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The galactose-binding and mannose-binding jacalin-related lectins are located in different sub-cellular compartments

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Abstract A galactose-specific and a mannose-specific lectin of the family of the jacalin-related lectins have been localized by immunofluorescence microscopy. The present localization studies provide for the first time unambiguous evidence for the cytoplasmic location of the mannose-specific jacalin-related lectin from rhizomes of *Calystegia sepium*, which definitely differs from the vacuolar location of the galactose-specific jacalin from *Artocarpus integrifolia*. These observations support the hypothesis that the galactose-specific jacalin-related lectins evolved from their mannose-specific homologues through the acquisition of vacuolar targeting sequences.

Key words: Calsepa; Jacalin; Lectin; Localization; *Artocarpus*; *Calystegia*

1. Introduction

During the last few years important advances have been made in the biochemistry, physiology and molecular biology of the family of the so-called jacalin-related lectins. This family of lectins comprises all carbohydrate-binding proteins that share sequence similarity with the Thomson-Friedenreich- or T-antigen-specific agglutinin from jack fruit (*Artocarpus integrifolia*) seeds, which received the trivial name 'jacalin' [1]. Until recently the jacalin-related lectins were considered a small family of lectins occurring in a few genera of the family Moraceae (namely *Artocarpus* and *Maclura*). However, evidence is accumulating now that the galactose-specific Moraceae lectins are just a small subgroup of an extended but heterogeneous family of evolutionary-related proteins, which comprises mainly mannose-binding lectins. During the last five years lectins with an exclusive specificity towards mannose have been identified and characterized in rhizomes of the hedge bindweed (*Calystegia sepium*) (Convolvulaceae family) [2], in tubers of the Jerusalem artichoke (*Helianthus tuberosus*) (Asteraceae family) [3], in leaves from salt-stressed rice (*Oryza sativa*) plants (Gramineae family) [4] and in the pulp of banana (*Musa acuminata*) fruits (Musaceae family) [5]. All these lectins share a reasonable sequence similarity with jacalin but exhibit a different specificity. In addition, these mannose-specific lectins consist of uncleaved protomers of about 150 amino acid residues whereas jacalin is built up of cleaved protomers consisting of a short (20 amino acids) β-chain and a long (133 amino acids) α-chain. Another major difference between the mannose-specific jacalin-related lectins and jacalin concerns their biosynthesis, processing and topogenesis. Jacalin is synthesized as a preproprotein, which undergoes a complex series of co- and post-translational processing steps [6] and is presumed to be located in the vacuolar compartment. In contrast, the mature polypeptides of the mannose-specific jacalin-related lectins correspond to the entire open reading frame of the respective lectin genes [3–5,7] and therefore are supposed to be synthesized and located in the cytoplasm.

Besides the above mentioned mannose-specific lectins, sequences encoding similar putative proteins have been identified in several other plant species like barley (*Hordeum vulgare*) [8], sweet potato (*Ipomea batatas*) [9] and arabidopsis. In addition, sequences encoding putative chimeric proteins containing a domain corresponding to a single protomer of the mannose-specific jacalin-related lectins have been identified in barley [10], wheat (*Triticum aestivum*) and also in arabidopsis. Finally, both in arabidopsis and *Brassica napus* [11,12] numerous sequences have been found encoding putative proteins consisting of 2–5 repeats showing sequence similarity to jacalin. Some of the putative *B. napus* multi-domain proteins have been identified as the polypeptides of the so-called myrosinase-binding proteins and have been shown to possess mannose-binding activity [11].

Though the physiological role of the jacalin-related lectins is still not understood there are several indications that especially the mannose-specific subfamily fulfils an important role in the plant. Most of the currently known mannose-specific jacalin-related lectins are apparently stress-related. For example, rice plants express the mannose-specific lectin only after induction with either NaCl or jasmonate [4]. Similarly, the putative chimeric wheat and barley proteins with a jacalin domain have been identified as jasmonate-induced proteins [10]. The same holds true for the extended family of jasmonate-inducible myrosinase-binding proteins in arabidopsis and other Brassicaceae species. It is also interesting to note that the putative protein encoded by the RTM1 gene, which controls restriction of long-distance movement of tobacco etch virus in arabidopsis corresponds to a single jacalin-related domain [13]. Taking into consideration that the physiological role of these (putative) lectins eventually relies on their mannose-binding activity it is of outermost importance to exactly define in which cell compartment these proteins are located. Based on the absence of a signal peptide it is tempting to speculate that all these proteins are located in the cytoplasm.

