The outer envelope protein OEP24 from pea chloroplasts can functionally replace the mitochondrial VDAC in yeast

Thomas Röhl, Michael Motzkus, Jürgen Soll*

Botanisches Institut, Universität Kiel, Am Botanischen Garten 1-9, D-24118 Kiel, Germany

Received 12 August 1999; received in revised form 4 October 1999

Abstract The chloroplastic outer envelope protein OEP24 from pea forms a high-conductance low specificity solute channel as shown by in vitro studies. In order to establish its function also in an in vivo-like system, the gene encoding OEP24 was transformed into a yeast strain which lacks the general mitochondria solute channel porin, also known as voltage-dependent anion channel (VDAC). Transformation of the yeast VDAC(−) strain with the OEP24 gene resulted in the recovery of a phenotype indistinguishable from the wild-type. The OEP24 polypeptide is targeted to the mitochondrial outer membrane in this heterologous system. We conclude that OEP24 forms a solute channel in pea chloroplasts in planta.

Key words: Chloroplast; Mitochondrion; Yeast complementation; Outer envelope protein; *Pisum sativum*; *Saccharomyces cerevisiae*

1. Introduction

The presence of different voltage-dependent solute channels in the outer envelope membrane of chloroplasts was described by using giant chloroplasts of *Nitellopsis* [1,2] or isolated envelope membranes from spinach [3,4] for electrophysiological studies. However, a general diffusion pore such as the mitochondrial porin or voltage-dependent anion channel (VDAC) has not been detected. The permeability of the outer mitochondrial membrane for hydrophilic solutes is due to the presence of VDAC, which is found in mitochondria from all organisms studied so far [5,6]. Each channel consists of a single polypeptide of about 30 kDa [7] that forms an aqueous pore of ~3 nm in diameter [8]. These proteins are unusual hydrophilic for membrane proteins (46–50% hydrophilic amino acids [9]) and form the pore with anti-parallel amphiphilic β strands. Yeast contains a second VDAC gene, encoding a protein (YVDAC 2) with 49% amino acid sequence identity to the previously identified yeast VDAC protein (YVDAC 1) [10,11]. YVDAC 2 can functionally complement defects present in a yeast YVDAC 1(−) mutant strain only when it is overexpressed [10]. When reconstituted in a lipid bilayer, in the absence of a transmembrane potential, VDACs form large-conductance 'open' ion channels (4–4.5 nS in 1 M KCl) with a greater permeability to anions than to cations [12]. When the transmembrane voltage exceeds either plus or minus 30–40 mV, the VDACs switch to a lower-conductance 'closed' substate (about 2 nS) in which they are cation-selective [13] and impermeant to nucleoside phosphates [14].

Only recently, two pore-forming proteins from the outer envelope (OEPs) of pea chloroplasts, OEP16 and OEP24, have been characterized on the molecular level [15,16]. In vitro electrophysiological measurements using heterologously expressed and reconstituted protein show that OEP16 forms a voltage-dependent, high-conductance solute channel selective for amino acids but excludes triosephosphates and uncharged sugars. Reconstituted OEP24 is slightly cation-selective, but it has a broad solute specificity. These results suggest that OEP24 could form a general solute channel. To establish the proposed role for OEP24 also in vivo, a YVDAC 1(−) yeast mutant was complemented with OEP24. Here, we report that OEP24 can functionally replace the mitochondrial YVDAC 1, as shown by similar growth kinetics of the complemented yeast strain and the wild-type. OEP24 is expressed and targeted to the outer mitochondrial membrane in this heterologous system.

2. Material and methods

2.1. Materials

The yeast expression vector p112A1 [17] was a kind gift of Dr W. Frommer, Botanisches Institut, Tübingen, Germany. Antibodies against VDAC from potato (αPOM36) which cross-react with the YVDAC [18] were a gift of Dr U. Schmitz, Institut für Angewandte Genetik (Hannover, Germany). Antibodies against yeast translocon inner mitochondrial membrane (TIM) 22 protein and translocon outer mitochondrial membrane (TOM) 40 protein were a gift of Dr N. Pfanner, Biochemisches Institut, University of Freiburg (Freiburg, Germany).

