The peptide HDEF as a new retention signal is necessary and sufficient to direct proteins to the endoplasmic reticulum

Karin Kaletta[a], Irene Kunze[b], Gotthard Kunze[b], Margret Köck[a,*]

[a] *Martin-Luther-Universität Halle-Wittenberg, Biozentrum, Weinbergweg 22, D-06120 Halle, Germany*
[b] *Institut für Pflanzengenetik und Kulturpflanzenforschung, Corrensstr. 3, D-06466 Gatersleben, Germany*

Received 6 July 1998; revised version received 31 July 1998

Abstract The key feature of tomato RNase LX localised solely outside the vacuole is the C-terminal peptide HDEF which is very similar to known endoplasmic reticulum (ER) retention signals. For functional testing of the ER-targeting ability of HDEF, different constructs including the complete RNase LX, two truncated forms without HDEF and the truncated chitinase FB7-1ΔVTP C-terminally flanked by HDEF, were expressed in *Saccharomyces cerevisiae*. The majority of RNase and chitinase, both containing HDEF, accumulates within the ER. However, the truncated constructs without the peptide are released into the medium. We provide compelling evidence that peptide HDEF at the C-terminus of secretory plant proteins is a new ER retention signal in yeast and most likely in plants.

Key words: Chitinase; Endoplasmic reticulum and retention; Heterologous expression; Ribonuclease; *Saccharomyces cerevisiae*; Tomato

1. Introduction

The secretory pathway provides a common route for both proteins destined to be secreted and proteins that reside in the various organelles of the pathway itself. The ability to discriminate between different protein populations requires specific recognition and sorting mechanisms to ensure correct compartmental localisation along this pathway [1]. One of the largest organelles and the first in the secretory pathway of eukaryotic cells is the endoplasmic reticulum (ER) [2]. Proteins that reside in the ER carry specific C-terminal retention signals. It has been shown that both KDEL and HDEL peptide motifs retain proteins in the ER of animal cells as well as of yeast (*Saccharomyces cerevisiae*) and plant cells [3–5]. Detailed studies using reporter proteins fused to different peptide extensions have revealed that the amino acids EL (Glu-Leu) seem to be essential for ER localisation [6–8]. But very recently, a Ca^{2+}-binding protein from mouse heart was isolated that is located in the ER and that carries as its most C-terminal amino acid phenylalanine instead of the leucine residue [9].

Retention of ER-resident proteins is thought to be the result of a continuous recycling process (retrograde transport) that is mediated by specific receptors, regulatory proteins and vesicles [10]. Fundamental processes associated with the endomembrane system are conserved between plants, mammals and yeasts. For many biological questions it is therefore possible to switch from one model system to another and conduct complementary experiments [11].

*Corresponding author. Fax: (49) (345) 552 7230.
E-mail: koeck@biozentrum.uni-halle.de

In cultivated tomato cells and plants (*Lycopersicon esculentum* Mill.), two RNase genes were characterised that are induced after the onset of phosphate starvation [12–17]. Cell fractionation and localisation studies revealed that the main intracellular enzyme activity, designated RNase LX, was exclusively found in microsomal fractions and was excluded from isolated and highly purified vacuoles [14]. The cDNA sequence contains an N-terminal secretory signal sequence of 24 amino acids conferring the co-translational transport into the endoplasmic reticulum [16]. By comparing the RNase LX protein sequence with those of homologous, but secreted RNases, it became obvious that RNase LX carries a unique sequence STNDDHDEF at its C-terminus [16]. Surprisingly, the most terminal amino acids HDEF are very similar to known ER retention signals. Taking into account the localisation of RNase LX in microsomal fractions, we suggest that the C-terminal peptide sequence can act as an ER retention signal. To prove this assumption we expressed different reporter protein constructs with and without the putative C-terminal signal peptide in yeast (*S. cerevisiae*) and analysed the final intra- or extracellular destination of protein constructs.

2. Materials and methods

2.1. Cultivation of yeast strains

S. cerevisiae strain INVSC2 (*MATa his3-D1 ura3-52*, Invitrogen) was cultivated in SD medium with either 1% D-glucose or 1% D-galactose at 30°C [18]. For induction experiments cells grown on SD medium with D-glucose for 24 h were harvested by sedimentation and resuspended in SD medium supplemented with D-galactose for 4–144 h.