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Abbreviations: Calsepa, *Calystegia sepium* agglutinin; PBS, phosphate-buffered saline

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To corroborate this presumed cytoplasmic location the subcellular distribution of the mannose-specific lectin in rhizomes of *C. sepium* was determined by immunofluorescence microscopy. Convincing evidence was obtained that this lectin is located, indeed, in the cytoplasm. Since parallel experiments indicated that jacalin is located in the vacuolar compartment it can be concluded that the mannose-specific and galactose-specific jacalin-related lectins are located in different sub-cellular compartments. This obvious differential location is discussed in view of the molecular evolution and physiological role of the family of jacalin-related lectins.

2. Materials and methods

2.1. Plant material

Rhizomes of hedge bindweed (*C. sepium* (L.) R.Br.) were collected in Leuven in winter. Jack fruit (*A. integrifolia* Lamark) seeds were collected from a mature fruit.

2.2. Isolation of C. sepium agglutinin (Calsepa) and jacalin

Calsepa was isolated as described previously [7]. Dry seeds (50 g) from jack fruit were imbibed overnight in distilled water at 2°C and homogenized in a Waring blender in 500 ml of 0.2 M NaCl. The homogenate was centrifuged ($3000\times g$; 5 min) and the pH of the supernatant adjusted to 7.5 with 1 N NaOH. The crude extract was centrifuged at $9000\times g$ for 15 min and the resulting supernatant filtered through filter paper (Whatman 3 MM). The cleared extract was applied onto a column (2.5×10 cm; approximately 50 ml bed volume) of galactose-Sepharose 4B equilibrated with phosphate-buffered saline (PBS; 1.5 mM KH_2PO_4/10 mM Na_2HPO_4/3 mM KCl/140 mM NaCl, pH 7.4). After loading the extract, the column was washed with PBS until the A_{280} fell below 0.01. The bound jacalin was eluted with a solution of 0.1 M galactose in PBS, dialyzed against PBS and stored at −20°C until use.

2.3. Preparation of monospecific antibodies against Calsepa and jacalin

Polyclonal antibodies were raised against Calsepa and jacalin. Male New Zealand white rabbits were injected with 1 mg purified protein dissolved in PBS and emulsified in 1 ml of Freund's complete adjuvant. Five booster injections with 1 mg purified protein in 1 ml of PBS were given with 10-day intervals. Ten days after the final injection, blood was collected from an ear marginal vein. After clotting, the crude serum was prepared by centrifugation ($3000\times g$ for 5 min) and processed immediately by affinity chromatography on the appropriate immobilized antigens.

Purified Calsepa and jacalin were coupled to Sepharose 4B using the divinylsulfone method. Sepharose 4B was extensively washed with distilled water on a Büchner funnel and equilibrated with a solution of 0.5 M Na_2CO_3 adjusted to pH 11 with HCl. Fifty ml of settled gel were suspended in 50 ml of the same carbonate buffer and supplemented with 5 ml divinylsulfone. The suspension was stirred for 3 h at room temperature, transferred to a Büchner funnel and extensively washed with carbonate buffer pH 11 followed by distilled water. Ten ml (settled gel) aliquots of the activated Sepharose 4B were subsequently suspended in 20 ml of a 0.5 M solution of Na_2CO_3/$NaHCO_3$ (pH 10) containing 50 mg of either Calsepa or jacalin. The mixtures were incubated at 37°C for 15 h under continuous agitation. Following this procedure >98% of the proteins was immobilized to the Sepharose 4B. After coupling, the Sepharose 4B was again transferred to a Büchner funnel and extensively washed with the Na_2CO_3/$NaHCO_3$ (pH 10) buffer and distilled water. Unreacted vinyl groups were blocked by incubation of the washed matrix in 50 ml of a 0.1 M solution of Tris–HCl (pH 8.7) for 6 h. After blocking, the coupled antigens were transferred to single fritted 25 ml columns (International Sorbent Technology, Mid Glamorgan, UK) and equilibrated with PBS.

Total serum was loaded on the column (filled with approximately 10 ml of the appropriate immobilized antigen) at a flow rate of 1 ml/min. After loading, the column was washed with PBS until the A_{280} fell below 0.01. Bound antibodies were desorbed with a solution of 20 mM unbuffered 1,3-propane diamine (pH 11) and collected (under continuous stirring) in 10 ml of 0.2 M Tris–HCl (pH 7.0) containing 0.5 M NaCl. Immediately upon completion of the elution, the affinity-purified antibodies were dialyzed against PBS for 15 h at room temperature.