2.2. Expression of OEP24 in yeast

The YVDAC 1(−) strain M22-2 used for the expression of OEP24 and the wild-type yeast strain M3 were a kind gift of Dr Forte (Portland, OR, USA) and have been described previously [19,20]. A 5′-*Xho*I site and a 3′-*Xba*I site were introduced by PCR amplification using the 5′-primer (GGGGGGCTCGAGATGAAGGCCGCTTTGAAGGGC) and the 3′-primer (GGGGGGTCTAGACTACATCTCAAAATTCAATGTGG), respectively, and the cDNA clone peaOEP24 [16] as a template. After restriction, the PCR product was ligated into the yeast expression vector p112A1. Competent cells were transformed as described [21] and grown on synthetic dextrose medium [22] with 2% glycerol instead of glucose.

2.3. Miscellaneous methods

Mitochondria from yeast were enriched according to [23,24]. Mitochondria were lysed in hypotonic buffer and by sonication exactly as described [25]. Mitochondrial membranes were separated by sucrose density gradient centrifugation (0.8–1.6 M sucrose, 16 h, 100 000×*g*) [25]. Outer envelope vesicles from chloroplasts were prepared as described [26]. The protein content was measured as in [27]. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and

*Corresponding author. Fax: (49) (431) 880-4222.
E-mail: jsoll@bot.uni-kiel.de

Abbreviations: OEP, outer envelope protein; VDAC, voltage-dependent anion channel; TOM, translocon outer mitochondrial membrane; TIM, translocon inner mitochondrial membrane

0014-5793/99/$20.00
PII: S0014-5793(99)01399-X

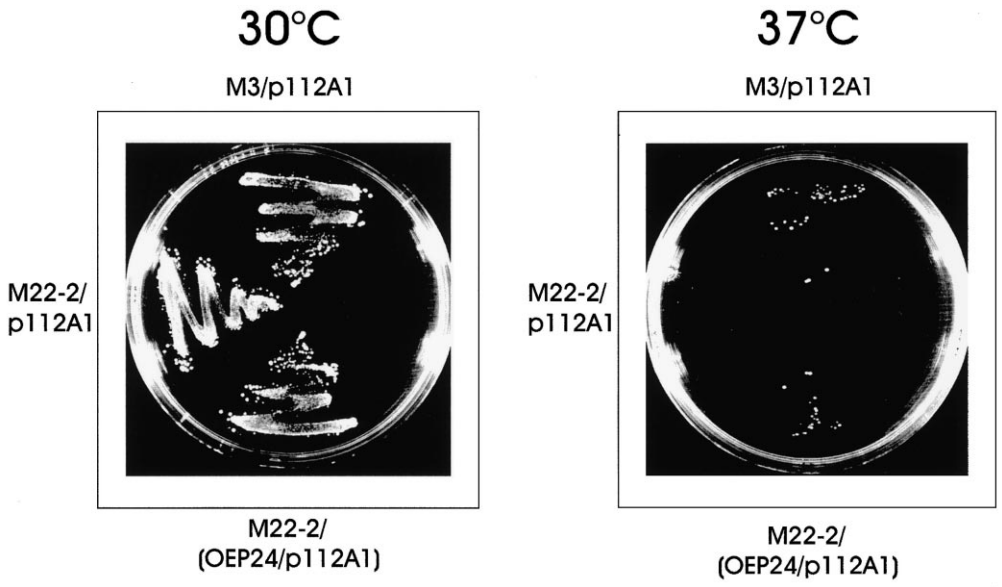

Fig. 1. Functional complementation of the YVDAC 1(−) strain by the pea OEP24 cDNA. The wild-type yeast strain M3 and the yeast strain M22-2, which chromosomal *vdac* gene is inactivated, were transformed with plasmids without insert (p112A1) or harboring the cDNA encoding OEP24 as insert (OEP24/p112A1). The transformants were grown on minimal media plates with 2% glycerol as the sole carbon source at 30 and 37°C for 7 days.

immunoblotting were done as described [28,29] using an antiserum against OEP24 [16], POM36 [18], TOM 40 or TIM 22 [30], respectively.