2.2. Construction of vectors and transformation procedure

Using the RNase LX gene described by [16] and the chitinase gene FB7-1 [19] as templates, modified or truncated constructs were amplified by in vitro mutagenesis (Fig. 1). To amplify RNase sequences the sense primer was used in combination with different reverse primers resulting in the wild-type RNase construct LX and those with modified C-terminal sequences, designated LXΔ4 and LXΔ9, missing the last four and nine amino acids, respectively. Oligonucleotides are given in the 5′ to 3′ direction (restriction enzyme sites are written in bold, initiation and stop codons are underlined):

LXs	**AAGCTT**ATGAAATCTCAAAAAAAATTGTTG;
LXas	**GGATCC**TTAAAATTCATCATGGTCATC;
LXΔ4as	**GGATCC**TTACTATTTGTGGAGAAA;
LXΔ9as	**GGATCC**TTAGAAAGAAGGGAATTCAAT.

To synthesise the truncated chitinase construct FB7-1ΔVTP+HDEF, the sense primer CG**GGATCC**CGGGATGAGGCTTTGTAAATTCACAGCT and the antisense primer TTAAAATTCATCATGATTTCCAAAAGACCTCTGGTTGCC were used. The antisense primer is homologous to C-terminal sequences preceding the vacuolar targeting peptide (VTP) and is fused to 12 nucleotides

PII: S0014-5793(98)01013-8

which encode the tetrapeptide sequence HDEF, followed by stop codon TAA.

The amplification consisted of 30 cycles of 95°C for 30 s, 56°C for 30 s, and 72°C for 90 s using one unit of *Taq* DNA polymerase (Invitrogen), 50 pmol of each primer and the buffer recommended by the manufacturer. Products were ligated into vector pCR2.1 (Invitrogen). Transformants were proven for correct amplification by DNA sequencing. For the heterologous expression of plant proteins in *S. cerevisiae* the yeast-*Escherichia coli* shuttle vector pYES2 (Invitrogen) was chosen. Using the appropriate restriction enzyme sites, *Hin*dIII/*Bam*HI and *Bam*HI/*Xho*I, the modified PCR constructs of RNase LX and chitinase, respectively, were ligated between the inducible promoter *GAL1* and terminator *CYC1* sequences of pYES2 that drives protein expression after induction with D-galactose in *S. cerevisiae*. The yeast strain INVSC2 was transformed according to [20]. All other experimental procedures were done according to standard recombinant DNA techniques [21].

2.3. Cell fractionation

For fractionation of yeast cell homogenates the method described in detail in [19] was used. Briefly, after incubation of cells with lysing enzyme from *Trichoderma harzianum* (Sigma), the emerging spheroblasts were resuspended in 0.4 ml 1.8 M sorbitol. Gentle lysis was achieved by adding 3.2 ml of chilled 10 mM Tris-HCl, pH 7.5. After centrifugation at $1000 \times g$ for 5 min the resulting supernatant (3.5 ml) was loaded onto the top of a continuous 15–55% sucrose density gradient (10 ml). Centrifugation was carried out at $100\,000 \times g$ for 2.5 h using the SW41Ti rotor (Beckman). 0.5 ml fractions were collected.

2.4. Enzyme assays, electrophoresis and RNase activity staining

In fractions of sucrose density gradients the marker enzymes α-mannosidase (vacuole) and cytochrome *c* reductase (ER) were assayed according to [19], alcohol dehydrogenase (cytosol) was measured following [22]. Using RNA as substrate, RNase activity was estimated from the release of ethanol-soluble A_{260} materials, as described previously [23]. The enzyme unit is defined as the amount of enzyme causing an increase in absorbance at 260 nm of 1.0/ml. Protein concentration was measured using the protein dye assay (Bio-Rad, Germany) with bovine serum albumin as standard.

Electrophoresis under denaturing conditions on 12% slab gels containing 2.4 mg/ml RNA, removal of SDS by isopropanol treatment and detection of RNase activity were carried out as described [24].