2.4. SDS–PAGE and Western blot analysis

Purified proteins were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) using 12.5 to 25% (w/v) acrylamide gradient gels as described by Laemmli [14]. After electrophoresis proteins were electroblotted on an Immobilon P membrane (Millipore, Bedford, MA, USA) for Western blot analysis. Before immunodetection the free binding sites on the membrane were blocked with 5% bovine serum albumin (BSA) in TSB (10 mM Tris, 150 mM NaCl, 0.1% Triton X-100, pH 7.6) for 1 h at room temperature. After washing the membrane with TSB for 5 min the membrane was consecutively treated with primary antibody (diluted 1/500; overnight incubation at room temperature), goat-anti-rabbit antibody (diluted 1/30; 1 h incubation at room temperature) and peroxidase–anti-peroxidase-complex (diluted 1/300; 1 h incubation at room temperature). After every treatment the membrane was washed three times with TSB for 5 min. Prior to the immunodetection the membrane was washed for 5 min with 0.1 M Tris–HCl, pH 7.6. The peroxidase reaction was carried out in a fresh solution of 0.1 M Tris–HCl pH 7.6 containing 0.7 mM 3,3′-diaminobenzidine tetrahydrochloride and 0.01% (v/v) H_2O_2. The reaction was stopped by washing the membrane in distilled water.

2.5. Immunocytochemistry

Small pieces of fresh *C. sepium* rhizomes were fixed with 4% paraformaldehyde/0.1% Triton X-100 in PBS, embedded in polyethylene glycol (PEG) and cut as described previously [15]. Cross-sections (2 μm thick) were immunolabeled by incubation with purified primary antibodies raised against Calsepa (diluted 1:250 in PBS containing 5% BSA and 1 mg/ml goat IgG) followed by a goat-anti-rabbit-IgG antibody conjugated with BODIPY (Molecular Probes, Eugene, OR, USA). After immunolabeling, sections were mounted in citifluor/glycerol. Control experiments were performed by omitting the primary antibody. The fluorescence of immunolabeled Calsepa was visualized with a Zeiss 'Axioskop' epifluorescence microscope using the proper filter combination. Micrographs were taken by a CCD camera (Sony, Japan) and processed through the Photoshop program (Adobe, Seattle, WA, USA).

Dry seeds of *A. integrifolia* were imbibed for 24 h on moistened filter paper. Small pieces were excised and fixed and embedded as described above for the *Calystegia* rhizomes. Cross-sections of 2 μm thick were immunolabeled by incubation with purified primary antibodies raised against jacalin (diluted 1:500 in PBS containing 5% BSA and 1 mg/ml goat IgG) and further treated as described above for the sections from the *Calystegia* rhizomes. To visualize subcellular structures immunolabeled sections were analyzed in parallel by differential interference contrast (Nomarski optics) using the Zeiss 'Axioskop' microscope.

3. Results and discussion

3.1. Nomenclature

Since there is some confusion about the terminology used to designate the different members of the family of jacalin-related lectins a brief survey is given of the nomenclature used here. Jacalin is the trivial name of the galactose-specific lectin from seeds of *A. integrifolia* (family Moraceae). All lectins sharing sequence similarity with jacalin are considered jacalin-related lectins. The family of jacalin-related lectins is subdivided in two subfamilies which according to their specificity are referred to as mannose-specific and galactose-specific jacalin-related lectins, respectively. Structural domains equivalent to a single jacalin protomer are usually referred to as 'jacalin-domains'.

3.2. Control of the monospecificity of the purified antibodies

Since a correct interpretation of the results obtained by immunofluorescence microscopy depends primarily on the

