Cloning and Characterization of Chloroplast and Cytosolic Forms of Cyclophilin from Arabidopsis thaliana*

(Received for publication, April 30, 1993, and in revised form, November 10, 1993)

Veronica Lippuner‡, Irene T. Chou‡, Sidney Varian Scott‡, William F. Ettinger‡*,
Steven M. Theg‡ and Charles S. Gasser‡‡

From the Sections of ‡Molecular and Cellular Biology, and ‡Plant Biology, Division of Biological Sciences,
University of California, Davis, California 95616

Cyclophilin (CyP), a protein with peptidyl-prolyl cis-trans isomerase (rotamase) activity, is the specific cellular target of cyclosporin A. We have isolated cDNA clones of two genes (designated ROC1 and ROC4) encoding CyP homologs from Arabidopsis thaliana (L.). The protein products of these genes are distinct from a previously identified Arabidopsis CyP, ROC1 is expressed in all tested plant organs and encodes a protein which is highly similar to previously described cytosolic CyP isoforms of other plants. In contrast, ROC4 is expressed only in photosynthetic organs and encodes a protein which includes an amino-terminal extension with properties of known chloroplast transit peptides. In vitro import experiments using the putative precursor protein to ROC4 showed that the protein is imported into chloroplasts where it is processed to the predicted mature size. Rotamase assays and immunoblot analysis of subcellular fractions indicate the presence of a CyP isoform in the stroma of chloroplasts but not in the thylakoid membranes or thylakoid lumen. Together, these data show that ROC4 is a novel CyP isoform which is located in the stroma of chloroplasts. In vitro chloroplast import of precursors of other chloroplast proteins was unaffected by concentrations of cyclosporin A which completely inhibit rotamase activity of chloroplast stromal CyP. Thus, this activity is not essential for protein import into chloroplasts.

Cyclophilin (CyP) is an abundant, highly conserved protein present in virtually all organisms (Koletsky et al., 1986). CyP was first identified as a high affinity binding protein for the immunosuppressive drug cyclosporin A (CsA) (Handschatcher et al., 1984). It has now been shown that the CyP-CsA complex suppresses the immune response through inhibition of the phosphoprotein phosphatase calcineurin (reviewed in Schreiber (1992) and Walsh et al. (1992)). A similar mechanism is responsible for CsA inhibition of recovery from α-factor arrest in yeast (Foor et al., 1992) demonstrating that this property of CyP is conserved in widely diverged organisms. Other naturally occurring ligands of CyP include a protein of unknown function identified in a mouse bone marrow-derived stromal cell line (Friedman et al., 1993), and the GAG protein of human immunodeficiency virus (Luban et al., 1993). However, the functional significance of these additional interactions remains unclear.

CyP has also been shown to be a peptidyl-prolyl cis-trans isomerase (“rotamase”) whose activity can be inhibited by CsA (Fischer et al., 1989; Takahashi et al., 1989). In vitro folding experiments have shown that isomerization around Xaa-Pro bonds is one of the slow, rate-limiting steps in the folding of many proteins (Brandts et al., 1975; Schmid and Baldwin, 1978; Lang et al., 1987) and that this process can be accelerated by the rotamase activity of CyP (Bächinger, 1987; Lang et al., 1987; Davis et al., 1989; Fischer et al., 1989). In vivo experiments support a physiological role of CyP rotamases in protein folding. Treatment of cultured fibroblasts with CsA slows the formation of collagen triple helix (Steinmann et al., 1991). Studies in transgenic flies show that the Drosophila NinaA protein (a membrane bound CyP homolog) is required for the folding of a subset of rhodopsin isoforms (Stamnes et al., 1991). CsA also slows an early step in transferrin folding in human hepatoma cells, and high concentrations (10 μM) block exit of this protein from the rough endoplasmic reticulum, suggesting that at this high concentration CsA inhibits correct folding of transferrin (Lodish and Kung, 1991).