2.5. Antibodies and Western blotting

Protein probes were separated on 12% SDS-PAGE and transferred onto nitrocellulose sheets using the semi-dry blotting procedure. Successful protein transfer to membranes was visualised by staining with Ponceau solution. Using standard procedures membranes were incubated with anti-RNase, anti-chitinase or anti-SEC61 antibodies for 1 h followed by incubation with anti-rabbit IgG antibody and ECL immunodetection (Amersham) of protein blots [25]. Production of anti-chitinase antiserum has been recently described [19], anti-SEC61 antiserum was obtained from Dr. S. Panzner (University of Halle). For production of polyclonal antiserum against RNase LX the 14 kDa C-terminal part which was synthesised as GST fusion protein (pGEX vector, Pharmacia) was used to immunise rabbit.

3. Results and discussion

3.1. The peptide HDEF is necessary to retain plant ribonuclease LX in the endoplasmic reticulum of yeast cells

Ribonuclease LX harbouring a unique nonapeptide on its C-terminus whose last four amino acids HDEF are similar to known ER retention signals is found in microsomal fractions of cultivated tomato cells. To analyse the influence of C-terminal amino acids on the targeting of RNase LX we expressed the wild-type RNase LX gene and truncated constructs missing the last four (LXΔ4) or nine amino acids (LXΔ9) in the heterologous yeast system (*S. cerevisiae*) under the control of the inducible *GAL1* promoter [26]. To target constructs into the lumen of the endoplasmic reticulum the endogenous signal peptide of RNase LX was used.

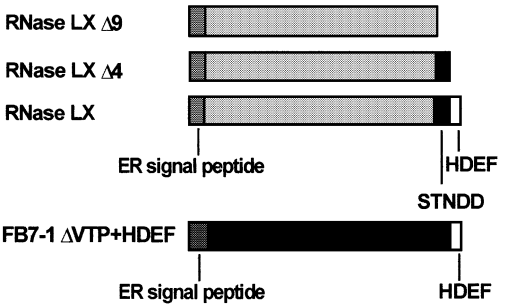

Fig. 1. Schematic representation of chimeric RNase LX and chitinase gene constructs with endogenous N-terminal signal sequences and modified C-terminal targeting information.

Heterologous expression of protein constructs does not influence growth parameters of yeast cells. As a control, strain INVSC2 transformed with the expression vector pYES2 was grown in D-glucose-containing SD medium. Growth was calculated from the increase in A_{600} for 96 h. The transformant LX cultivated in the same medium for 24 h was transferred either to fresh medium or to D-galactose-containing medium in which expression of RNase LX is induced. Both of the cultures show similar growth rates and reach almost the same cell density in the stationary phase. RNase LX protein from plant cells and from the yeast transformant LX migrates identical in SDS-PAGE (data not shown). This result indicates that the N-terminal ER signal sequence of RNase LX is functional in yeast and is properly cleaved off. Although the expression system (vector pYES2) has been described as tightly repressed by glucose [26], a small amount of RNase activity was already expressed in cells cultivated in D-glucose-supplemented SD medium. This leakage of *GAL1* promoter activity did not interfere with our experiments. The highest expression was found after 24 h of induction with D-galactose. Therefore, cells harvested at this time point were chosen for further experiments.

Expressing RNase constructs LX, LXΔ4 and LXΔ9 in yeast cells, we compared the distribution of total RNase activity between cell extracts and medium probes. 78% of the total RNase activity was found in the cell homogenate of construct LX (Fig. 2) whereas the remaining activity was found in the cultivation medium. When the truncated RNase LXΔ4 and

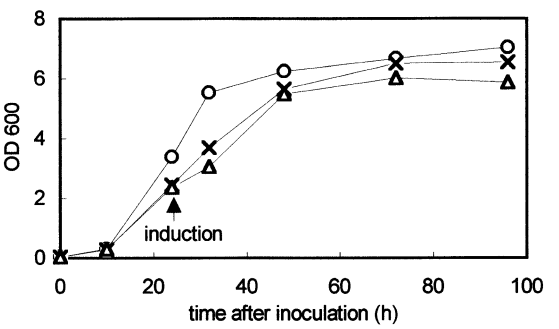

Fig. 2. Comparison of the relative distribution of RNase activity in cell extracts (□) and in adequate medium probes (■) of the constructs LX, LXΔ4 and LXΔ9 after 24 h induction by D-galactose. Results are means ± S.D. ($n = 6$).