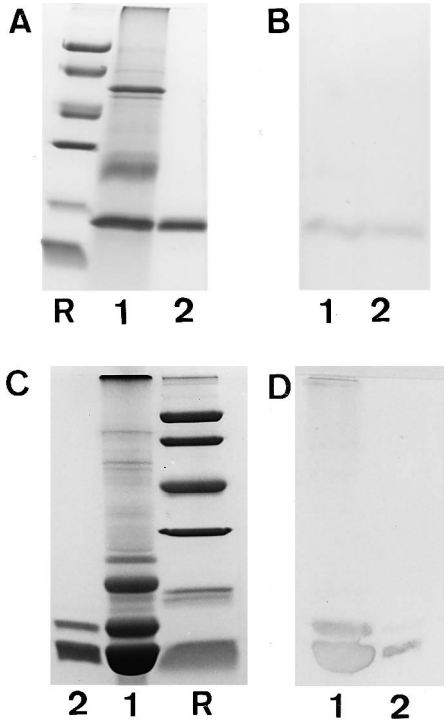

Fig. 1. Control of the monospecificity of the purified antibodies against Calsepa and jacalin. SDS–PAGE and Western blot analysis of a crude protein extract from *C. sepium* rhizomes developed with affinity-purified antiserum against Calsepa are shown in (A) and (B), respectively. Samples were loaded as follows: lanes 1, 100 μl total extract from *Calystegia* rhizomes; lanes 2, 20 μg purified Calsepa; lane R, molecular mass reference proteins. SDS–PAGE and Western blot analysis of a crude protein extract from jack fruit seeds developed with affinity-purified antiserum against jacalin are shown in (C) and (D), respectively. Samples were loaded as follows: lanes 1, 100 μl total extract from jack fruit seeds; lanes 2, 20 μg purified jacalin; lane R, molecular mass reference proteins. Note that jacalin is the predominant protein in the crude extract. The upper (minor) band in the jacalin preparation corresponds to the α-chain of the glycosylated isoforms, whereas the major band represents the α-chain of the unglycosylated isolectins. The β-chain is not visible. Molecular mass reference proteins (lane R) were lysozyme (14 kDa), soybean trypsin inhibitor (20 kDa), carbonic anhydrase (30 kDa), ovalbumin (43 kDa), BSA (67 kDa) and phosphorylase b (94 kDa).

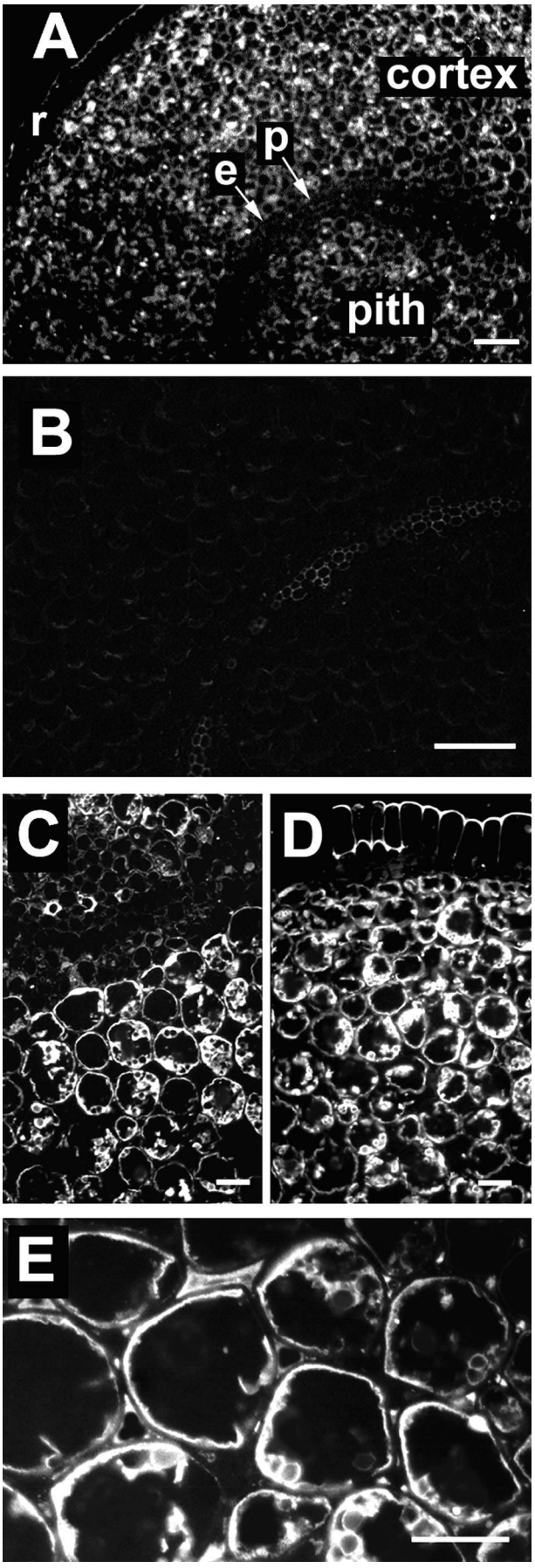

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Fig. 2. Immunolocalization of Calsepa in cross-sections of rhizomes of *C. sepium*. The lectin is visualized by immunodecoration with the corresponding purified anti-Calsepa antibody followed by a BODIPY-conjugated secondary antibody. A: Micrograph showing an overview of a cross-section through the rhizome labeled with anti-Calsepa. Note the label restricted to the cortex and the pith. The vascular tissues, the pericycle (p), the endodermis (e) as well as the rhizodermis (r) exhibit only some autofluorescence of the lignin containing cells. B: Control performed by omitting the first antibody. Minor autofluorescence is visible due to the cell walls of xylem-related cells. C: Inner part of a cross section of a rhizome labeled with anti-Calsepa. The pith as well as the cortex are labeled, whereas the vascular tissues, the pericycle and the endodermis do not show any label. D: Marginal part of a cross section of a rhizome. In contrast to the strong label within the cortex the rhizodermis is free from any labeling. E: Detail of (C) showing cells of the pith. Fluorescent signals of immunodecorated Calsepa are mainly restricted to the cytoplasm, whereas some label occurs within the intercellular space. Bars represent 100 μm in (A) and (B) and 25 μm in (C)–(E), respectively.

