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ORIGINAL ARTICLE

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Studies on the function of TM20, a transmembrane protein present in cereal embryos

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Abstract The possible function of the maize transmembrane protein TM20 in hormone transport has been investigated using immunological methods and by microinjection of TM20 cRNA in *Xenopus* oocytes. The existence of a similar gene in rice indicates that the overall structure of the deduced protein is conserved between these two cereals. An antibody raised against a conserved motif, in a loop between two transmembrane domains, locates the protein (TM20) in differentiating provascular cells in maize embryo. The protein has a polarized distribution within the cell in the most differentiated stages of development. *Xenopus laevis* oocytes microinjected with TM20 appear to modify transport activities across the plasma membrane. These results are discussed in relation to other transport proteins that influence plant development.

Keywords Embryo development · Microinjection · *Oryza* · Transmembrane protein · *Xenopus* · *Zea*

Abbreviations DAP: Days after pollination · IAA: Indol acetic acid · NPA: n-1-naphthylphtalamic acid · PVDF: Polyvinylidene difluoride · TBA: Tert-butanol

Introduction

During embryogenesis in higher plants, the essential features of the individual such as the shoot–root body pattern or the primary cell types are specified. How the embryo acquires its three-dimensional shape with specialized organs and tissues, and what gene networks control and regulate the multiple processes of embryonic development, are still largely unanswered questions. After initial morphological studies of plant embryos during development (Johansen 1950; Wardlaw 1955), genetics and molecular biology are being applied to the study of plant embryogenesis, including the generation of mutants and their analysis at a molecular level (Meinke 1991, 1995). A variety of mutants with defective and altered embryos have been generated and characterized in plants such as *Arabidopsis thaliana* and *Zea mays*, which represent the two major groups of flowering plants. The analyses of these mutants have identified genes that play an essential role in the correct development of the plant embryo (reviewed Bommert and Werr 2001; Souter and Lindsey 2000; Jürgens 2001).

In maize, the first stages of development lead to a proembryo formed by irregular cell divisions that produce the embryo proper, the suspensor, and the protoderm. The suspensor disappears during later stages of development, while the embryo proper develops into the embryo with root and shoot meristems, leaf primordia and the scutellum, giving rise to a switch from radial to bilateral symmetry. Several mutants blocked during embryogenesis have been described in maize. These have been grouped in two major classes: defective kernel (*dek*) and embryo-specific (*emb*) mutants (Sheridan and Clark 1993; Sheridan and Neuffer 1982). While in the *dek* mutants the endosperm and the embryo are defective, the mutation in the *emb* mutants only affects the embryo.

The maize mutant *lachrima* is a lethal homozygous *dek*-mutant, which was found while screening for embryogenesis mutants generated by *Ac*-tagging (Stiefel et al. 1999). The development of *lachrima* embryos is blocked before the transition stage, preventing the formation of bilateral symmetry, in the same way as in cultured embryos by inhibitors of polar transport of hormones (Fischer and Neuhaus 1996). The molecular

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analysis of *lachrima* leads to the identification of a gene with an *Ac* insertion in the promoter region, linked to the *lachrima* phenotype, which codes for a new class of proteins with 20 transmembrane spanning domains, called TM20 (Stiefel et al. 1999). Based on the phenotype of *lachrima*, phenocopied by the blockage of polar auxin transport, and the transporter-like structure of TM20, it was proposed that TM20 is involved in processes that regulate or mediate the movement of auxin through the plant (Stiefel et al. 1999).

In the last few years several proteins in *Arabidopsis* have been described, which are thought to play an active role in the transport of auxin (Müller et al. 1998; Gälweiler et al. 1998; Marchant et al. 1999; Friml et al. 2002a, 2002b). They support the model that proposes the action of specific influx and efflux carriers, located at the apical and basal end of the cells respectively and responsible for the distribution of auxin throughout the plant (Rubery and Sheldrake 1974; Raven 1975). Auxin transport involves a variety of molecules, still only partially identified, to guarantee the finely regulated transport of auxin during embryogenesis and plant development (Gil et al. 2001; Muday and DeLong 2001). In order to assess the possible function of TM20 related to transport processes, three types of studies have been carried out. First, the rice genome database has been searched to demonstrate that the protein is generally present in other cereals. Second, immunological methods have been used to show the presence of the protein in different cell types. Finally, the *Xenopus* oocyte system has been used to study the ability of the TM20 protein to alter the properties of transport across the membrane and IAA has been used as an example to test this hypothesis.

Material and methods

Plant material

Wild-type and mutant maize plants were grown in the greenhouse of the Departament de Genètica Molecular (IBMB-CSIC, Barcelona) under temperature and light conditons that correspond to field conditions in summer. The maize material used pertains to the *Z. mays L* line W64A+/+. Maize mutants were originated by Dr. Dellaporta (Yale University) as previously described (Dellaporta and Moreno 1994)

Database searches

For the comparison of nucleotides and amino acid sequences with the databases the BLASTN, TBLASTN and FASTA algorithm were used (Altschul et al. 1990; Pearson 1990). For protein prediction and nucleotide analysis the DNAStar software package (Madison, WI, USA) was employed. The transmembrane predictions were done by the TMHMM-v2.0 server (http://www.cbs.dtu.dk/services/TMHMM-2.0/).

Antibody generation and purification

The anti-TM20 antiserum was raised in rabbits injected with a synthetic peptide (YNAGLTPPGGFWSKN-C) coupled via disulphide bonding to the Keyhole limpet haemocyanin (KLH) protein used as a carrier. The polyclonal anti-TM20 antibody was purified from serum by immunoaffinity using TM20-peptide immobilized in SulfoLink^{TM}Coupling Gel (Pierce). For dot-blots, solutions with known protein concentrations were loaded onto a PVDF membrane (Immobilon P, Millipore) that was blocked with 5% low fat milk in phosphate-buffered saline (PBS) for 1 h at RT. Subsequently the membranes were incubated with the primary anti-TM20 antibody at different dilutions in PBS for 1–2 h at RT or overnight at 4°C, followed by incubation with a secondary anti-rabbit antibody coupled to horseradish peroxidase or biotin at different dilutions in PBS for 45 min at RT. Signal detection was achieved either by chemiluminescence (ECL, Pierce) or by colourimetric reaction (BCIP/NBT-Blue Liquid Substrate System, Sigma) on the membrane.