3. Results and discussion

There are several proteins localized in the outer envelope membrane of chloroplasts that entail the permeability of this membrane to solutes. A function of these proteins as general diffusion pore in vivo has not been described so far. Nevertheless, one of the pore-forming proteins, namely OEP24, shows some similarities to the general diffusion pore of the mitochondrial outer membrane, i.e. secondary structure predictions reveal a high content of β sheets, and the heterologously expressed protein forms a high-conductance solute channel in bilayer experiments in vitro [16]. Therefore, we wanted to examine if OEP24 can function as a general solute channel in vivo and replace the VDAC of the mitochondrial outer membrane. The cDNA for OEP24 was cloned in the yeast expression vector p112A1 and transformed into the yeast strain *Saccharomyces cerevisiae* M22-2. The M22-2 strain, whose endogenous *vdac* gene is disrupted [19], cannot grow at 37°C in the presence of glycerol as the sole carbon source, whereas it grows well at 30°C [19]. The presence of the second yeast YVDAC 2 gene product or there may be even other channel proteins present in the mitochondrial outer membrane that are not able to rescue the YVDAC 1(−) phenotype [10]. The M22-2 strain can therefore be used to search for proteins which are able to rescue the phenotype at 37°C on glycerol media. Fig. 1 shows the growth phenotype of the different yeast strains at 30 and 37°C on minimal medium in the presence of glycerol as the sole carbon source. Both the wild-type strain *S. cerevisiae* M3 and the strain M22-2 complemented with the OEP24 grew at 37°C, whereas the strain M22-2 harboring only the plasmid p112A1 did not. We conclude that OEP24 could functionally replace the VDAC in mitochondria and rescue the growth inhibition on glycerol.

To get a more quantitative information about the growth rate, the yeast cells were grown on glycerol in liquid culture at 30 and 37°C and the optical density (OD) of the cultures at 600 nm was monitored for 9 days. The strain M22-2 harbor-

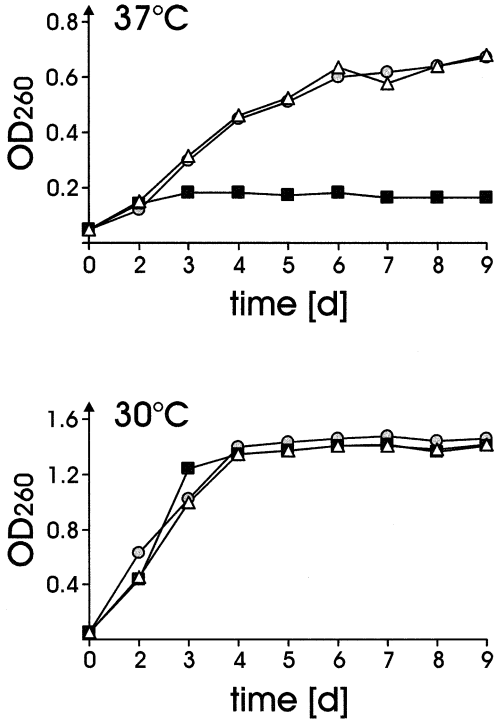

Fig. 2. Growth kinetic of the VDAC(−) strain complemented by the OEP24 cDNA. The yeast expression vector p112A1 without insert was transformed in the wild-type yeast strain M3 (●) and the yeast strain M22-2 (■), which chromosomal *vdac* gene is inactivated. The vector p112A1 with the OEP24 cDNA as insert was also transformed in the strain M22-2 (△). Growth on liquid media with 2% glycerol as the sole carbon source at 30 and 37°C was monitored over 9 days.