monospecificity of the antibody preparations, the specificity of the final preparation of anti-Calsepa antibodies was carefully checked by Western blot analysis. A total protein extract from the rhizomes was analyzed by SDS–PAGE, blotted and incubated with the presumed monospecific antibodies against Calsepa. To trace the running position of the lectin, purified Calsepa was run in adjacent slots. The antibody preparation reacted exclusively with Calsepa and showed no cross-reaction with any other protein when the antiserum was used at the same concentration as for the immunocytochemistry (Fig. 1A,B). Accordingly, one can reasonably assume that that the results of the immunofluorescence are reliable.

The monospecificity of the anti-jacalin was assayed in the same way. A total protein extract from the jack fruit seeds was analyzed by SDS–PAGE, blotted and treated with the purified antibodies against jacalin. The antibodies reacted exclusively with the lectin and did not cross-react with other proteins in the seed extract (Fig. 1C,D).

3.3. Localization of Calsepa in the cells of the rhizomes and jacalin in the cells of jack fruit seeds

The cellular and sub-cellular localization of Calsepa within rhizomes of *C. sepium* was studied by an immunocytological technique. Rhizomes embedded in PEG were sectioned and immunolabeled with purified antibodies raised against Calsepa. Calsepa could be detected in the cortex and the pith of rhizomes (Fig. 2A,C,D). Other tissues of rhizomes like vascular tissues, pericycle, endodermis and rhizodermis did not exhibit any label. Control experiments performed by omitting the first antibodies showed that there was no unspecific binding of the secondary antibody (Fig. 2B). However, some fluorescence was visible in the vascular tissues and epidermal cells. This was caused by the autofluorescence of cell walls containing phenolic compounds. In a cross-section of cells of the pith and the cortex the vacuole is the dominant organelle. The cytoplasm is visible only as a thin layer adjacent to the cell wall. As shown in Fig. 2E, Calsepa is predominantly located in the cytoplasm. A less prominent staining is also visible at some positions in the intercellular space. No label was detectable within the large vacuoles, which appeared in this case as a dark area in the center of the cells. According to these results Calsepa can be considered a cytoplasmic protein. The origin of the lectin present in the intercellular space is not clear. Possibly, it is released in the apoplast from in vivo damaged cells.

To compare the location of Calsepa with that of a presumed vacuolar galactose-specific jacalin-related lectin imbibed jack fruit seeds were processed for immunolabeling of the well-characterized jacalin. As shown in Fig. 3A, jacalin is present in all living cells of the cotyledons except the xylem-related cells such as tracheids, which did not exhibit any label (arrows in Fig. 3B). Controls performed by omitting the first antibody exhibited only some autofluorescence (Fig. 3C). Because the label within the cells appeared equally distributed, cross-sections of jack fruit cotyledons were analyzed by differential interference contrast to visualize subcellular structures