Preparation of the microsomal fractions

Immature maize embryos were homogenized in a precooled mortar adding non-denaturizing extraction buffer (100 mM Tris–HCl pH7.9, 12% sucrose, 1 mM EDTA, protease inhibitors). After cell debris removal, the microsomal fraction was separated from the soluble fraction by ultracentrifugation at 150,000 *g* for 1.5 h at 4°C. The pellet containing the membrane fraction was resuspended in PBS, while the proteins of the soluble fraction were precipitated with 15% trichloroacetic acid (TCA, Merck). Proteins were quantified by Bradford assay (BioRad)

Immunohistochemistry

Immunohistochemistry was performed on immature maize embryo sections of paraffin embedded samples and whole mount embryos. Maize embryos used for sections were fixed with EtOH-acetic-acetic-acid (80:3.5:5, by vol.) for 1 h at RT under vacuum and were incubated for 1 week in fresh EtOH-acetic-acetic acid solution. Fixed embryos were then incubated for 30–60 min at RT in 70% EtOH, dehydrated in steps of 80%EtOH, 90%EtOH, 100%EtO (twice), 25% TBA in EtOH,50% TBA in EtOH, 100% TBA, 100% TBA of 30–60 min at RT each step, embedded in paraffin and sectioned on a microtome (Reichert-Jung, Supercut, Germany). Paraffin-embedded sections of maize embryos were deparaffined in two wash steps for 10 min at RT with xylol and rehydrated in an decreasing ethanol series (100%, 100%, 90%, 70%, 50%, 30%) followed by two incubation steps with distilled water. Sections and whole mount preparations were then incubated with

0.5% Triton X-100 in PBS containing 1% BSA for 5 min at room temperature, and blocked in 4–6 mg of goat serum (Jackson Immunoresearch, West Grove, PA, USA) in PBS containing 1% BSA. The samples were incubated with the primary anti-TM20 antibody at different dilutions in PBS containing 1% BSA for 1–2 h at room temperature, or overnight at 4°C. For the fluorescence labelling, the samples were incubated with a secondary anti-rabbit antibody conjugated to biotin (Jackson Immunoresearch) for 1 h at room temperature and a third incubation with Streptravidin labelled with Oregon-green 488 or Rhodamin Red (Jackson Immunoresearch, West Grove, PA, USA) for 1 h at room temperature. The sections were visualized by fluorescence light microscopy (Axiophot Zeiss) or confocal laser scanning microscopy (Leica TCS SP, Germany).

In the case of whole mount preparations, 10DAP maize embryos were placed in 1.5 ml microfuge tubes and fixed with 3.5% paraformaldehyde (Merck) in PBS for 1 h at RT under vacuum. All treatments and incubation steps were performed by applying a vacuum for 1 min, to guarantee the access of reagents into the tissues. After three wash steps with PBS for 10 min at room temperature, the embryos were treated with 1% cellulysin cellulase (Calbiochem) and 1% pectolyase Y-23 (Kikkoman, Tokyo, Japan) in PBS for 30 min. Incubations with anti-TM20 antibody and secondary antibodies were performed as described above. Finally the samples were washed as described above and mounted with Mowiol on microscope slides for further visualization.

Oocytes, injections and uptake measurements

Oocyte origin, management and injections were as described elsewhere (Bertran et al. 1992a, 1992b). Defolliculated stage VI *X. laevis* oocytes were injected with 20 ng/oocyte of cRNA of maize TM20. Maize TM20 cRNA was synthesised using the standard transcription protocol of the Ambion in-vitro transcription kit. Influx rates of 3-[5(*n*)−^{3}H] indolacetic acid (^{3}H-IAA) (Amersham Pharmacia Biotech) were measured in choline-Cl medium (100 mM choline-Cl, 2 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM Hepes, adjusted to pH 5.5, 7.5 or 8.5) three days after injection. Amino acid transport rates obtained with oocytes injected with water (50 nl) were similar to those with non-injected oocytes (data not shown). For each determination, groups of seven oocytes were washed once in choline-Cl buffer and then incubated at room temperature in a 5-ml polypropylene tube containing 100 µl of 2 µM ^{3}H-IAA ($1{,}11\times10^6$ Bq/ml). Transport was stopped after the appropriate interval by washing oocytes three times with 4 ml of 2 mM IAA in ice-cold choline-Cl buffer. Single oocytes were placed in scintillation vials and lysed by addition of 200 µl 10%SDS. After lysis, 3 ml scintillation fluid was added and the radioactivity determined. The procedure for the transport inhibition by NPA was performed as described previously adding 5 µM NPA to the choline-Cl buffer. After 60 min of incubation the radioactivity in oocytes incubated in the presence of NPA and without NPA in parallel was determined.

For the localization of TM20, groups of five oocytes were prepared for immunofluorescence 3 days after injection with 20 ng/oocyte human TM20 cRNA. Oocytes were placed in 500 mm^3 cryomolds (Tissue-Tek, Miles Inc., Elkhart, IN, USA), sliced, fixed, and permeabilized as described elsewhere (Estevez et al. 1998). Slices were incubated with polyclonal anti-TM20 antibody, diluted in 10% phosphate-buffered saline, at room temperature for 1 h. Slices were washed three times in phosphate-buffered saline, incubated with 7.5 µg/ml Texas red-conjugated goat anti-rabbit (Molecular Probes) at room temperature for 1 h, washed three times in phosphate-buffered saline, and mounted in Immunofluore (ICN, Madrid, Spain).

Results

TM20 in Oryza sativa

In the maize genome TM20 appears to be encoded by a single gene (Stiefel et al. 1999). The search for a similar sequence in the rice genome was carried out for two main reasons, first, to confirm that the gene exists in at least another cereal and second, to look for the conserved features in the protein sequences. The search of the entire amino acid sequence of TM20 (1389aa) against the six possible open reading frames (ORF) of the rice genome database brought up only one genomic clone (accession number OSM117231). When the sequence was fully analyzed it appeared that OsTM20 is encoded by two exons of 445 bp and 2,948 bp respectively, separated by an intron of 1,708 bp. The amino acid sequences of TM20 and OsTM20 deduced from DNA (Fig. 1) are 46% identical and the structure of both proteins is very similar. With the program TMHMM v2.0, for the prediction of transmembrane domains, (http://www.cbs.dtu.dk/services/TMHMM-2.0/) a protein with an equal number and similar distribution of transmembrane domains as that observed in TM20 can be deduced (Fig. 1). The rice protein has 20 transmembrane spanning segments grouped together in five groups of four transmembrane elements. The first transmembrane domain of every group is separated from the other three domains. The central loop described for TM20 (509aa-817aa, see Fig. 1), located between the second and third group of transmembrane domains, is much shorter in the OsTM20 protein than in TM20 from maize and it is the region with the lowest similarity between the two proteins. Besides the shorter central loop of the OsTM20, several gaps of 3–15 amino acids length are distributed throughout the last 450 amino acids (Fig. 1). Five phosphorylation sites (GSC/SR) were identified in the rice protein located in the same position as described for the maize TM20 protein (Stiefel et al. 1999). The