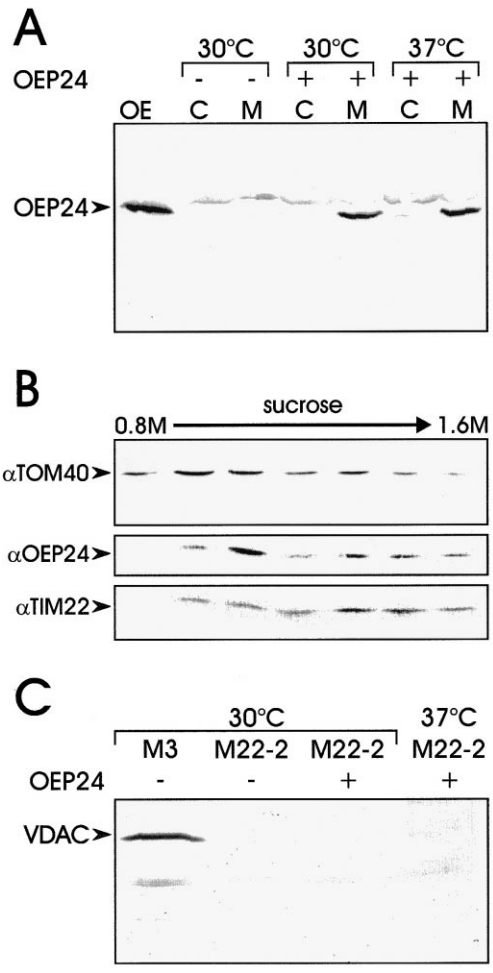

Fig. 3. (A) Expression of OEP24 in yeast and its localization in mitochondria. The YVDAC 1(−) strain M22-2 was transformed with p112A1 without insert (lanes −) or with p112A1 harboring the OEP24 cDNA (lanes +). The cells were grown on liquid media with 2% glycerol at 30 and 37°C, respectively. Cells corresponding to 500 μl of liquid culture with an OD_{600} of 0.6 (lanes C) or enriched mitochondria equivalent to 15 μg protein (lanes M) of these cultures were analyzed. Lane OE shows proteins of outer envelope vesicles equivalent to 5 μg protein. The proteins were separated by SDS-PAGE. An immunoblot decorated with αOEP24 is shown. (B) Mitochondrial membranes were separated by sucrose density gradients. The gradient was fractionated into 400 μl fractions, polypeptides precipitated with trichloroacetic acid and separated by SDS-PAGE. Proteins were blotted on nitrocellulose filters and incubated with the different antisera (α) as indicated. An immunoblot is shown. (C) VDAC deficiency of the yeast strain M22-2. The wild-type strain M3 and the YVDAC 1(−) strain M22-2 were transformed with p112A1 without insert (lanes −) or with p112A1 harboring the OEP24 cDNA (lanes +). After growth on minimal media with 2% glycerol at 30 or 37°C, mitochondria were enriched and the immunoblot was decorated with an antiserum raised against the potato outer membrane protein POM36.

ing the OEP24 cDNA shows a wild-type-like growth rate at 37°C (Fig. 2), whereas the YVDAC 1(−) strain M22-2, which was transformed with the vector p112A1, could not grow on glycerol at 37°C. The wild-type strain M3 and strain M22-2 containing the OEP24 cDNA grew up to an OD_{600} of 0.7 at 37°C over a period of 9 days (Fig. 2). This finding is in line with the growth rate on minimal medium plates, suggesting that the protein OEP24 is stable expressed in the YVDAC 1(−) yeast strain M22-2 and that OEP24 is localized to the mitochondrial outer membrane.