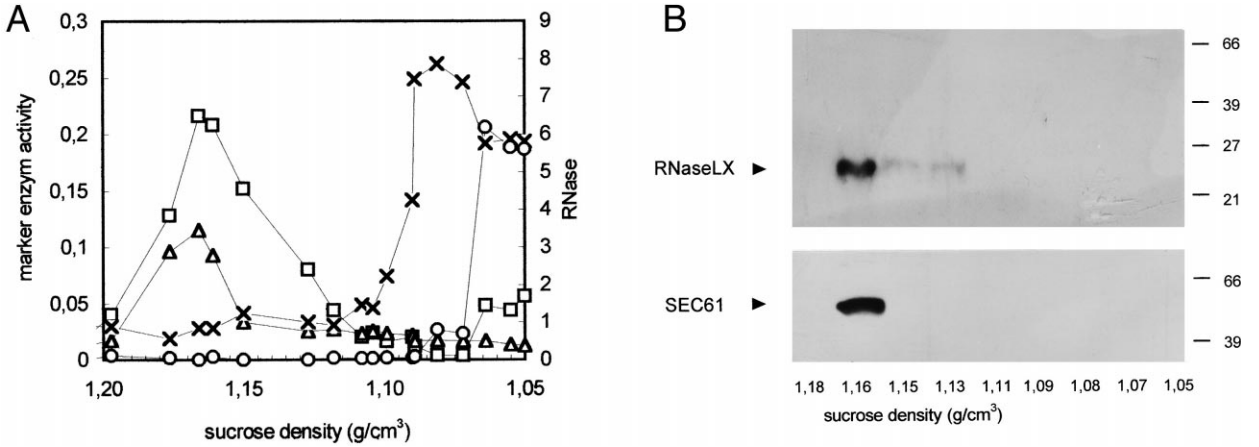

Fig. 3. Subcellular localisation of the complete, wild-type RNase LX in *S. cerevisiae*. A: Determination of marker enzymes and RNase activity in sucrose gradient fractions of yeast transformant LX. After centrifugation sucrose gradients were separated into 0.5 ml fractions and assayed for cytochrome *c* reductase (Δ, pkat/ml), α-mannosidase (×, nkat/ml), alcohol dehydrogenase (○, 10 nkat/ml) and RNase (□, U/ml). B: Western blot analysis of sucrose gradient fractions of yeast transformant expressing RNase LX. 20 μl each were analysed and probed with anti-RNase LX antibody and anti-SEC61 antibody (indicated by arrows). Sizes of marker proteins are indicated on the right (in kDa).

LXΔ9 constructs were used, the opposite relation was found. About three-fourths (72% and 73%, respectively) of the total activities were present in the culture medium and only one-fourth were found in cells of LXΔ4 and LXΔ9 constructs. Both constructs result in the same ratio of intra- and extracellular enzyme activity revealing that the last four amino acids (HDEF) are responsible for the intracellular localisation and their absence targets proteins to the extracellular space in *S. cerevisiae*.

Although most of the wild-type RNase LX has been accumulated in yeast cells it remained unknown where the protein is intracellularly localised. To address this question, fractionation experiments were performed using isopycnic density gradient centrifugation. Marker enzymes frequently used in yeast cell fractionation were estimated to characterise collected fractions (Fig. 3A). Determination of RNase activity in fractions of the continuous density gradient revealed that the enzyme is found colocalised with the ER-marker enzyme cytochrome *c* reductase (Fig. 3A). To further substantiate the characterisation as ER fractions we conducted Western blot experiments with anti-SEC61 antibody that is only detectable in this fraction (1.16 g/cm^3 sucrose density, Fig. 3B). The ER membrane protein SEC61 is the key component of the protein translocation apparatus [27,28]. In agreement with the estimated RNase LX activity the RNase protein was exclusively found in ER fractions as revealed by immunodetection (Fig. 3B).

We conclude that the tetrapeptide HDEF is necessary to accumulate the mature RNase protein in the endoplasmic reticulum of yeast cells.

