Intracellular Localization of Lipid Transfer Proteins in *Brassica oleracea*

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Summary

We have isolated a protein (cryoprotectin) from the leaves of cold acclimated cabbage (*Brassica oleracea)*, that protects thylakoids from freeze-thaw damage *in vitro*. Sequencing of cryoprotectin showed the presence of at least three isoforms of WAX9 proteins, which belong to the class of nonspecific lipid transfer proteins. The localization of endogenous WAX9 protein in cabbage leaf cells was investigated using gold-labelled anti-WAX9 antibodies. Label was detected in chloroplasts, cytosol, mitochondria and the nucleus of cold acclimated plants. Conversely, when WAX9 fusion proteins expressed in *E. coli* were gold labelled, binding to chloroplasts of nonacclimated cabbage leaves was observed for isoforms WAX9A and WAX9D, but not for isoform WAX9E.

Introduction

Nonspecific lipid transfer proteins (LTPs) are organized as multigene families in higher plants. Nonspecific lipid transfer was demonstrated *in vitro,* suggesting that LTPs might have a similar function *in vivo* (Kader 1996). The *in vivo* function of LTPs is, however, not yet clear. LTPs generally contain a signal peptide sequence for the secretory pathway (Arondel and Kader 1990) and the proteins can be found in the extracellular space (Kader 1996; Sterk *et al.* 1991; Thoma *et al.* 1993). There are, however, reports that considerable amounts of LTPs are also found within cells (Tsuboi et al. 1992, Douady et al. 1986).

We have isolated a protein (cryoprotectin) from cold acclimated cabbage

leaves, that protects spinach thylakoids from freeze-thaw damage *in vitro* (Hincha *et al.* 1990). Although we have purified this protein to electrophoretical homogeneity (Sieg *et al.* 1996), this fraction still contained at least three homologous polypeptides, as revealed by protein sequencing (Hincha *et al.* 2001). All peptides belong to the family of WAX9 proteins (Pyee and Kolattukudy 1995), which show a high degree of sequence homology to other plant lipid transfer proteins (Hincha 2002). To gain insight into a possible site of action of cryoprotectin, we have studied its localisation using gold-labelled anti-WAX9 antibodies. In addition, we have investigated the affinity of members of the WAX9 family to cellular structures by labelling WAX9 fusion proteins with gold.

Materials and Methods

Plant material and purification of cryoprotectin

Cabbage (*Brassica oleracea* L. cv Grüfewi) was grown in the garden for several months. For cold acclimation, plants were held at a constant temperature of 4°C with a 10 h light/14 h dark cycle for two weeks.

Cryoprotectin isolation followed the method devised by Sieg *et al.* (1996). For all experiments, reported here, the crude homogenate was only fractionated by heat, acetic acid, and ammonium sulfate precipitations as described.

Cloning of wax9 *genes and expression of recombinant WAX9 proteins*

The genes were cloned into pET32a(+) and transformed in *Origami*(DE3)LysS cells (Novagen) according to the manufacturer's manual. The cloning strategy and protein expression and purification have been described by Schilling *et al.* (2002). The WAX9 fusion proteins posses an N-terminal tag consisting of an Trx-tag, His-tag, S-tag and an enterokinase cleavage site. Anti-WAX9 antibodies were raised in rabbits against synthetic peptides as described (Hincha *et al.* 2001).

Gold-labelling of proteins

The recombinant WAX9 fusion proteins and antibodies were labelled as described by Roth (1982). The recombinant WAX9 fusion proteins was labelled with 10 nm gold colloids, while a mixture of 5 to 20 nm colloids was used for labelling the antibodies.

On-section labelling of cabbage leaf tissue

Pieces of cabbage leaf tissue (approximately 2 mm^2) from acclimated

or nonacclimated plants were fixed for 2 h in 1% glutaraldehyde in phosphate buffer at room temperature. After washing with buffer the specimens were fixed for 12 h with osmium tetroxide in the same buffer, washed again in buffer and then dehydrated in an ascending series of ethanol. The dehydrated specimens were cryoembedded in K4M. Ultrathin sections from all samples were incubated with gold-labelled proteins for 12 h in phosphate buffer, with 0.25% gelatin and 0.5% bovine serum albumin to block unspecific protein binding sites. The labelled sections were stained with uranyl acetate and lead citrate and examined in a Siemens 101 transmission electron microscope.

Results

Immunogold localization of WAX9 proteins in cabbage leaves

The localization of endogenous WAX9 protein in cabbage leaf cells was investigated using gold-labelled anti-WAX9 antibodies. Sections from cold acclimated leaves showed gold particles in chloroplasts (Figure 1A, B, C) and the cytoplasm, nucleus and mitochondria (Figure 1D). There was no label detectable in cell walls or vacuoles. There was also no detectable label on sections from nonacclimated leaves (Figure 1E). Likewise, incubation of sections from cold acclimated leaves with a gold-labelled preimmune serum did not result in any labelling (Figure 1F).