Most proteins of the outer membranes of chloroplasts and mitochondria are synthesized without a N-terminal cleavable sequence but are targeted to the membrane by internal information. Since yeast cells contain only mitochondria but no plastid type organelles, we assumed that a protein of the outer envelope of chloroplasts like OEP24 could be targeted and inserted into the mitochondrial outer membrane in this heterologous system. Therefore, we investigated the subcellular localization of the OEP24 polypeptide in transformed yeast cells and as a control, the YVDAC protein. The YVDAC 1(−) yeast strain M22-2 containing only the vector was grown at 30°C and M22-2 cells harboring the OEP24 cDNA were grown at 30 or 37°C, respectively. From all cultures, intact cells and fractions enriched in mitochondria were prepared and analyzed by immunoblotting with an antiserum specific for OEP24 [16]. After growth at 30 and 37°C, OEP24 is detected in the mitochondrial fraction, whereas it is not present in the cells transformed only with vector p112A1. In intact yeast cells, OEP24 is barely detectable, probably due to a low protein content (Fig. 3A).

To obtain further evidence about the submitochondrial distribution of OEP24, mitochondria were isolated from yeast cells harboring the OEP24 cDNA in p112A1. The mitochondria were ruptured by hypotonic lysis and sonication (see Section 2) and membranes fractionated by centrifugation on a sucrose density gradient. OEP24 protein co-fractionates with the outer membrane marker TOM 40 and is recovered in the low buoyant density fractions of the gradient (Fig. 3B). TIM 22, a marker for the inner mitochondrial membrane, is predominantly found at higher buoyant density fractions of the sucrose gradient. No soluble form of OEP24 was found (not shown). We conclude that OEP24 is indeed localized in the yeast mitochondrial outer membrane.

To eliminate the possibility that authentic YVDAC 1 protein was responsible for the observed complementation, mitochondria were checked for the presence of this protein. The wild-type yeast strain M3 and the YVDAC 1(−) strain M22-2 were transformed with p112A1 and grown at 30°C in minimal media with 2% glycerol. The strain M22-2 was also transformed with p112A1 containing the cDNA for OEP24 and was grown at 30 and 37°C, respectively. From all cultures, mitochondria were enriched [23] and an immunoblot was decorated with an antiserum against a VDAC homologue from potato, POM36. This antiserum was shown to cross-react with mitochondrial porin from yeast [18]. Our results demonstrate that only the wild-type strain expresses the *vdac* gene whereas the strain M22-2 does not (Fig. 3C). This means that the wild-type-like growth of the yeast transformed with the OEP24 cDNA is indeed achieved by a functionally complementation with the pea chloroplast protein OEP24 and not for example by a temperature-induced VDAC expression.

4. Conclusions

Because OEP24 can complement the YVDAC 1(−) deficiency in vivo completely and is found in the enriched mitochondrial outer membrane fraction, we conclude that OEP24 is targeted to the outer mitochondrial membrane and assembled in a manner which allows for a YVDAC equivalent function.

Although the integral membrane protein OEP24 shows neither homologies to bacterial nor to mitochondrial porins in the primary sequence, a content of 49% hydrophilic amino acids is similar to the composition of mitochondrial porins (46–50%, [9]). Structure prediction algorithms and circular dichroism spectra indicate seven amphiphilic β strands for OEP24 [16]. Chemical crosslinking experiments with isolated chloroplast envelope vesicles suggest that OEP24 forms homodimers in its functional active state in the outer envelope membrane and there were no other proteins detected in close physical proximity (K. Pohlmeyer, unpublished results). Therefore, the pore formed by OEP24 could consist of 14 amphiphilic β strands. This structure is similar to the VDAC structure with 12–16 β strands [9,20,31]. The low selectivity of reconstituted OEP24 in bilayer experiments strongly suggests that it forms a general solute channel also in vivo, like the VDAC protein does in the outer mitochondrial membrane. Reconstituted OEP24 shows a voltage-dependent gating mechanism with the highest open probability at 0 mV, similar to VDAC.

The methods presented here might be valuable tools to establish the function of different solute transporters from plants in vivo.

Acknowledgements: This work was supported in part by grants from the Deutsche Forschungsgemeinschaft and Fonds der Chemischen Industrie. We thank Drs Forte, Frommer, Schmitz and Pfanner for gifts of the yeast strains, plasmid and antisera, respectively.