Studies in higher plants have revealed the presence of cytosolic CyPs (Gasser et al., 1990; Marivet et al., 1992). Recently, Breiman et al. (1992) reported evidence of the presence of CsA-sensitive rotamase activity in plant subcellular fractions enriched in components of chloroplasts and mitochondria. However, the isolation of neither the corresponding proteins nor their genes has yet been reported. Because of the numerous advantages of Arabidopsis thaliana (L.) as an experimental organism (Meyerowitz, 1989) we have chosen to focus our efforts on the CyP genes of this plant. Here we describe the isolation of cDNA clones derived from two different genes encoding cytosolic and chloroplast stromal forms of CyP. We additionally provide evidence that the rotamase activity of the stromal form is not required for efficient import of proteins into chloroplasts.

EXPERIMENTAL PROCEDURES

Plant Material—A. thaliana (L.) Heynh. ecotypes Landsberg erecta and Columbia were grown at 20–25 °C under continuous fluorescent and incandescent light as described by Kranz and Kirchheim (1987). Peas (Pisum sativum cv. Progress #9) were grown as described by Ettinger and Theg (1991).
RNA Isolation and Characterization—Total RNA isolation and Northern blotting experiments were performed as described by Gasser et al. (1989).

Isolation of Arabidopsis CyP Clones—Approximately 50,000 plaques from a ZAP (Stratagene) cDNA library (a gift of J. Callis) of leaves from 3-week-old Arabidopsis ecotype Columbia were screened for cDNA clones encoding CyP. The screening was performed at low stringency (final wash = 2 x SSPE, 0.1% SDS at 55°C; 1 x SSPE = 150 mM NaCl, 10 mM NaH2PO4, 1 mM EDTA, pH 7.4) using the coding region of the Brassica napus CyP cDNA (Gasser et al., 1990) which had been 5'-labeled by random oligonucleotide priming (Feenbarg and Vogelstein, 1983). Standard plaque hybridization procedures were used (Bissis et al., 1980). Plasmid subclones were made from the isolated phage by coinfection with the helper phage M13K07 (Vieira and Messing, 1987) according to instructions supplied with ZAP by the manufacturer. The resulting plasmids carry the inserts in the EcoRI site of the vector plBluescript II (Stratagene). Three independent clones containing cDNA sequences encoding ROC1 were isolated. The clone with the longest insert, pCl1, was used for all subsequent experiments.

The ROC1 coding sequence was used to screen plates representing 2.4 x 107 plaques from an independently amplified aliquot of the same Arabidopsis leaf cDNA library (see above) at high stringency (final wash = 0.3 x SSPE, 0.1% SDS at 65°C). Twenty-seven independent clones were isolated and all contained the same size insert encoding ROC4. Plasmid subclones were prepared as described above, and one of the clones, designated pCl43, was selected for use in further experiments.

In Vitro Protein Synthesis and Chloroplast Import—Plasmids containing either a cDNA clone encoding the putative precursor of ROC4 (pre-ROC4), or the precursor of the small subunit of ribulose-1,5-bisphosphate carboxylase (pre-rbcS) were CoCl2 purified and linearized by restriction endonuclease digestion at sites 3' of the coding regions. Transcriptions from the linearized plasmids were performed using T3 (Stratagene Cloning Systems) or SP6 (Promega) RNA polymerase as described by Titus (1991). Precursor proteins were synthesized in the presence of [35S]methionine and [35S]cysteine in wheat germ cell-free lysate (Cline et al., 1985). Chloroplast import reactions were performed for 20 min (unless otherwise indicated) at 25°C as described by Ettinger and Theg (1991), except that 2 x 10^5 dpm of each radiolabeled precursor protein was used per 60 l of reaction. When no further treatments were required for loading import, chloroplasts were repurified by spinning through silicone oil into 1.5 ml perchloric acid (Theg et al., 1989). In some cases, after import and prior to repurification, the chloroplasts were treated with 200 µg/ml protease K (Boehringer Mannheim) for 25 min on ice, followed by inhibition of the protease reaction with 1 mM phenylmethylsulfonyl fluoride. Samples which were treated with Triton X-100 were first washed in an import buffer (10 mM Tris-HCl, pH 8.0, 0.33 M sorbitol) following the import reaction, and were then resuspended in 60 µl of IB containing 0.1% Triton X-100, 200 µg/ml protease K for 25 min on ice. These reactions were stopped by addition of 10% perchloric acid and an equal volume of 2 x SDS-PAGE sample buffer. Samples were separated by SDS-PAGE and visualized by fluorography (Theg et al., 1989).