Binding of WAX9 proteins to sections from nonacclimated and cold acclimated cabbage leaves

In order to have a cryoprotective effect *in vivo*, cryoprotectin must be able to bind to cellular membranes of cabbage leaves. To test this, we labelled *E. coli* expressed fusion proteins with gold particles and incubated the labelled protein with sections from embedded leaves from nonacclimated and cold acclimated cabbage.

WAX9A and WAX9D fusion proteins bound to chloroplasts, especially to the thylakoid membranes in non-acclimated plants as examplified for WAX9D in Figure 1G. When the closely related, but not cryoprotective, fusion protein WAX9E (Hincha 2002) was gold-labelled and incubated with sections from cold acclimated and nonacclimated cabbage leaves, there was no significant label visible (Figure 1H). This indicates that cabbage leaves possess cellular binding sites for some WAX9 proteins.

Conclusions

Using gold-labelled anti-WAX9 antibodies, WAX9 proteins were

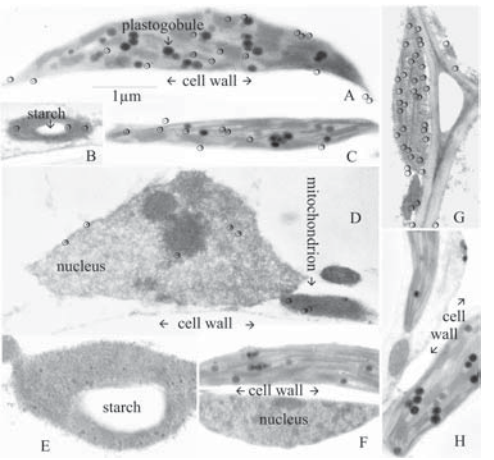

Figure 1. Immunolocalization of WAX9 proteins on cabbage leaf slices. Sections of cabbage leaves from either cold acclimated (A, B, C, D, F) or nonacclimated (E) plants were incubated with gold-labelled anti-WAX9 antibodies. As a control, sections were also incubated with a gold-labelled pre-immune serum (F). Labelling of WAX9 proteins was found in chloroplasts (A, B, C), nucleus and mitochondria (D) from cold acclimated plants, but there was no label on sections obtained from nonacclimated plants (E). There was also no label on sections from cold acclimated plants incubated with a gold-labelled pre-immune serum (F). All figures are shown at the same magnification. The bar in (D) represents 1 µm. For searching WAX9 binding sites, cabbage leave sections were incubated with gold labelled fusion protein WAX9D (G, nonacclimated plants) or WAX9E (H, cold acclimated plants)

detected in chloroplasts, cytoplasm, mitochondria and the nucleus of cabbage leaf cells (Figure 1). Label was only detectable on sections from cold acclimated leaves, but not on sections from nonacclimated leaves. This is in accordance with the notion that cryoprotectin is a cold regulated protein. Cryoprotective activity could only be isolated from cold acclimated plants, and not from nonacclimated tissues (Hincha *et al.* 1990).

The localization of LTP homologues such as WAX9 proteins in chloroplasts and nucleus was unexpected, because LTPs are generally considered extracellular proteins (Kader 1996). This assumption is based on the presence of an amino-terminal extension, that is thought to function as a sorting signal for the secretory pathway, in all LTP genes cloned to date. In addition, the WAX9E protein is localized in the external wax layer of leaves (Hincha *et al.* 2001). On the other hand, there are also reports on the intracellular localization of LTPs. In maize seeds, LTPs were detected in the cytoplasm (Sossountzov *et al.* 1991), and in mitochondria and microsomes (Douady *et al.* 1986). In cotyle-

dons from castor bean, LTP was found in glyoxysomes (Tsuboi *et al.* 1992), and in sugar beet leaves, LTPs were detected both intra- and extracellularly (Nielsen *et al.* 1996). We therefore conclude, that an intracellular localization of cryoprotectin is in agreement with several reports in the literature. It seems possible that the different WAX9 isoforms found in our cryoprotectin preparations (Hincha *et al.* 2001) may be localized in different cellular compartments. The mechanism of the differential targeting of LTPs to different cellular compartments, however, remains to be analyzed.

WAX9 fusion proteins A and D bind to thylakoid membranes of non-hardy plants while isoform E does not bind. WAX9E has lipid transfer activity *in vitro* whereas sequences from both A and D were identified in highly purified cryoprotectin preparations (Hincha *et al.* 2001). Protection against freezing is achieved by a mechanism involving stable membrane binding (Sror *et al.* unpublished) so the binding of WAX9A and D constitutes evidence that these isoforms may indeed have cryoprotective activity. In contrast, stable membrane binding would preclude lipid transport, so the lack of binding of WAX9E is in accordance with its known function.

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