tetrapeptides GSC/SR are known to be linked to the phosphorylation activity of protein kinase C (Kishimoto et al. 1985; Woodgett et al. 1986). This type of motifs is present in proteins such as the sodium dependent dopamine transporter from *Bos taurus* and *Homo sapiens* (Usdin et al. 1991; Giros et al. 1992). The results of these analyses suggest that a protein structurally similar to TM20 also exists in *Oryza sativa*, sharing the characteristics of a transmembrane protein and that might fulfill the same function as TM20.

TM20 antibody generation and characterization

To study the protein TM20 in maize embryos, antibodies against TM20 were generated. The antibodies were raised against a synthetic peptide that corresponds to a motif present in a loop existing in the five repeated domains of the TM20 protein. The sequence of this peptide is shown in Fig. 2. Results are also presented of dotblot analysis where the antiserum against TM20 recognizes the antigen specifically (Fig. 2 a), whereas the negative control (1 μg of a synthetic peptide with a different sequence) was not detected by the antibody and the negative control with pre-immune serum (Fig. 2a, NI) did not show any cross-reaction. In addition, dotblot analysis was carried out with the purified antibody α-TM20 incubated with different concentrations of the peptide TM20 for 1 h prior to the use in immunodetection. As shown in Fig. 2b, the ability of the antibody α-TM20 to detect the peptide TM20 on the membrane decreases in relation to an increasing concentration of

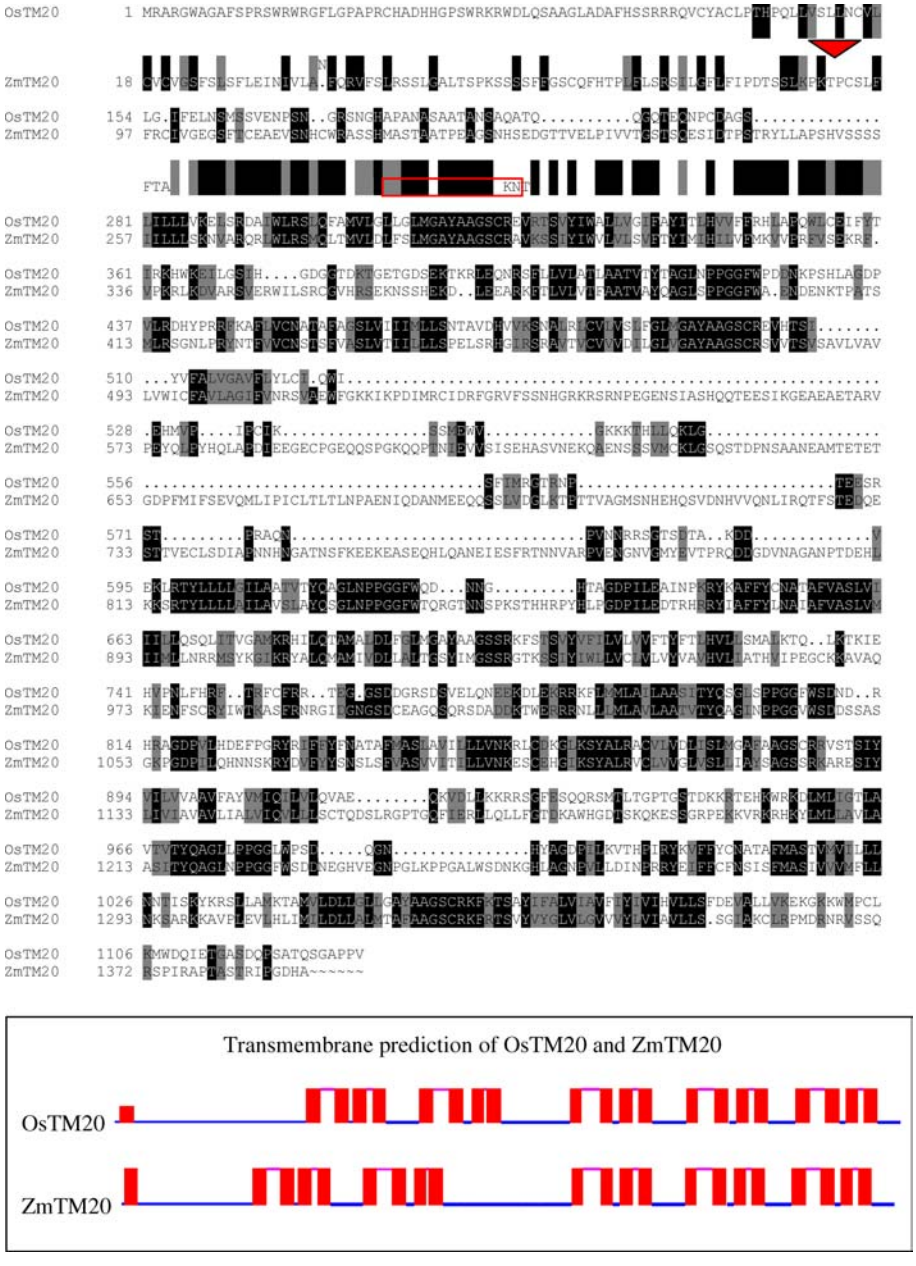

Fig. 1 Alignment of the deduced amino acid sequences of ZmTM20 and OsTM20. Identical amino acids marked by *black boxes* and similar amino acids shaded in *grey*; the identity of the aligned sequences is about 46% and the similarity about 57%. The alignment was performed after Clustal W algorithm (Higgins and Sharp 1989). The comparison of the structure of predicted transmembrane domains is presented in the *box* below. The transmembrane predictions were performed using the TMHMM-v2.0 server (http://www.cbs.dtu.dk/services/TMHMM-2.0/). Peptide sequence for Antibody generation is marked by a *red box*. The *arrow* marks the position of the intron in the sequence of OsTM20

intercepting peptides. In dot-blot assays the antibody α-TM20 recognizes the peptide TM20 in the native state (Fig. 2a). The immunodetection of the peptide TM20 in the presence of 0.5% SDS was clearly reduced. Therefore, the α-TM20 antibody could be used for in-situ immunolocalization but not in Western blot analyses.