RESULTS

Cloning cDNAs Encoding Arabidopsis CyP Proteins—Screening of an Arabidopsis leaf cDNA library with the coding region of a B. napus CyP cDNA clone (Gasser et al., 1990) resulted in the isolation of three independent clones encoding identical proteins. The gene from which these cDNAs derive was designated ROC1. The predicted sequence of the 172-amino acid ROC1 protein is compared to previously reported plant CyPs and to human cytosolic CyP in Fig. 1. ROC1 exhibits a high degree of sequence identity to the cytosolic CyPs of other plants, showing 81% identity to that of tomato (another dicot) and 78% identity to that of maize (a monocot). ROC1 is also 71% identical to human cytosolic CyP. Like these known cytosolic proteins, the ROC1 cDNA clones do not encode presequences that would provide information necessary for translocation of the protein out of the cytosol. As seen in the previously described plant CyPs, ROC1 includes a seven amino acid insertion beginning at position 47 relative to the human cytosolic and most other known CyP proteins (Fig. 1) (Gasser et al., 1990). On the basis of the sequence similarities and lack of apparent targeting information we conclude that ROC1 is a cytosolic form of CyP.

Subsequent screening resulted in isolation of cDNA clones encoding a unique CyP isoform. The gene from these cDNAs derive was designated ROC4. The predicted protein product of the ROC4 gene includes an extension of 91 amino acids amino-terminal to the region homologous to known cytosolic CyPs (Fig. 1). The sequence of the first 80

Chlorophyll and Protein Assays—Chlorophyll was assayed according to Arnon (1949). Protein assays were determined using the bichinchonic acid method (Smith et al., 1985). SDS-Polyacrylamide Gel Electrophoresis—SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using 15% acrylamide gels and the Laemmli (1970) buffer system.
### Arabidopsis Cyclophilins

| 1 | ROC4 | MALSSMOMY MTSTRSIAQIG PGKSVQLVSA KRTTQVCFG ARSSGIALS |
|---|------|--------------------------------------------------|
| 51 | ROC4 | RLYASAPIKQ FSGYATTHK QRTACVRSMA AEEEEVIEFP AKVTVKVYFD |
| ROC1 | MAFP.. .. |
| Tomato | MANP.F.. |
| Maize | MANPR.F.. |
| ATHCyc | MAHRF.. |
| Human | M.NFT.. .. |
| CON | FD |

**Fig. 1. Alignment of CyP protein sequences.** The amino acid sequences of ROC1 and ROC4 deduced from the corresponding cDNA sequences are aligned with the published sequences of tomato and maize CyP (Gasser et al., 1990), the previously isolated Arabidopsis CyP (ATHCyc) (Bartling et al., 1992), and human CyP A (Haendler et al., 1987). Only amino acids that differ from ROC4 are shown. Dots indicate amino acids which are identical to the ROC4 sequence, and dashes indicate gaps introduced to allow for optimal alignment of the sequences. The consensus (CON) sequence shows those positions where all six sequences are identical. Single-letter codes for amino acid residues are used and stars indicate termination codons for translation.

In addition to the presence of the amino-terminal extension, the product of the ROC4 gene shows other significant differences from previously described plant CyPs. ROC4 lacks the seven amino acid insertion characteristic of the other plant CyPs, and in this respect is more similar to human cytosolic CyP (Fig. 1 and see above). However, ROC4 shares only 66% sequence identity with human cytosolic CyP, and is thus less similar to this protein than are the plant cytosolic CyPs. On the basis of the presence and nature of its amino-terminal extension and the sequence of the putative mature protein, pre-ROC4 represents a novel class of plant CyP which has not been previously described.