3.2. The peptide HDEF is able to retain a chimeric reporter gene construct in the endoplasmic reticulum

To test whether ER retention signals are functional, these signals were often fused to reporter or passenger proteins which are genuine vacuolar, cytosolic or extracellular pro-

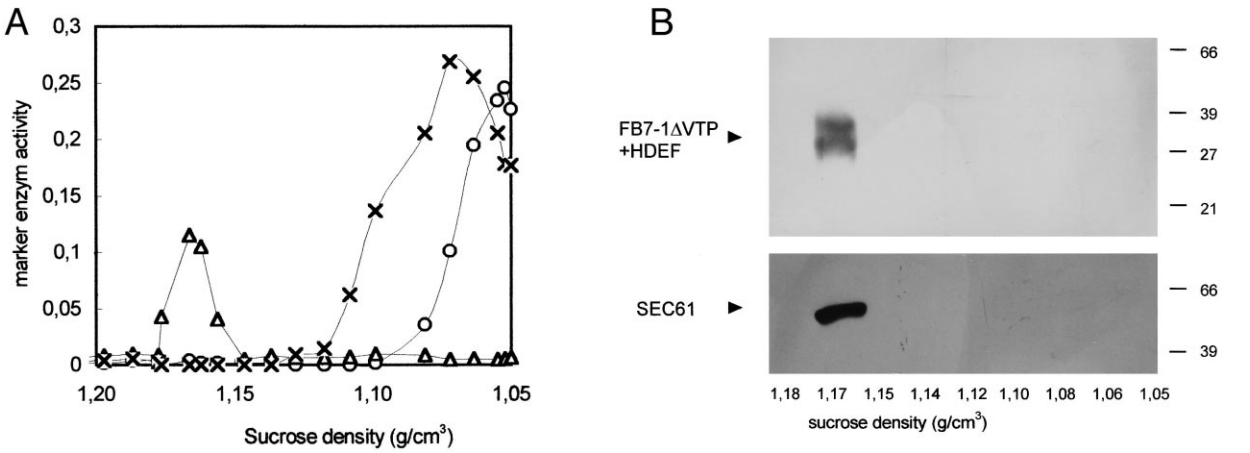

Fig. 4. Subcellular localisation of the modified reporter protein, the HDEF-tagged truncated chitinase construct FB7-1ΔVTP+HDEF, in *S. cerevisiae*. A: Determination of marker enzymes in sucrose gradient fractions. After centrifugation sucrose gradients were separated in fractions of 0.5 ml and assayed for cytochrome *c* reductase (Δ, pkat/ml), α-mannosidase (×, nkat/ml), alcohol dehydrogenase (○, 10 nkat/ml). B: Western blot analysis of sucrose gradient fractions of yeast transformant expressing modified chitinase. 20 μl each were analysed and probed with anti-chitinase antibody and anti-SEC61 antibody (indicated by arrows). Sizes of marker proteins are indicated on the right (in kDa).

teins. Consecutively, intracellular targeting and final localisation were determined by cell fractionation methods and immunolocalisation.

As passenger protein can characterise the targeting ability of the isolated peptide sequence HDEF, we used the vacuolar class I chitinase gene FB7-1 from *Nicotiana tabacum* [29,30] whose targeting was recently investigated in two *S. cerevisiae* strains [19]. Prochitinase expressed with the 7 amino acids long C-terminal VTP has been localised by subcellular fractionation as well as immunohistochemical experiments in yeast vacuoles. In the strain INVSC2 the VTP is proteolytically cleaved at a late stage during the transport to vacuoles. This result is in agreement with studies of a homologous chitinase in the plant system [31,32]. When the VTP sequence was removed, truncated chitinase FB7-1ΔVTP was transported into the culture medium [19].

On the basis of these results we investigated cellular localisation of the chimeric chitinase construct FB7-1ΔVTP+HDEF in which the VTP was replaced by the putative signal peptide HDEF. The modified protein construct was expressed under the control of the *GAL1* promoter as described. First, we analysed by immunodetection after SDS electrophoresis the distribution of chitinase in the cell extract and in adequate volumes of culture medium in probes during a 48 h induction period. In contrast to the secreted construct FB7-1ΔVTP [19] the protein tagged with the HDEF sequence was effectively accumulated inside the cells. No chitinase protein was found in medium probes by immunodetection.

