the most effective approaches for understanding the molecular mechanisms involved in microsporogenesis is to isolate and characterize genes that are expressed specifically in meiocytes. Previously, 18 meiosis-associated cDNAs were isolated from microsporocytes of the monocotyledonous lily (*Lilium longiflorum*) and their corresponding genes designated as LIM genes (Kobayashi et al. 1994). Of their gene products, LIM15, thought to take part in meiosis-specific homologous chromosome recombination, is well characterized (Kobayashi et al. 1993; Terasawa et al. 1995). LIM cDNAs also contain novel clones which exhibit no homology to known genes and gene products. Among them, the deduced amino acid sequence of LIM5 and LIM13 show very interesting features in their predicted structures. First, they have an N-terminal hydrophobic region, which might serve as an extracellular targeting signal. Second, both contain clusters of basic amino acids which might function as nuclear localization signals (NLSs) (Raikhel 1992). These two contradictory features of LIM5 and LIM13 proteins prompted us to study their intracellular localization and function. To obtain information concerning the cellular targeting signals embedded within the amino acid sequence of LIM5 and LIM13, we carried out a series of experiments described in this study.

Transport of proteins into the nucleus is a tightly regulated process within eukaryotic cells and is mediated by NLSs. Entry of proteins into the nucleus through the nuclear pore complexes on the nuclear envelope requires hydrolysis of ATP or GTP (Sweet and Gerace 1995). Most NLSs can be categorized into three groups (Raikhel 1992). First, simian virus 40 large T-antigen-type NLS (PKKKRKV) contains a single basic amino acid region. Second, Mata2-type NLS (KIPIK) consists of short hydrophobic amino acids that contain one or more basic amino acids. Third, bipartite-type NLS from nucleoplasmin (KRPAAATKKAGQAKKKK) and many other NLSs which consist of two basic amino acid clusters separated by a spacer of typically ten amino acids. NLSs have been studied in a variety of plant proteins including plant pathogenic viruses (Carrington et al. 1991), *Agrobacterium* (Citovsky et al. 1992; Howard et al. 1992), the SV40 large T-antigen (van der Krol and Chua 1991), and transcription factors (van der Krol and Chua 1991;
Varagona et al. 1992, 1994; Shieh et al. 1993; Hiratsuka et al. 1994; Dehesh et al. 1995). In these studies, the subcellular localization of interested proteins was determined by translational fusion using Escherichia coli β-glucuronidase protein as a reporter molecule. Recently, the green fluorescent protein (GFP) of Aequorea victoria has been reported to be an alternative sensitive reporter molecule for intracellular localization of plant proteins (Pang et al. 1996; Grebenok et al. 1997). In this study, we identified targeting signals embedded within the novel gene products and examined their intracellular localization.

Materials and methods

Plasmid construction

Standard molecular cloning techniques were performed as described previously (Ausubel et al. 1990). The full-length LIM5 and LIM13 cDNA fragments and their deleted cDNA fragments were synthesized by PCR using the following primers:

LIM5::GFP and LIM13::GFP, 5'CCCCCCCCTCTAGATCAT-GAATACCCCTCTGCTCATCACTGTCGAC-3' and 5'-CCCGGATCCGAGTGACGGCGTTGGAATCACACAC-3';

LIM13-41::GFP, 5'-CCCTCTAGACCATTGGGCT-GAGCTTCCGGCTGCCGGA-3' and 5'-CCCGGATCCGGAGTGGCATCGTACACACAC-3';

LIM13-42::GFP, 5'-CCCTCTAGACCATTGGGCT-GAGCTTCCGGCTGCCGGA-3' and 5'-CCCCCGATCCGGAGTGGCATCGTACACACAC-3';

LIM13-43::GFP, 5'-CCCTCTAGACCATTGGGCT-GAGCTTCCGGCTGCCGGA-3' and 5'-CCCCCGATCCGGAGTGGCATCGTACACACAC-3';

Transformation of onion cells and microscopic observation

Onion epidermal cells were bombarded with 1.6-mg/ml particles coated with a few drops of DAPI solution (1 μg/ml). A cover glass was then placed on the samples for observation. Intracellular localization of GFP fusion proteins was observed using a Olympus BHT fluorescence microscope and photographed with a PM-10ADS camera (Olympus, Tokyo, Japan). For macroscopic observation of the GFP fluorescence, samples were observed directly with a Nikon SMZ-U-GFP stereoscopic microscope and photographed with a model H-III camera (Nikon, Tokyo, Japan).

Results and discussion

Nuclear localization of LIM5 and LIM13

The LIM5 and LIM13 cDNAs described previously (Kobayashi et al. 1994) are not full length and lack the 5' coding regions. We have recently isolated their 5' coding regions by the 5'-RACE method. Sequence analysis of these clones indicates that both LIM5 and LIM13 cDNAs have open reading frames encoding proteins of 139 amino acids and their amino acid compositions are 98% identical with each other (S.-i. Ogata, M. Minami, R. Hiratsuka, H. Takase, K. Hiratsuka, Y. Hotta, unpublished data).

Onion epidermal cells were used because their large size facilitated subcellular localization and provided a useful system for high-velocity microprojectile bombardment (Klein et al. 1987). Unlike the β-glucuronidase assay, GFP requires no substrate for detection and provides very clear cellular localization especially in onion epidermal cells. In addition, we could detect GFP fluorescent signals as early as 4 h after bombardment. These advantages enabled us to carry out a series of experiments within a relatively short time. Although GFP has been used as a reporter protein in both N- and C-terminal fusion, to investigate the intracellular localization of LIM5 and LIM13 proteins, we tagged the C-terminus of each protein with GFP because of the presence of a hydrophobic N-terminal region which might function as a signal peptide.

The stereoscopic observation shown in Fig. 1 clearly demonstrated the high efficiency of the transfection system and the specific cellular localization of GFP fluorescence. There are problems associated with the GFP tag strategy. The most critical point is that GFP is naturally dual-targeted to the cytosol and the nucleus, perhaps because its small size allowed passive diffusion through the nuclear pore (Grebenok et al. 1997). In our assay system, relatively strong GFP fluorescence was observed in the nucleus even without NLS, but the lack of a fluorescent signal in the cytosol was a good indi-

**Fig. 1** Macroscopic views of onion epidermal cells transfected with GFP (A) and LIM13::GFP constructs (B). Several transformed onion epidermal cells can be seen (bars 500 μm). A Accumulation of GFP protein is observed not only in the cytosol but also in the nucleus. B The green fluorescence derived from the LIM13-GFP fusion protein is confined to the nucleus.