#### Spatial Distribution of ROC1 and ROC4 Gene Expression

To determine the pattern of expression of ROC1 and ROC4 in mature plants, northern blots of root, leaf, and flower total RNA were hybridized with sequences from each of the cDNA clones (Fig. 2). Analysis of previously identified higher plant cytosolic CyP genes showed that these genes are expressed at relatively high levels in all tested parts of plants (Gasser et al., 1990; Marivet et al., 1992). mRNA from ROC1 was readily detected in all tested organs of Arabidopsis plants (Fig. 2A), consistent with ROC1 being the Arabidopsis homolog of the previously reported plant cytosolic CyPs. In contrast, while ROC4 mRNA was readily detected in RNA from leaves and flowers, no mRNA was detected in root RNA (Fig. 2B). Leaves are the primary photosynthetic organs of Arabidopsis, and Arabidopsis flowers also include leaf-like photosynthetic sepals. Thus, the observed expression pattern is consistent with a role for ROC4 in chloroplasts. The fact that the ROC1 and ROC4 probes detected different sets of bands demonstrates that the conditions used provided for gene specific hybridization.

**CyP Is Present in the Stroma of Chloroplasts**—The presence of a putative chloroplast transit peptide in the predicted ROC4 protein product prompted us to investigate the presence and localization of CyP in chloroplasts. Rotamase activity in pea chloroplast fractions was assayed using a coupled assay with chymotrypsin first developed by Fischer et al. (1984a, 1984b). Chloroplast stromal extracts were shown to contain detectable rotamase activity which showed a linear relationship with the amount of stroma added (Fig. 3). The rotamase activities of all
clones. The migration of the hybridizing band in comigration of one of the chloroplast ribosomal RNAs. Approximate experimental Procedures.

 FIG. 2. Northern blots hybridized with ROC1 and ROC4 cDNA clones. Total RNA (20 μg/lane) from Arabidopsis roots (lane 1), flowers (lane 2), and leaves (lane 3) were hybridized as described under "Experimental Procedures." A, blot hybridized with a 332-base pair BglII-BamHI fragment of the protein coding region of the ROC1 cDNA clone. B, blot hybridized with the insert (938 base pairs) of the ROC4 cDNA clone. The migration of the hybridizing band in lane 3 is altered due to comigration of one of the chloroplast ribosomal RNAs. Approximate sizes of hybridizing bands are indicated to the left in bases. The final wash for both blots consisted of 0.5 x SSPE, 0.1% SDS at 65 °C. Blots were exposed for (A) 2 days and (B) 4 days. Numbers to the left of the figures indicate the sizes of the hybridizing bands in bases.

 FIG. 3. Effect of CsA on rotamase activity of chloroplast stromal extracts. Rotamase activity assays of the stroma of chloroplasts were performed as described under "Experimental Procedures." Rotamase activity is shown as a function of stromal protein quantity (μg) in the presence (○) or absence (●) of 10 μM CsA. Assays were performed at least in duplicate for each concentration tested.

 known eukaryotic CyPs and some prokaryotic CyPs are inhibited by CsA (Herler et al., 1992; Walsh et al., 1992). Addition of CsA (10 μM) to the stromal extract abolished the rotamase activity dropping the rate into the range of the uncatalyzed reaction (Fig. 3). These results demonstrate the presence of a CyP homolog in the chloroplast stroma in agreement with previous findings (Breiman et al., 1992).

 Material from the thylakoid lumen was also analyzed for rotamase activity in the thylakoid membrane fraction because chlorophyll associated with these membranes interferes with the rotamase assay.