To localise the modified protein FB7-1ΔVTP+HDEF in cell compartments, fractionation experiments were performed as already described for RNase constructs. Marker enzymes were measured to characterise collected fractions (Fig. 4A). In parallel, all fractions were applied to SDS-PAGE followed by immunodetection of chitinase using the anti-chitinase antibody as described in [19]. It became obvious that chimeric chitinase FB7-1ΔVTP+HDEF is exclusively found in ER fractions identified by the marker enzyme cytochrome *c* reductase (Fig. 4A). For additional confirmation that the interesting fractions contain indeed proteins associated with the endoplasmic reticulum, the anti-SEC61 antibody was applied on Western blots. The SEC61 protein is detectable in the same fractions of the sucrose density gradient (Fig. 4B). This result strongly supports that the peptide is able to change the targeting route of the formerly secreted chitinase construct. It also excludes the possibility that the HDEF containing construct is targeted to vacuoles. Experiments done following the described procedures with the C-terminal nonapeptide STNDDHDEF fused to the construct FB7-1ΔVTP gave the same result (not shown). We conclude that peptide HDEF is sufficient to redirect a secreted reporter protein to the endoplasmic reticulum of yeast cells.

A comparison of known ER retention signals K/R/HDEL and the peptide characterised in this study, HDEF, elucidates that the main difference concerns the last amino acid residue. In studies performed to prove the functionality of modified tetrapeptides, in which single amino acids were substituted, this amino acid combination was never tested [1,8,10,11]. The two C-terminal amino acid residues were considered to be critical for retention whereas the first and second position were exchangeable [6–8]. The only report that phenylalanine can replace the amino acid leucine at the C-terminus of proteins without loss of the retention capacity came from studies performed with calumenin, a novel Ca^{2+}-binding protein strongly expressed in mouse heart [9]. It was found that this protein carries the C-terminal peptide HDEF whose removal resulted in the secretion of the truncated calumenin. Transfection of COS-7 cells with FLAG-tagged calumenin and visualisation by immunofluorescence as well as deglycosylation assays revealed the localisation of this protein in the endoplasmic reticulum. Taken together, these results indicate that the peptide acts as a new ER retention signal [9].

Here, we provide strong evidence that the same C-terminal peptide HDEF containing Phe as the last amino acid residue is responsible for efficient retention of plant RNase LX in the endoplasmic reticulum of yeast cells. Experiments with plant cells confirming these findings are in progress. Additionally, we can show that the isolated signal HDEF is able to redirect completely a secretory reporter protein to the ER thereby fulfilling the second important attribute of targeting signals. We conclude that the C-terminal sequence HDEF is not only functional in animal cells but also in yeast and plant cells suggesting the ubiquitous nature of the described peptide signal. Therefore, it seems very likely that new ER resident proteins carrying this sequence will be found.

The accumulation of lumenal ER proteins harbouring ER retention signals such as KDEL or HDEL is mediated by a continuous retrieval from *cis*-Golgi and needs the interaction with specific receptors. Two receptors for HDEL sequence, ERD1 and ERD2, have been identified in yeast [33,34]. Human and plant genes homologous with the ERD2 gene were also isolated [35,36]. It is worth to note that the gene from *Arabidopsis thaliana* is able to complement ER retention-deficient yeast mutants whereas the human gene, which probably encodes a KDEL-specific receptor, is not. Whether the new ER retention signal HDEF can interact with the known receptors or needs a new receptor type for the retrieval process has to be proven. It is not ruled out that a receptor exists which interacts specifically.

Acknowledgements: This work was supported by the Deutsche Forschungsgemeinschaft (SFB 363) and by the Kultusministerium of the Land Sachsen/Anhalt. We thank Dr. Panzner, Halle, for providing the SEC61 antiserum. We gratefully acknowledge the CSIRO Division of Plant Industry (Canberra) for the kind gift of the cDNA gene FB7-1 of tobacco.

References

[1] Vitale, A., Ceriotti, A. and Denecke, J. (1993) J. Exp. Bot. 44, 1417–1444.
[2] Staehelin, L.A. (1997) Plant J. 11, 1151–1165.
[3] Munro, S. and Pelham, H.R.B. (1987) Cell 48, 899–907.
[4] Pelham, H.R.B., Hardwick, K.G. and Lewis, M.J. (1988) EMBO J. 7, 1757–1762.
[5] Napier, R.M., Fowke, L.C., Hawes, C., Lewis, M.J. and Pelham, H.R.B. (1992) J. Cell Sci. 102, 261–271.
[6] Andres, D.A., Dickerson, I.A. and Dixon, J.E. (1990) J. Biol. Chem. 265, 5952–5955.
[7] Haugejorden, S.M., Srinivasan, M. and Green, M. (1991) J. Biol. Chem. 266, 6015–6018.
[8] Denecke, J., DeRycke, R. and Botterman, J. (1992) EMBO J. 11, 2345–2355.
[9] Yabe, D., Nakamura, T., Kanazawa, N., Tashiro, K. and Honjo, T. (1998) J. Biol. Chem. 272, 18232–18239.
[10] Gomord, V. and Faye, L. (1996) Plant Physiol. Biochem. 34, 165–181.
[11] Denecke, J. (1996) in: Membranes: Specialized Functions in