TM20 detection in microsomes of maize embryos at different developmental stages

The 20 transmembrane domains of TM20 are an indication of its membrane localization. Microsome preparations of maize embryos to enrich membrane fractions in relation to soluble proteins can help to resolve the relationship between TM20 and membranes. To this end protein extracts from maize embryos were separated into fractions of soluble proteins (S150) and microsomes (P150) by ultracentrifugation (150,000 *g*). The membrane-enriched protein fractions (P150) of maize embryos of 10 DAP, 15 DAP and 20 DAP (whole embryo and isolated embryo axis) were placed onto a PVDF membrane. Soluble proteins (S150) were precipitated and also placed onto the PVDF membrane. The results obtained using the purified antibody α-TM20 are shown in Fig. 3a. A clear signal was observed in fraction P150 (1–4) as well as traces in fraction S150 (5–8). As expected, TM20 detection was observed mainly in the microsomal fraction. The traces of TM20 detected in the soluble protein fractions could be due to contamination of microsomal proteins during the process of membrane preparation. The negative controls (Fig. 3b) gave no signal. This result indicates that TM20 is present in the microsomal fraction and that the antibody can detect native TM20 in protein extracts of maize embryos. Furthermore, the result demonstrated the presence of TM20 at different developmental stages of the maize embryo in accordance with the mRNA accumulation analyses (Stiefel et al. 1999).

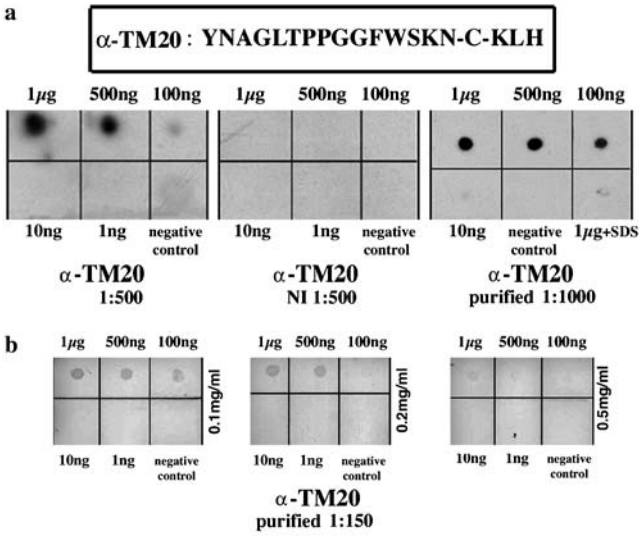

Fig. 2 Dot-blot immuno analysis of antibodies against a synthetic peptide of 15aa coupled to the carrier protein KLH (*box*). **a** Dot-blots of different peptide concentrations incubated with the antibody α-TM20, secondary antibody and α-TM20 purified by immunoaffinity with SulfoLink columns, respectively. The incubation with the preimmune serum α-TM20 NI 1:500 was used as negative control for possible cross-reaction of the antibody. Note, the SDS-treated peptide sample on the membrane that was incubated with purified α-TM20, has no signal. α-TM20 detects the antigen only in its native state. **b** Dot-blots of different peptide concentrations. The purified antibody α-TM20 was incubated for 1 h at RT with different concentrations of intercepting peptide TM20 prior to the incubation of the membranes

Immunohistochemistry on sections of immature maize embryos

RNA dot-blots and immunoblots provide general information on the accumulation of RNA or protein. In order to observe the localization of the protein in specific cell types, the α-TM20 antibodies were used in immunocytochemical experiments. RNA blot has shown that TM20 is expressed in embryos up to 31 days after pollination (Stiefel et al. 1999). For this reason the first immunolocalizations were carried out with 20 DAP embryos where the accumulation of TM20 transcripts is maximum. Figure 4a shows the immunolocalization of TM20 in the section of a whole maize embryo 20 DAP labeled with Oregon-green 488. Specific fluorescence is mainly found in the young foliar primordia and in the apical meristem. In the lower epicotyl, the so-called mesocotyl, two strands of labeled cells can be observed. The scutellar node, or first node, of the embryo axis (Esau 1960) displays only a trace of labeled cells, but this is more intense in the radicular region. The labeling of TM20 in the root seems to be more intense in the outer cell layers of the central vascular cylinder. However,

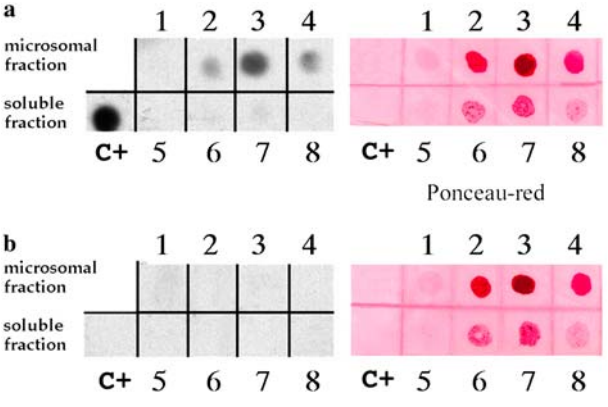

Fig. 3 Dot-blot analysis of microsomal fractions of maize embryos of ten DAP, 15 DAP, 20 DAP and 20 DAP without scutellum. **a** Dot-blot incubated with purified α-TM20 (1:250) and Ponceau-red staining of the PVDF membrane. **b** Dot-blot incubated with the secondary antibody (α-rabbit) as negative control. 1–4 microsomal fractions of P150 of maize embryos and 5–8 soluble protein fractions S150 precipitated with TCA 1 and 5:10 DAP; 2 and 6:15 DAP; 3 and 7:20 DAP; 4 and 8:20 DAP only the embryo axis, C+: positive control 1 μg peptide TM20

both the apical and the basal part of the embryo show more fluorescence than the scutellar node or the mesocotyl. No fluorescence was detected in the scutellum.