 To further determine the intracellular localization of CyP, immunoblot analysis was performed using antiserum raised against ROC1. Anti-ROC1 serum reacted strongly with purified ROC1 (Fig. 4A, lane 2) and cross-reacted with recombinant ROC4 obtained from solubilized inclusion bodies (Fig. 4A, lane 3). It also detected recombinant cytosolic tomato CyP (data not shown; Gasser et al. (1990)) and even reacted weakly with E. coli CyP (data not shown; Liu and Walsh (1990)) indicating cross-reactivity to a broad range of CyP isoforms. Immuno- staining of total Arabidopsis (Fig. 4A, lane 1) and total pea (Fig. 4A, lane 4) extracts showed at least three cross-reacting bands ranging from 18 to 20 kDa, consistent with the sizes of known CyP proteins. Pea chloroplast extract contained a single immunoreactive band (Fig. 4A, lane 5) which comigrated with recombinant ROC4 and the 20-kDa immunoreactive band in both total pea and total Arabidopsis extracts.

 To determine the intramembrane location of ROC4, chloroplast stroma, thylakoid membrane, and thylakoid lumen fractions representing equal amounts of chloroplasts were reacted with anti-ROC1 serum (Fig. 4A, lanes 6–8). An immunoreactive protein was detected in the stromal fraction (Fig. 4A, lane 6), but not in thylakoid membrane or thylakoid lumen fractions (Fig. 4A, lanes 7 and 8). The chloroplast stromal CyP comigrated with the immunoreactive band in total extract of pea chloroplasts and was of equal intensity. Thus, the CyP protein detected in pea chloroplasts is located in the stroma.

 ROC4 Precursor Protein Is Imported into Chloroplasts—Transit peptides of chloroplast proteins are necessary and sufficient for targeting these proteins to chloroplasts. Since this targeting reaction is organelle-specific, import of pre-ROC4 into chloroplasts would prove that ROC4 is a plastid-resident protein. Incubation of radiolabeled pre-ROC4 (Fig. 5, lane 1) with freshly isolated pea chloroplasts followed by repurification of the chloroplasts resulted in a decrease in size of the majority of the pre-ROC4 band to that predicted for the mature form of this protein (Fig. 5, lane 2). Control experiments carried out with the well characterized precursor of the small subunit of ribulose-1,5-bisphosphate carboxylase (pre-rbcS) showed identical results (Fig. 5, lanes 5 and 6).

 Protease digestions were performed to demonstrate that the processed form of ROC4 was inside the chloroplasts. Following import, all residual precursor was eliminated by digestion with proteinase K, but the processed protein was protected from digestion (Fig. 5, lane 3). In contrast, when Triton X-100, which lyses chloroplasts, was included during the protease digestion both the residual precursor and processed ROC4 were digested.
**Abbildung**

**Fig. 5.** In vitro chloroplast uptake of pre-ROC4. 3H-Labeled pre-ROC4 (lane 1) and pre-rbcS (lane 5) were produced by in vitro transcription and translation as described under "Experimental Procedures." These precursor proteins were incubated with chloroplasts (20 μg of chlorophyll per reaction) for 20 min at 25 °C as described under "Experimental Procedures." After the incubation period, chloroplasts were either repurified immediately (lanes 2 and 6), or treated with 200 μg/ml proteinase K in the absence (lanes 3 and 7), or in the presence (lane 4) of 0.1% Triton X-100 for 25 min on ice and then processed as described under "Experimental Procedures." Proteins were resolved on 15% SDS-polyacrylamide gels and visualized by fluorography. The positions of the precursor (pr) and mature (m) forms of ROC4 (left) and rbcS (right) are indicated. Molecular masses (in kDa) and relative positions of protein standards are indicated on the right.

(Fig. 5, compare lanes 3 and 4). Thus, the mature-sized ROC4 was protected inside the chloroplasts. We conclude that pre-ROC4 was competent for import into chloroplasts.