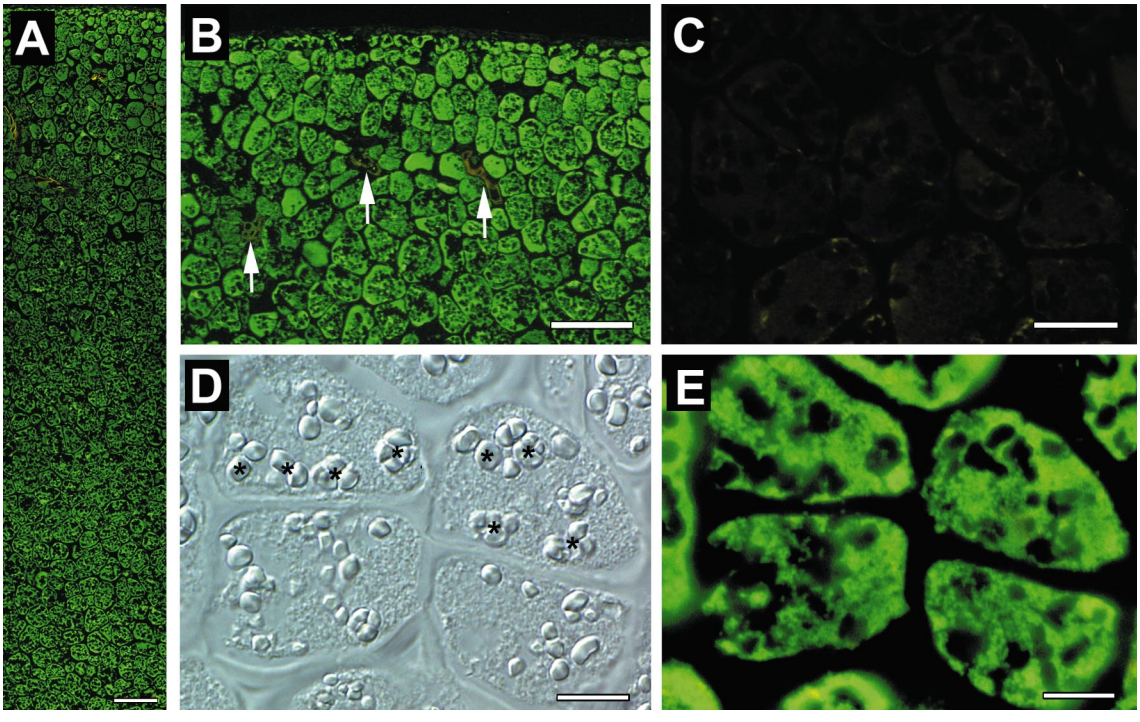

Fig. 3. Immunolocalization of jacalin in sections of seeds of *A. integrifolia*. Jacalin is visualized by immunodecoration with a purified anti-jacalin antibody followed by a BODIPY-conjugated secondary antibody. A: Micrograph showing an overview of a section through a cotyledon. Note the label distributed over the whole area of the section. B: Detail of (A) showing the epidermal cell layer and its adjacent region of a cotyledon. The tracheids exhibit only some brownish autofluorescence of the lignin (arrows), whereas the parenchymatic cells exhibit strong fluorescence due to the presence of immunolabeled jacalin. C: Control performed by omitting the first antibody. The concomitant region to (D) and (E) is shown. A very low autofluorescence is visible. D: Detail of a cross-section of the cotyledon. The image is taken by differential interference contrast to visualize subcellular structures as starch grains (*) and storage vacuoles. Note the vesicular appearance of small storage vacuoles. E: Same section as in (D) visualizing the sub-cellular distribution of jacalin. The cell area containing storage vacuoles exhibited label, black dots were caused by starch grains which are not labeled by the antibody. Bars represent 100 μm in (A), 50 μm in (B), 25 μm in (C) and 10 μm in (D) and (E), respectively.