Plants (Smallwood, M., Knox, J.P. and Bowles, D.J., Eds.), pp. 367–382, BIOS, Oxford.
[12] Nürnberger, T., Abel, S., Jost, W. and Glund, K. (1990) Plant Physiol. 92, 970–976.
[13] Jost, W., Bak, H., Glund, K., Terpstra, P. and Beintema, J.J. (1991) Eur. J. Biochem. 198, 1–6.
[14] Löffler, A., Abel, S., Jost, W., Beintema, J.J. and Glund, K. (1992) Plant Physiol. 98, 1472–1478.
[15] Löffler, A., Glund, K. and Irie, M. (1993) Eur. J. Biochem. 214, 627–633.
[16] Köck, M., Löffler, A., Abel, S. and Glund, K. (1995) Plant Mol. Biol. 27, 477–485.
[17] Bosse, D. and Köck, M. (1998) Plant Cell Environ. 21, 325–332.
[18] Rose, M.D., Winston, F. and Hieter, P. (1990) Methods in Yeast Genetics, A Laboratory Course Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
[19] Kunze, I., Nilsson, C., Adler, K., Manteuffel, R., Horstmann, C., Bröker, M. and Kunze, G. (1998) Biochim. Biophys. Acta 1395, 329–344.
[20] Dohmen, R.J., Strasser, A.W.M., Dahlems, U. and Hollenberg, C.P. (1991) Yeast 7, 691–692.
[21] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
[22] Bergmeyer, U. (1984) Methods of Enzymatic Analysis, 2nd edn., Vol. 1, pp. 428–429, Verlag Chemie, Weinheim.
[23] Abel, S. and Glund, K. (1987) Planta 172, 71–78.
[24] Yen, Y. and Green, P. (1991) Plant Physiol. 97, 1487–1493.
[25] Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A. and Struhl, K. (1997) Current Protocols in Molecular Biology, John Wiley and Sons, New York.
[26] Johnston, M. and Davis, R.W. (1984) Mol. Cell. Biol. 4, 1440–1448.
[27] Panzner, S., Dreyer, L., Hartmann, E., Kostka, S. and Rapoport, T.A. (1995) Cell 81, 561–570.
[28] Rapoport, T.A., Jungnickel, B. and Kutay, U. (1996) Annu. Rev. Biochem. 65, 271–303.
[29] Meeks-Wagner, D.R., Dennis, E.S., Van, K.T.T. and Peacock, J.W. (1989) Plant Cell 1, 25–35.
[30] Neale, A.D., Wahleitner, J.A., Lund, M., Bonnett, H.T., Kelly, D.R., Meeks-Wagner, D.R., Peacock, J.W. and Dennis, E.S. (1990) Plant Cell 2, 673–684.
[31] Neuhaus, J.-M., Sticher, L., Meins Jr., F. and Boller, T. (1991) Proc. Natl. Acad. Sci. USA 88, 10362–10366.
[32] Sticher, L., Hofsteenge, J., Neuhaus, J.-M., Boller, T. and Meins Jr., F. (1993) Plant Physiol. 101, 1239–1247.
[33] Hardwick, K.G., Lewis, M.J., Semenza, J., Dean, N. and Pelham, H.R.B. (1990) EMBO J. 9, 623–630.
[34] Semenza, J., Hardwick, K.G., Dean, N. and Pelham, H.R.B. (1990) Cell 61, 1349–1357.
[35] Hsu, V.W., Shan, N. and Klausner, R.D. (1992) Cell 69, 625–635.
[36] Lee, H.I., Gal, S., Newman, T.C. and Raikhel, N.V. (1993) Proc. Natl. Acad. Sci. USA 90, 11433–11437.