**Rotamase Activity Is Not Required for Chloroplast Protein Import**—To investigate whether rotamase activity is necessary for efficient import into chloroplasts, in vitro import reactions were carried out in the presence of CsA, which is known to penetrate readily through membranes (Schreiber, 1992). 10 μM CsA added to an import reaction containing pre-rbcS did not significantly decrease the rate of protein import (Fig. 6). This concentration of CsA was sufficient to eliminate activity of the chloroplast stromal CyP (Fig. 3), as well as activity of cytosolic CyP rotamases carried over from the wheat germ translation system used to produce the precursor proteins (data not shown). Similar results were obtained with the precursor to the 33-kDa protein of the oxygen evolving complex, a thylakoid lumen protein (data not shown). This indicates that CyP rotamase activity is not essential for efficient import of these two proteins.

**DISCUSSION**

CyP homologs have been identified in the cytoplasm of bacteria (Hayano et al., 1991) and in the cytosol of animals (Handschumacher et al., 1984), fungi (Tropschug et al., 1988; Haendler et al., 1989) and higher plants (Gasser et al., 1990; Marivet et al., 1992). Toward our goal of defining the rotamase activities and the in vivo roles of CyPs in plants, we have isolated cDNAs for two CyPs from Arabidopsis. Based on amino acid identity, presence of a seven amino acid insertion that is found in other plant cytosolic CyPs (Fig. 1), absence of NH$_2$- or COOH-terminal extensions which are usually necessary for translocation of proteins out of the cytosol, and constitutive high level expression, we conclude that ROC1 is the major cytosolic CyP in this species. Bartling et al. (1992) recently described the isolation of a cDNA clone, designated ATHCYC, encoding a CyP from Arabidopsis. While ROC1 has ~80% amino acid identity to other plant cytosolic CyPs, ATHCYC has only ~70% identity to ROC1 or to the previously described plant CyPs (Fig. 1). Thus, ATHCYC is more diverged from all previously described monocot and dicot cytosolic CyPs (including ROC1), than the previously described proteins are from each other (Gasser et al., 1990). In fact, ATHCYC is as different in sequence from ROC1 and the other known plant cytosolic CyPs as is human CyP A (~70% amino acid identity). ATHCYC, therefore, appears to represent a member of a class of plant CyPs that has not previously been described. No information on the pattern of expression of this gene is yet available. However, because ATHCYC lacks apparent targeting information it may represent an additional cytosolic CyP in Arabidopsis.

Protein folding occurs in several subcellular compartments in addition to the cytosol. Consistent with an important role for CyP in this process, members of the CyP family have been found in the endoplasmic reticulum (Friedman and Weissman, 1991; Hasel et al., 1991; Price et al., 1991; Stannes et al., 1991) and mitochondria (Tropschug et al., 1988; Walsh et al., 1992) of different eukaryotic species, and in the periplasm of E. coli (Liu and Walsh, 1990). The chloroplast is a unique subcellular compartment which is present in all photosynthetic eukaryotes. While chloroplasts have their own genomes, most chloroplast proteins are encoded in the nucleus, synthesized in the cytosol, and imported post-translationally into the organelle (Theg and Scott, 1993). Targeting information is contained on an aminoterminal extension called a transit peptide.

ROC4 is expressed only in parts of the plant containing photosynthetic tissues, and encodes an NH$_2$-terminal extension having an amino acid composition consistent with chloroplast transit peptides. We have demonstrated the presence of CyP in the stroma of chloroplasts by enzyme activity assays and immunoblot analysis. These results support the previous observation of CsA-sensitive rotamase activity in chloroplast extracts (Breiman et al., 1992). Our results differ from those of Breiman et al. in that we were unable to detect immunoreactive CyP in the thylakoid fraction. Because the purity of their fractions was not assessed, stromal contamination could account for the observed thylakoid activity. Regarding our immunoblot experiments, the possibility remains that a thylakoid CyP may be too diverged in sequence to be recognized by our CyP antiserum. The ability of pre-ROC4 to enter the chloroplasts in vitro import assays, together with the finding that the major immunoreactive CyP in chloroplasts is in the stroma, demonstrate that ROC4 is a novel CyP which functions in the stroma of chloroplasts.