At higher magnification it is possible to observe the label restricted to some cell layers in the leaf primordia (Fig. 4b). At this stage of development it is still difficult to distinguish different cell types. However, the labeled cells may form part of the provascular tissue. The same pattern was observed in the root (Fig. 4f) and the mesocotyl (Fig. 4d). These files of cells link the coleoptile with the root but also connect the embryo axis to the scutellum, necessary for the transport of nutrients. At the cellular level, TM20 seems to be present only in the apical part of the cells and no fluorescence is found in the lateral or basal cell parts. This polarization effect was not found in the apical meristem where the label was observed throughout the cell cytoplasm with the exception of the nucleus. Controls without primary antibody confirm the specificity of the antibody by comparing the labeled tissues to sections of 20 DAP embryos (Fig. 4c, e, g).

The provascular TM20 localization was confirmed on transversal sections of 20 DAP embryos (Fig. 4h–j). The TM20 labeling pattern consists of spots located in the central part of the young leaves (arrows), whereas in the meristem the signal can still be observed all over the tissue without any obviously defined pattern (Fig. 4h–j). The result of the immunolocalization on transversal sections of maize embryo is in accordance with the data

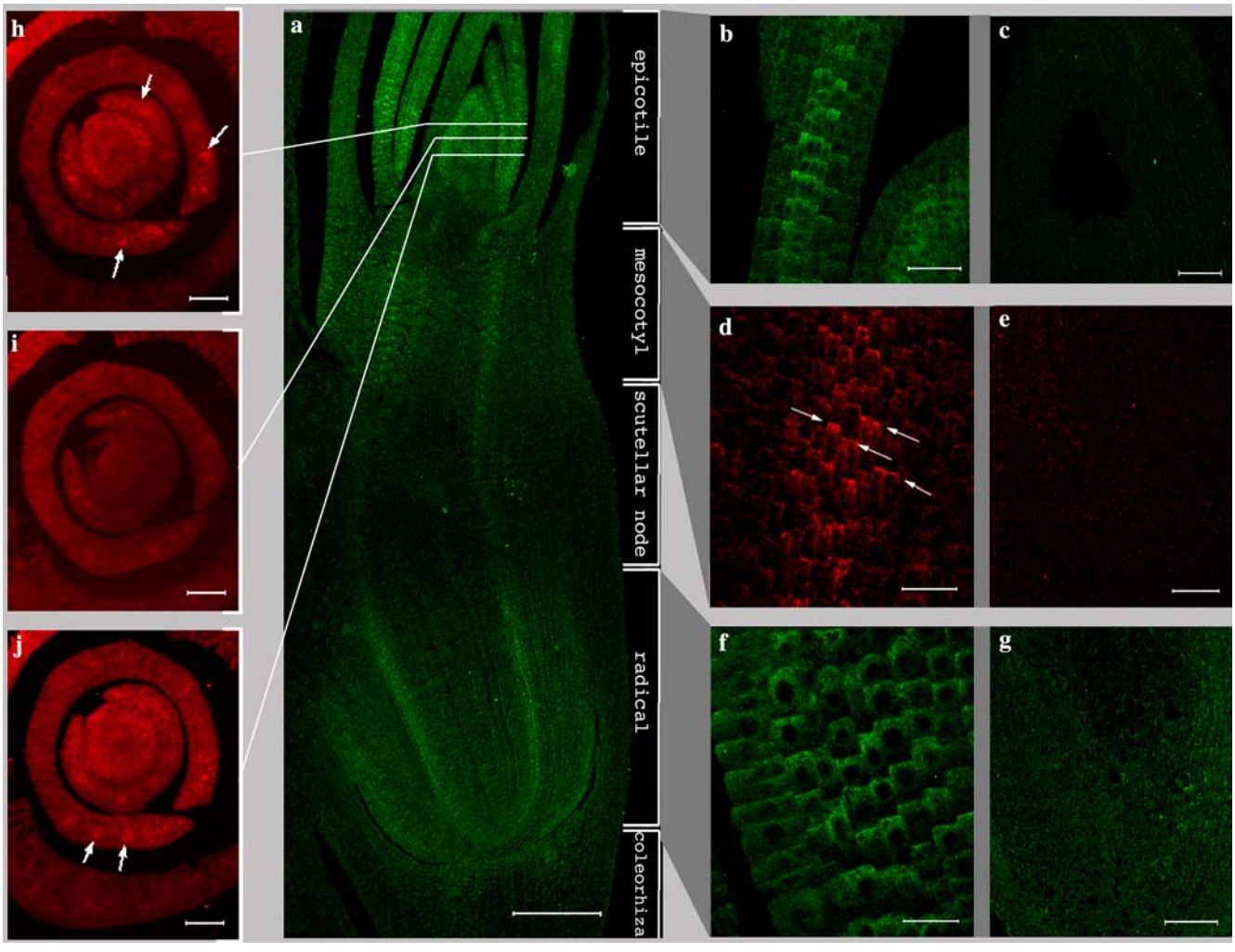

Fig. 4 Immunolocalisation of TM20 in paraffin-embedded sections of 20 DAP maize embryos applying the antibody α-TM20 purified by immunoaffinity. Visualization was achieved by confocal or fluorescence light microscopy. The secondary α-rabbit antibody used for the indirect labeling was conjugated to biotin. The fluorochrome (oregon-green 488 or rhodamine-red) was coupled to streptavidin. The negative controls were incubated only with the secondary antibody. **a** Longitudinal section of a 20 DAP embryo axis. TM20 label can be observed in the leaves, the shoot meristem, the mesocotyl and the radical. **b** Detail of young leaf primordia from 20 DAP embryo. TM20-label was found in the central cells of the leaf primordia in a polarized manner, accumulating fluorescence in the apical part of the cell. **c** Negative control of **b. d** Detail of the mesocotyl region. Specific cells of this region have polarized TM20-labeling. **e** Negative control of **d**. **f** Detail of the outer layers of the radical. The cells have the same polarized labeling as in the leaves and the mesocotyl. **g** Negative control of **f**. **h–j**: Transversal sections of the apical region of a 20 DAP maize embryo including the shoot apical meristem and two leaves. *Bar* = 200 μm (**a**), 41 mm (**b**), 50 μm (**c**), 20 μm (**d, f**), 40 μm (**e**), 80 μm (**g**), 100 μm (**h, j**)

of the in-situ mRNA hybridization of *TM20* (Stiefel et al. 1999). TM20 transcript accumulation was found in the leaf primordia, the vascular system of the coleoptile and the shoot meristem of transversal sections of 20 DAP maize embryos.