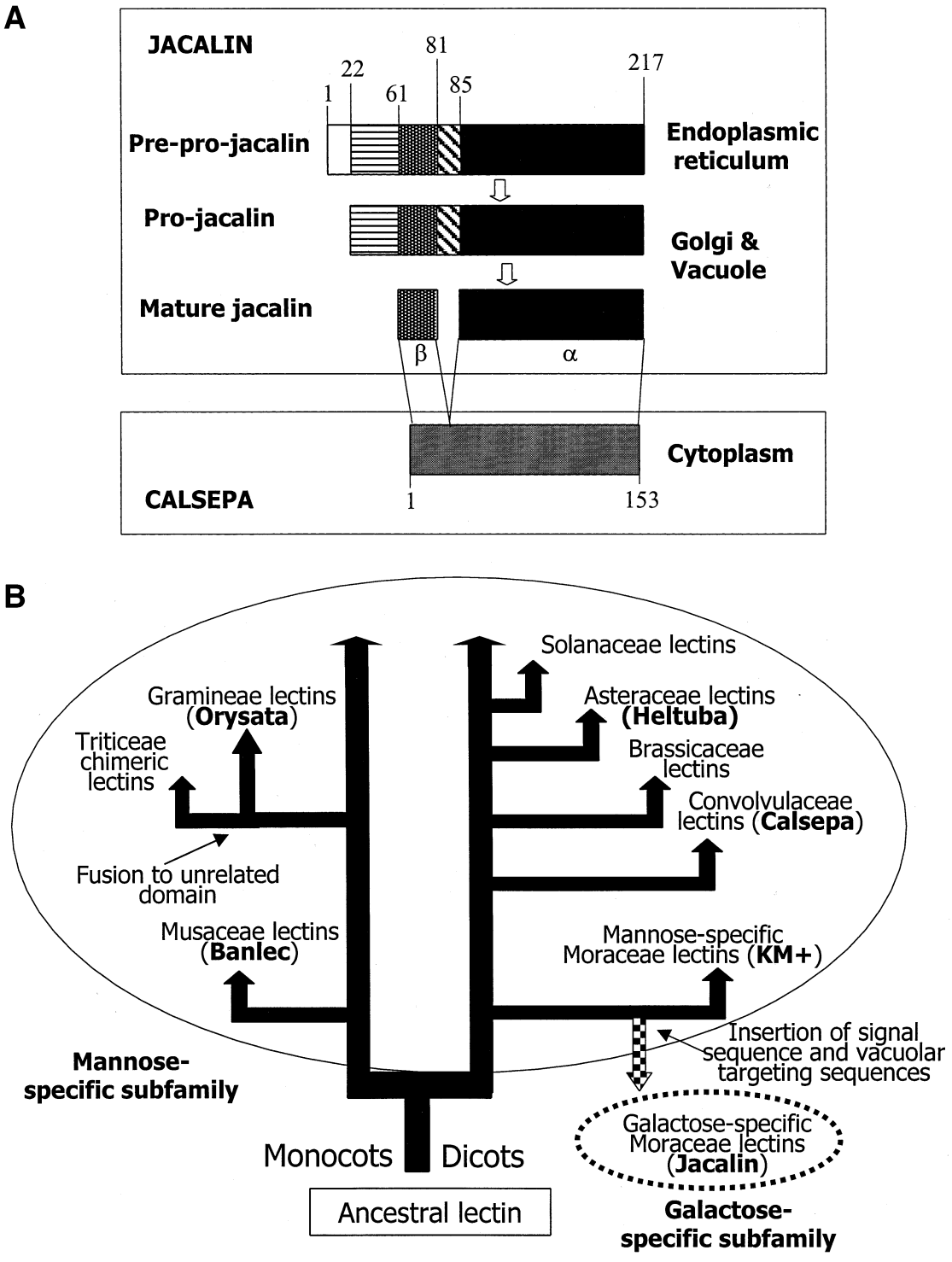

Fig. 4. A: Differences in biosynthesis, processing and targeting of mannose- and galactose-specific jacalin-related lectins as exemplified by Calsepa and jacalin, respectively. Calsepa is synthesized in the cytoplasm and undergoes no processing. The mature protein corresponds to the entire open reading frame. Jacalin is synthesized on the ER as a preproprotein. Cotranslational cleavage of 21 amino acid residues yields projacalin. Removal of a 39 amino acid residue N-terminal propeptide and a linker tetrapeptide yields the mature lectin protomer consisting of an N-terminal β-chain and a C-terminal α-chain of 20 and 133 amino acid residues, respectively. The numbering corresponds to the position of the amino acids in the primary translation products of the jacalin and Calsepa genes. The alignment shows that the overall structure of the mature jacalin protomer corresponds exactly to that of Calsepa (except the fact that jacalin protomer is cleaved). At the level of the primary translation products jacalin possesses three extra sequences: a signal peptide, an N-terminal propeptide and a linker tetrapeptide located between the α- and β-chain of the mature protein. B: Model of the molecular evolution of the jacalin-related lectins. An ancestral mannose-specific jacalin-related lectin gave rise to a large family of both monocot and dicot mannose-specific jacalin-related lectins. An evolutionary event, which took place somewhere in the evolution of the Moraceae family and involved the insertion of a signal peptide and vacuolar targeting sequences gave rise to the small subfamily of galactose-specific jacalin-related lectins.

in parallel to immunofluorescence analysis. As shown in Fig. 3D, storage vacuoles (protein bodies) appear as small vesicles in the cell and are clearly distinguished from the starch grains (* in Fig. 3D). The immunofluorescence labeling of jacalin is clearly visible in small dots distributed over the whole area of the cells (starch grains appeared as black dots within the cells) indicating that this lectin is located within the small storage vacuoles (Fig. 3E).

To the best of our knowledge no structural or ultra-structural studies have been made of jack fruit seeds. However, since these seeds are rich in protein (comparable to most legume seeds) and starch one can reasonable assume that the cellular and sub-cellular organization of the storage parenchyma cells of jack fruit seeds is comparable to that of, for example, pea and jack bean seeds, which have been studied in detail at the light and electron microscopy level. A comparison of the sub-cellular location of jacalin as shown in Fig. 3D to that of, for example, concanavalin A and phytohemagglutinin, which were both localized in protein bodies of jack bean (*Canavalia ensiformis*) and bean (*Phaseolus vulgaris*) seeds, respectively [16,17], reveals a high degree of similarity between the overall sub-cellular distribution of jacalin and the legume lectins. Accordingly, one can reasonably assume that jacalin is located, indeed, in storage protein vacuoles.