della-Cioppa and Kishore (1988) obtained evidence indicating that proteins must undergo a change in conformation to be translocated across the chloroplast envelope membranes. Molecular chaperones are likely to act as catalysts for import (Waegemann et al., 1990; Theg and Scott, 1993). Given the known function of CyP in the protein folding process, we questioned whether, like other chaperones, the rotamase activity of CyP might facilitate the import process. Addition to import reactions of CsA sufficient to inhibit both cytosolic and chloroplast stromal CyPs did not block uptake of the tested chloro-
plast precursors, demonstrating that the rotamase activity is not essential for efficient translocation of these proteins across the chloroplast envelope. However, these experiments do not preclude an in vivo role of CyP rotamase activity in the chloroplast stroma. The rotamase activity may be required for import of other proteins not tested here, or for proper folding and assembly of proteins inside the plastid.

Acknowledgments—We thank Eric P. Beers for helpful discussions, Robert F. Standaert and Lynne D. Zydowsky for gifts of plasmids, Judy Callis for the Arabidopsis leaf cDNA library and revision of the manuscript, and Victoria A. Vucich for helpful comments. The gift of radiolabeled leucine for in vitro translation reactions from Du Pont Company is gratefully acknowledged.

REFERENCES

Arnon, D. I. (1949) Plant Physiol. 24, 1-15

Biecheler, H. P. (1987) J. Biol. Chem. 262, 17144-17148

Berrill, D., Heene, A., and Weiler, E. W. (1992) Plant Mol. Biol. 19, 529-530

Beers, E. P., Moreno, T. N., and Callis, J. (1992) J. Biol. Chem. 267, 15432-15439

Brandts, J. F., Halvorsen, H. R., and Brennan, M. (1975) Biochemistry 14, 4953-4963

Breitman, A., Fawcett, T. W., Ghirardi, M. L., and Matteo, A. K. (1992) J. Biol. Chem. 267, 21293-21296

Cline, R., Werner-Washburne, M., Lubben, T. H., and Keegstra, K. (1985) J. Biol. Chem. 260, 3691-3696

Crouse, G. F., Frischauf, A., and Lehrach, H. (1983) Methods Enzymol. 101, 78-89

Davis, J. M., Boswell, B. A., and Bachinger, H. P. (1985) J. Biol. Chem. 260, 8906-8912

Davis, R. W., Botstein, D., and Roth, J. R. (1980) Advanced Bacterial Genetics, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

della-Cioppa, G., and Kishore, M. (1988) EMBO J. 7, 1299-1305

Fetters, W. F., and The, T. M. (1981) J. Cell Biol. 115, 312-318

Feinberg, A. P., and Vogelstein, B. (1983) Anal. Biochem. 132, 6-13

Fischer, G., Bang, H., Berger, B., and Schellenberger, A. (1984a) Biochem. Biophys. Acta 781, 87-97

Fischer, G., Bang, H., and Mech, C. (1984b) Biochim. Biophys. Acta 43, 1011-1111

Fischer, G., Wittmann-Liebold, B., Lang, K., Kaechabut, T., and Schmid, F. X. (1989) Nature 337, 476-478

Foor, F., Parent, S. A., Morin, N., Dahl, A. M., Ramadan, N., Chrebet, G., Bestian, K. A., and Nielsen, J. B. (1993) Nature 369, 882-894

Friedman, J., Trahey, M., and Weissman, I. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 6815-6819

Friedman, J., and Weissman, I. (1991) Cell 66, 799-806

Gasser, C. S., Budelser, K., Smith, A. G., Shah, D. M., and Fraley, R. T. (1989) Plant Cell 1, 15-24

Gasser, C. S., Gunning, D. A., Budelser, K. A., and Brown, S. M. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 8519-8523

Haendler, B., Hofer-Warbinek, R., and Hofer, E. (1987) EMBO J. 6, 947-950

Haendler, B., Keller, R., Hiestand, P. C., Kocbor, H. P., Wegmann, G., and Marva, N. R. (1989) Gene 83, 39-46