The cellular localization of the TM20 protein depends on the differentiation state of the cell

To study the pattern of TM20 localization in embryos a few days after the establishment of bilateral symmetry, immunohistochemistry on ten DAP embryos was performed. Due to the small size of the ten DAP embryos, whole mount immunocytochemical labeling was used, taking advantage of the virtual sectioning of confocal microscopy (Fig. 5). As in 20 DAP embryos, TM20 label was detected in the apical region of the ten DAP embryo (Fig. 5a). Strong fluorescence was observed in the central layers of the coleoptile and in the shoot apical meristem. The shoot meristem appears in the middle of the coleoptile and protrudes from the surrounding coleoptile tissue. In the basal part of the embryo axis (Fig. 5a) there was slight labeling, indicating the presence of TM20 in the early root. The polarization of the TM20 labeling at the cellular level observed in 20 DAP embryos (Fig. 4b, f) was not found in more immature embryos. Instead, the protein was present in nearly the whole cell in ten DAP embryos except in the cell nucleus (Fig. 5b). In all the cases where the resolution could be optimized, no indication for the presence of the protein in the cell wall was found (see Fig. 5b, bottom left, showing a straight and very thin cell wall). The observations indicate that the presence and the localization of TM20 may be dependent on the developmental stage of the embryo.

As a control of the homogeneous cytoplasmic localization of the TM20 protein in ten DAP embryos, monoclonal mouse antibodies against ATPase (Villalba et al. 1991), a known plasma membrane located protein, were used. Whole mount immunolocalization was carried out in ten DAP embryos and the antibodies detected the ATPase in the scutellum (Fig. 5e) and in the embryo axis (Fig. 5d). In scutellum cells (Fig. 5e) the label of the ATPase was restricted to the peripheral cytoplasm including the plasma membrane. The wavy form of the scutellum cells was traced by the red fluorescence emitted by Cyanine 3.

The ATPase labeling in cells of the embryo axis (Fig. 5d), however, gave the same pattern as the

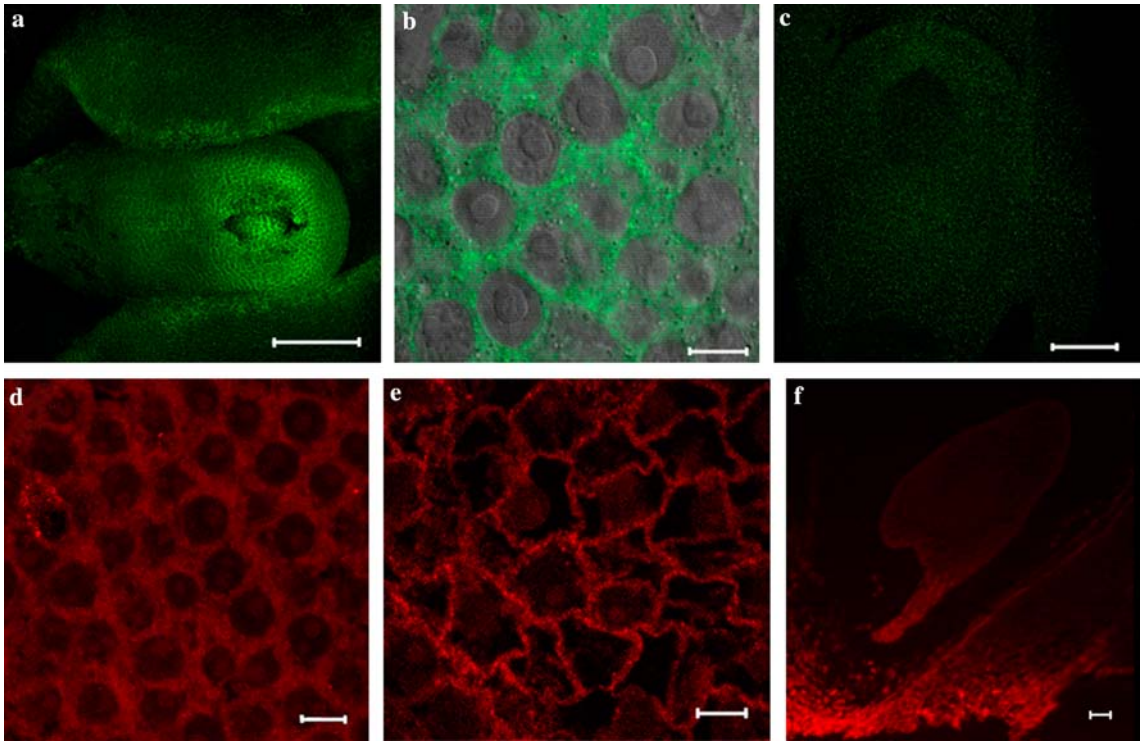

Fig. 5 Images of confocal microscopy of whole-mount immunolocalisation on ten DAP maize embryo. The secondary α-rabbit antibody used for the indirect labeling of TM20 was conjugated to biotin. The fluorochrome oregon-green 488 was coupled to streptavidin. **a** Immunolocalisation of TM20 on a maize embryo, clearly distinguishable are the embryo axis, the suspensor and the scutellum. **b** Overlay of cells from the shoot apical meristem: confocal and transmission image from a ten DAP embryo. Clearly seen is the fluorescence of the TM20-label all over the cell except inside the cell nucleus. No TM20-label in the cell wall could be observed (see bottom left, thin wall between cells) **c** Negative control; maize embryo incubated only with the secondary antibody. **d, e** Whole-mount immunolocalisation of a membrane-specific ATPase in the shoot apical meristem (**d**) and the scutellum (**e**) of a ten DAP maize embryo using antibodies α-ATPase and α-mouse conjugated with cyanine 3. **f** Immunolocalisation of TM20 in the null mutant *lachrima* as control for the specificity of the antibody α-TM20. No fluorescence label could be detected in the null mutant. *Bar* = 200 μm (**a**), 8 μm (**b**, **d**, **e**), 80 μm (**c**), 50 μm (**f**)

immunolocalization of TM20 in the same material (Fig. 5b). There was labeling throughout the cytoplasm but not in the nucleus, nor in the cell wall as seen in appropriate sections. This similarity of the labeling-pattern between TM20 and ATPase may provide evidence for similar processing and trafficking of these two proteins. It cannot be excluded that the stage of differentiation of the cells also plays a role in the localization of the ATPase as seen before for TM20. As an additional control for the antibody specificity the null mutant *lachrima* was incubated with α-TM20 antibody (Fig. 5f). The lack of fluorescence label in the mutant embryo indicates that no other embryo specific proteins are apparently detected by the antibody.