References

[1] Pottosin, I.I. (1992) FEBS Lett. 308, 87–90.
[2] Pottosin, I.I. (1993) FEBS Lett. 330, 211–214.
[3] Flügge, U.-I. and Benz, R. (1984) FEBS Lett. 169, 85–89.
[4] Heiber, T., Steinkamp, T., Hinnah, S., Schwarz, M., Flügge, U.-I., Weber, A. and Wagner, R. (1995) Biochemistry 34, 15906–15917.
[5] Colombini, M. (1989) J. Membr. Biol. 111, 103–111.
[6] Manella, C.A. (1992) Trends Biochem. Sci. 17, 315–320.
[7] Thomas, L., Kocsis, E., Colombini, M., Erbe, E., Trus, B.L. and Steven, A.C. (1991) J. Struct. Biol. 106, 161–171.
[8] Manella, C.A., Guo, X.W. and Cognon, B. (1989) FEBS Lett. 253, 231–234.
[9] Benz, R. (1994) Biochim. Biophys. Acta 1197, 167–196.
[10] Blachly-Dyson, E., Song, J., Wolfgang, W.J., Colombini, M. and Forte, M. (1997) Mol. Cell Biol. 10, 5727–5738.
[11] Lee, A.C., Xu, X., Blachly-Dyson, E., Forte, M. and Columbini, M. (1998) J. Membr. Biol. 161, 173–181.
[12] Manella, C.A. (1998) J. Struct. Biol. 121, 207–218.
[13] Benz, R., Kottke, M. and Brdiczka, D. (1990) Biochim. Biophys. Acta 1022, 311–318.
[14] Rostovtseva, T. and Colombini, M. (1996) J. Biol. Chem. 271, 28006–28008.
[15] Pohlmeyer, K., Soll, J., Steinkamp, T., Hinnah, S. and Wagner, R. (1997) Proc. Natl. Acad. Sci. USA 94, 9504–9509.
[16] Pohlmeyer, K., Soll, J., Grimm, R., Hill, K. and Wagner, R. (1998) Plant Cell 10, 1207–1216.
[17] Riesmeier, J.W., Willmitzer, L. and Frommer, W.B. (1992) EMBO J. 11, 4705–4713.
[18] Heins, L., Mentzel, H., Schmid, A., Benz, R. and Schmitz, U.K. (1994) J. Biol. Chem. 269, 26402–26410.
[19] Blachly-Dyson, E., Peng, S., Colombini, M. and Forte, M. (1989) J. Bioenerg. Biomembr. 21, 471–483.
[20] Blachly-Dyson, E., Songzhi, P., Colombini, M. and Forte, M. (1990) Science 247, 1233–1236.
[21] Dohmen, R.J., Strasser, A.W.M., Höner, C.B. and Hollenberg, C.P. (1991) Yeast 7, 691–692.
[22] Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A. and Struhl, K. (1987) Current Protocols In Molecular Biology, Chapter 13, Green Publishing Associates, New York.
[23] Daum, G., Böhni, P.C. and Schatz, G. (1982) J. Biol. Chem. 257, 13028–13033.
[24] Glick, B.S. and Pon, L.A. (1995) Methods Enzymol. 260, 213–219.
[25] Glick, B.S. and Pon, L.A. (1995) Methods Enzymol. 260, 236–237.
[26] Keegstra, K. and Youssif, A.E. (1986) Methods Enzymol. 118, 316–325.
[27] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
[28] Laemmli, U.K. (1970) Nature 227, 680–685.
[29] Towbin, H., Staehelin, T. and Gordon, J. (1979) Proc. Natl. Acad. Sci. USA 76, 4350–4354.
[30] Pfanner, N., Craig, E.A. and Hönliger, A. (1997) Annu. Rev. Cell Dev. Biol. 13, 25–51.
[31] De Pinto, V.D. and Palmieri, F. (1992) J. Bioenerg. Biomembr. 24, 21–26.