Handschumacher, R. E., Harding, M. W., Rice, J., Druge, R. J., and Speicher, D. W. (1984) Science 226, 544-546

Harlow, D., and Lane, D. (1988) Antibodies: A Laboratory Manual, pp. 61-63, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

Hase, P. W., Glass, J. R., Godbeet, M., and Sutcliffe, J. G. (1991) Mol. Cell. Biol. 11, 3484-3491

Hayano, T., Takaishi, N., Kato, S., Maki, N., and Suzuki, M. (1991) Biochemistry 30, 5041-5048

Herrler, M., Bang, H., Brune, K., Fischer, G., and Marshals, M. A. (1992) FEBS Lett. 309, 231-234

Kolotyko, A. J., Harding, M. W., and Handschumacher, R. E. (1986) J. Immunol. 137, 1054-1059

Kranz, A. R., and Kirchheim, R. (1987) Arabidopsis Inf. Serv. 24

Kunkel, T. A. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 488-492

Laemmli, U. K. (1970) Nature 227, 680-685

Lang, K., Schmidt, F. X., and Fischer, G. (1987) Nature 329, 288-290

Liu, J., and Walsh, C. T. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 4028-4032

Ludish, H. F., and Kong, N. (1991) J. Biol. Chem. 266, 14835-14838

Luban, J., Bosshart, R. L., Franke, E. K., Kalpana, G. V., and Gooff, S. P. (1993) Cell 73, 1067-1076

Marivet, J., Frendo, P., and Burkard, G. (1992) Plant Sci. 84, 171-178

Meyerowitz, E. M. (1985) Cell 55, 263-269

Price, R., Zydowsky, L. D., Jim, M., Baker, C. H., McKeon, F. D., and Walsh, C. D. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 1903-1907

Sanger, F., Nicklen, S., and Coulson, A. R. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 5463-5467

Schmid, F. X., and Baldwin, R. L. (1978) Proc. Natl. Acad. Sci. U. S. A. 75, 4764-4768

Schreiber, S. L. (1992) Cell 70, 365-368

Standaert, F. R., Bruckner, P., and Superti-Furga, A. (1993) J. Biol. Chem. 268, 21293-21296

Tai, M., Kroc, R. L., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olsen, B. J., and Klenk, D. C. (1985) Anal. Biochem. 150, 76-85

Tamura, M. A., Shish, B. H., Chuman, L., Harris, G. L., and Zuker, C. S. (1991) Cell 65, 219-227

Standzaer, R. F., Galat, A., Verdin, G. L., and Schreiber, S. L. (1990) Nature 346, 671-674

Steinmann, B., Bruckner, P., and Superti-Furga, A. (1991) J. Biol. Chem. 266, 1299-1303

Studer, F. W., Rosenberg, A. H., Dunn, J. J., and Dubendorff, J. W. (1999) Methods Enzymol. 328, 60-69

Takashahi, N., Hayano, T., and Suzuki, M. (1989) Nature 337, 473-475

Theg, S. M., and Scott, S. V. (1993) Trends Cell Biol. 3, 186-190

Theg, S. M., Bauerle, C., Olsen, L. J., Selman, B. R., and Keegstra, K. (1989) J. Biol. Chem. 264, 6730-6736

Titus, D. E., Ed. (1991) Promega Protocols and Applications Guide, p. 59, Promega Corporation, Madison, WI

Tropashch, M., Nicholson, D. W., Hartl, F. U., Kohler, H., Pfannen, W., Wachter, E., and Neupert, W. (1990) J. Biol. Chem. 263, 14433-14440

Vieira, J., and Messing, J. (1987) Methods Enzymol. 153, 3-11

von Heijne, G., Stemhel, J., and Herrmann, R. G. (1989) Eur. J. Biochem. 180, 535-545

Waegegmans, K., Paulsen, H., and Sol, J. (1990) FEBS Lett. 216, 89-92

Walsh, C. T., Zydowsky, L. D., and McKeon, F. D. (1992) J. Biol. Chem. 267, 13115-13118