Microinjection in oocytes of *Xenopus laevis*

In addition to the identification of putative homologues and the immunological characterization of TM20, a cellular approach to study the possible function of TM20 was chosen. To demonstrate that TM20 could be involved in polar hormone transport, cRNA of TM20 was microinjected in oocytes of *Xenopus laevis*. For this purpose the entire coding sequence of the TM20 gene was cloned into a transcription vector and transcribed in vitro. TM20 injected oocytes were used for transport assays with radiolabeled indolacetic acid (^{3}H-IAA) as an example of a possible molecule that could be transported by the protein. Confocal immunofluorescence detected TM20 at the oocyte plasma membrane (Fig. 6a). The signal is absent in non-injected oocytes (Fig. 6b).

The TM20 injected oocytes and the control oocytes were placed in choline-Cl sample buffer at pH 5,5 and were incubated in the presence of ^{3}H-IAA for 5 min, 15 min, 30 min and 60 min. The oocytes were washed with sample buffer and the amount of ^{3}H-IAA inside the oocytes was measured. Figure 6c shows the ^{3}H-IAA accumulation in oocytes injected with TM20 and in control oocytes in relation to the incubation time. A difference in IAA accumulation between TM20 injected oocytes and control oocytes could be observed, which increased depending on the incubation time. The decrease in auxin accumulation in TM20-expressing oocytes compared to non-injected oocytes was 33–50% after 60 min of incubation in five independent experiments ($P \leq 0.05$). The accumulation of auxin at pH 7.5 and 8.5 was about 20 and 25 times lower than at pH 5.5 in non-injected oocytes. No significant differences in auxin accumulation were observed between TM20 and non-injected oocytes at these pHs (data not shown). The fact that auxin has a pKa of 4.75, suggests that auxin at pH 5.5 enters the oocyte in its neutral form. Moreover, the uptake in non-injected oocytes is not saturated up to 500 μM auxin concentration (data not shown). This suggests that the uncharged auxin could enter the oocyte by diffusion or by a low affinity mechanism. To show that the observed difference in IAA accumulation between oocytes injected with TM20 and not injected is due to the auxin transport activity of TM20, the same experiment was performed adding the known inhibitor of polar auxin transport NPA to the incubation buffer. As shown in Fig. 6d a statistically significant accumulation difference can be observed in the absence of NPA,

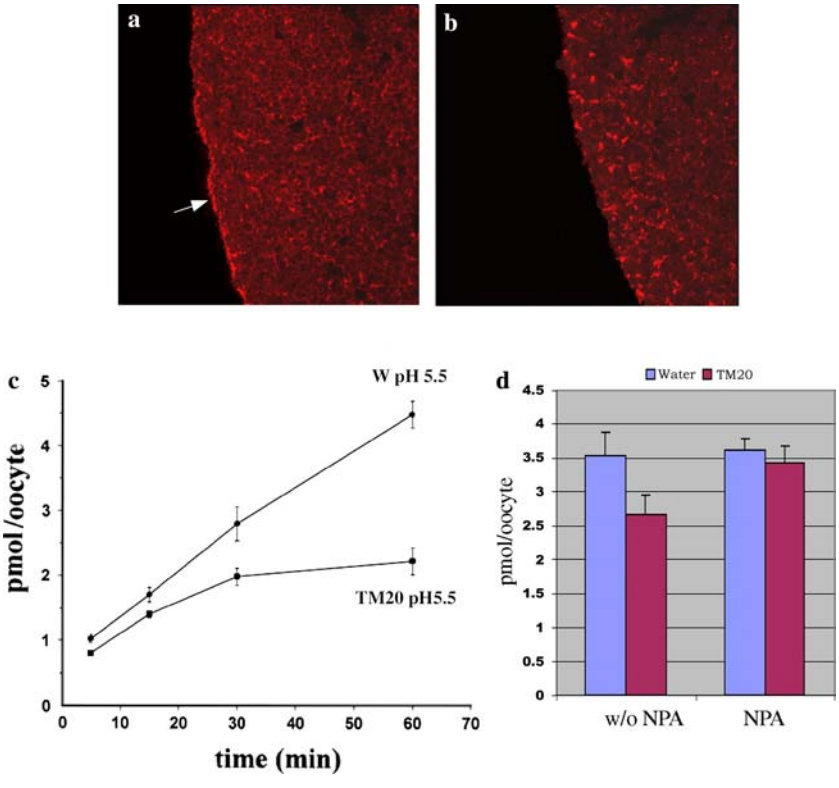

Fig. 6 Expression of TM20 in oocytes of *Xenopus laevis* and transport assay with radiolabeled ^{3}H-IAA: immunolocalization of TM20 in oocytes. cRNA of TM20 was injected in oocytes and incubated for three days. Subsequently the oocytes were processed for immunocytochemistry. **a** Oocytes injected with cRNA of TM20, **b** Oocytes injected with water. **c** Accumulation of ^{3}H-IAA in oocytes injected with cRNA of TM20 and control oocytes injected with water. At pH 5.5 the accumulation difference of ^{3}H-IAA between TM20 expressing oocytes and control oocytes, after one hour of incubation, is up to 50%. Data presented represent the mean and SD of seven individual oocytes for each point of measurement. **d** Change of IAA accumulation by addition of 5 μM NPA. The addition of NPA modifies the accumulation of IAA in oocytes injected with TM20

meanwhile the levels of auxin accumulated in injected and non-injected oocytes in the presence of NPA are almost the same.

The data obtained point to a possible auxin transport activity of TM20. However, it is not clear whether this accumulation difference is caused by a reduced uptake of IAA or efflux activity produced by TM20.

Discussion

The protein encoded by *TM20* is a new class of transmembrane plant proteins. In order to identify its possible function different strategies have been used. This has involved the production of antibodies against a region of the protein that is conserved in the two cereal species analyzed, maize and rice. No protein with a similar structure was found when analyzing the Arabidopsis genome although a group of proteins having four transmembrane domains sharing similarity to the cereal proteins has recently been described (Becerra et al. 2004), pointing towards different specific proteins or groups of proteins having homologous functions in dicot plant species.

The observed localization of the TM20 protein using affinity purified polyclonal antibodies in sections and in whole mount immunocytochemical studies in maize embryos at different developmental stages (10, 15 and 20 DAP) agrees with the described accumulation of TM20 transcripts observed by in-situ hybridisation (Stiefel et al. 1999). At 10 and 15 DAP, TM20 is located mainly in the apical region of the embryo axis, including the developing coleoptile and the shoot apical meristem, and also in the radicle. At 20 DAP, TM20 is localized mainly in the young leaves and the shoot apical meristem, but it is also present in cell layers surrounding the vascular tissue along the embryo apical-basal axis. In the radicle, TM20 was observed in vascular and provascular cells and in cortical cells. It has been shown that the vascular tissue is the main route of apical-basal polar auxin transport in plants, and even though the molecular details remain hypothetical, the apical-basal transport of IAA itself is experimentally well established (Jones 1990; Berleth et al. 2000). The role of auxin in the formation of vascular strands and vein patterning in plants is widely documented (Mattsson et al. 1999; Sieburth 1999; Berleth and Mattsson 2000). The localization of TM20 in the tissues of the vascular system correlates with the main flux of polar auxin transport. Several putative auxin carrier proteins have been cloned from *Arabidopsis* in the last few years. The *AUX1* gene codes for a presumptive auxin influx carrier, while it has been suggested that members of the AtPIN gene family act as auxin efflux carriers, providing the plant with a system to control the establishment and direction of an auxin gradient required for the different stages of development of the plant (Müller et al. 1998; Gälweiler et al. 1998; Friml 2003; Friml et al. 2002a, 2002b, 2003). In indirect immunolabeling studies the polar localization of these proteins at the apical or basal part of the cell has been observed. The subcellular localization of TM20 in cells of maize embryos, reported here, is partly similar to the localization observed for AtPIN2 and AUX1 in *Arabidopsis* roots (Müller et al. 1998; Marchant et al. 1999). In contrast to AtPIN2 and AUX1, TM20 localization is not restricted exclusively to the apical plasma membrane, but is also observed in the cytoplasm below the plasma membrane in a more diffused manner, indicating that TM20 may be found, for example, in vesicles directed to the plasma membrane. This suggests that the transport of TM20 by vesicles towards the plasma membrane may be an important step in the regulation of its activity. TM20 has no sequence similarity to the putative auxin carriers of *Arabidopsis*, and may be part of an auxin transport system that operates in an alternative pathway not described up to now, or it may act in the same transport pathway interacting with the PIN proteins.

The cellular distribution observed for TM20 is dependent on the developmental stage of the maize embryo and the differential state of the cells. In cells of the coleoptile and the shoot apical meristem of ten DAP embryos; TM20 is distributed over the whole cell lumen, except in the nucleus. The same distribution is found in non-differentiated meristematic cells of more developed embryos (20 DAP). The asymmetric distribution observed in more differentiated tissues seems to take place parallel to the formation of the apical basal asymmetry of the embryo and cell differentiation. Even though little is known about the auxin levels in embryos at the transition and coleoptile stage, polar auxin transport is an essential process for the formation of apical-basal asymmetry (Liu et al. 1993) and has to be established before or during this developmental stage. The definition of a polar localization for TM20, dependent on the developmental stage, may overlap with the formation of the auxin gradient and relate the localization of TM20 to this process. A similar pattern was also found in 10 DAP maize embryos, using specific antibodies against a plasma membrane located ATPase (Villalba et al. 1991). In the more differentiated scutellum cells the ATPase is found in the plasma membrane, while ATPase distribution in cells of the embryo axis is the same as the localization of TM20 in the same cell type. These results indicate that some membrane integral proteins are positioned in the plasma membrane depending on the state of differentiation of the cells.

The expression of membrane transport genes in heterologous systems such as oocytes of *X. laevis*, yeast, insect or mammalian cells is an important approach to study the functional properties of the corresponding transporter proteins. Here we report the expression of cRNA of TM20 in oocytes of *X. laevis* to test the ability of TM20 to stimulate hormone transport through membranes. As an example IAA was used and the results obtained from these studies indicate that radiolabeled IAA can enter the oocytes by diffusion and is

trapped and accumulated in the cells. Comparing the accumulation of IAA in oocytes injected with TM20 and control oocytes, it was observed that the control oocytes accumulated up to 50% more radiolabeled IAA than the oocytes injected with TM20 at pH 5.5. These results suggest that the presence and the activity of TM20 in the plasma membrane could facilitate the efflux of IAA from the oocyte, which works against the incoming IAA molecules. This model is consistent with the increase in the difference in IAA accumulation dependent on the incubation time. The IAA transport observed in oocytes expressing TM20 indicates that these oocytes can provide the machinery for the transport of IAA across the membrane. This fact is supported by the observation that the known auxin transport inhibitor NPA is able to neutralize the described accumulation difference of IAA. Nevertheless, *X. laevis* oocytes are an animal system and distinct activity of TM20 in plant cells cannot be excluded. It has also to be pointed out that no specificity in the transport of auxin can be concluded from our studies, but that the protein has an effect on the transport across membranes of small molecules including auxin. The difficulties of the functional study of plant-specific transport systems are reflected in the lack of data on the functional characterization of the putative auxin carriers described in *Arabidopsis*. In addition, alternative auxin transport systems may operate not only in distinct cell types, but also at different stages of development.

The possible function of TM20 in relation to polar auxin transport correlates with the phenotype of *lachrima* mutants. These mutants have arrested embryo development in the transition stage and they do not develop bilateral symmetry. The same phenotype occurs in embryos cultured in the presence of auxin transport inhibitors, or growing with an excess of exogenous auxin (Fischer and Neuhaus 1996; Liu et al. 1993). However, due to the limited number of maize *lachrima* embryos and alleles of these mutants, the genetic identity between *TM20* and *lachrima* cannot be confirmed. Nevertheless, the clear inhibition of *TM20* expression in the *lachrima* mutants and the transporter-like characteristics of TM20 suggest the involvement of the TM20 protein in the processes disturbed in *lachrima*. The arrest at early stages of embryogenesis and the failure in the transition to a bilateral symmetrical structure has also been described for *Arabidopsis* mutants defective in the *ABP1* gene that binds auxin and it has been suggested that it mediates auxin-induced cell processes (Chen et al. 2001). The phenotypes produced by perturbations involving auxin action may interfere at different levels such as auxin synthesis, polar auxin transport, auxin perception and auxin metabolism (Berleth and Sachs 2001). This is the case for mutants such as *bodenlos*, *monopteros*, *pin1-1* and *emb30*/*gnom* which are affected at distinct levels of auxin action. If we consider the main expression of *TM20* in the very early stages of embryogenesis shown by northern blot analysis (Stiefel et al. 1999), TM20 may form part of a hormone transport system active during embryogenesis and adapted to the necessities of the developing embryo, different to the transport system in the adult plant.

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