The results of the localization studies of Calsepa and jacalin are of great importance because they demonstrate for the first time that the mannose-specific and galactose-specific jacalin-related lectins are located in the cytoplasm and the storage vacuoles, respectively. Though previous biochemical and molecular studies already indicated that jacalin is synthesized as a preproprotein on the endoplasmic reticulum (ER) and follows the secretory pathway [6], the presumed vacuolar location of the lectin was never determined. The same holds true for the mannose-specific jacalin-related lectins, which due to the absence of a signal peptide [3–5,7] and possible other targeting sequences have been considered putative cytoplasmic proteins. It appears, therefore, that the mannose-specific and galactose-specific jacalin-related lectins not only differ with respect to their specificity and molecular structure but also for what concerns their intra-cellular location. The particular location of the two subfamilies of jacalin-related lectins also explains the obvious differences in biosynthesis and processing. Calsepa, and probably all other mannose-specific jacalin-related lectins are synthesized in the cytoplasm and undergo no processing (Fig. 4A). Accordingly, the mature lectin polypeptides comprise the entire open reading frame of the corresponding genes. In contrast, the conversion of the primary translation product of jacalin-mRNA into the mature jacalin protomer includes a complex series of co- and post-translational modifications including the removal of a signal peptide, a (partial) glycosylation, removal of an N-terminal propeptide and the excision of a linker tetrapeptide [6]. As a result, the mature jacalin protomer consists of a short (20 AA) N-terminal β-chain non-covalently linked to a long (133 AA) C-terminal α-chain (Fig. 4A). At present, the role of the N-terminal propeptide and the linker tetrapeptide is still unclear. Since the formation of the carbohydrate-binding site of jacalin requires a correct excision of the linker tetrapeptide, the role of this linker possibly consists of a temporal inactivation of the lectin (e.g. to prevent jacalin to interact with glycoproteins in the ER and/or Golgi compartment). Comparison of the sequence of the N-terminal propeptide of jacalin to that of propeptides with a presumed vacuolar targeting role like in sporamin from sweet potato and aleurin from barley reveals little similarity. However, part of the sequence, which is believed to constitute the core of the vacuolar sorting determinant ($N^{26}PIRL^{30}$) of the propeptide of sporamin [18] is apparently conserved in jacalin ($N^{35}PI^{37}$). It is well possible, therefore, that the N-terminal propeptide of projacalin contains a vacuolar targeting signal.

The localization of the mannose- and galactose-specific jacalin-related lectins in the cytoplasm and vacuole, respectively, also contributes to our understanding of the molecular evolution of the family of jacalin-related lectins. Taking into consideration the wide distribution of the cytoplasmic mannose-specific jacalin-related lectins in both monocots and dicots one can reasonably assume that this subfamily represents the main evolutionary line (Fig. 4B). Accordingly, the subfamily of vacuolar galactose-specific jacalin-related lectins, which is confined to a few genera of the family Moraceae should be considered a small side group resulting from an evolutionary event whereby a gene encoding a cytoplasmic mannose-specific jacalin-related lectin acquired a signal peptide and a vacuolar targeting sequence. It is worth mentioning in this context that jack fruit seeds contain, besides the very abundant vacuolar galactose-specific jacalin, also small amounts of a typical mannose-specific jacalin-related lectin called KM+ [19]. Though the physiological role of jacalin and KM+ is not fully understood it seems likely that the abundant vacuolar jacalin is a major seed storage protein whereas the minor presumed cytoplasmic KM+ fulfils a specific endogenous role. Accordingly, it is tempting to speculate that a gene encoding a physiologically active ancestor of KM+ has been used as a template for the generation of a gene encoding a typical storage protein (in casu an ancestor of jacalin). This is another illustration of how storage proteins may be derived from genes that originally encoded proteins with a well-defined enzymatic or other biological activity [20,21].

The unambiguous demonstration of the cytoplasmic location of Calsepa is also important in view of the search for the physiological role of the mannose-specific jacalin-related lectins because it indicates that the (endogenous) site of action of these carbohydrate-binding proteins is most likely the cytoplasm. Evidently, this particular location raises the question of the possible cytoplasmic glycan-receptors for these lectins. At present, only the gross specificity of a few mannose-specific jacalin-related lectins has been determined. However, more detailed specificity studies done with the Jerusalem artichoke lectin clearly indicated that at least this lectin exhibits a preferential specificity towards Manα(1-3)Man and Manα(1-2)-Man [3]. It is tempting to speculate, therefore, that the putative endogenous glycan receptors for the mannose-specific jacalin-related lectins contain α(1-3) and/or α(1-2) dimannosides. The proposed cytoplasmic site of action of the mannose-binding jacalin-related lectins is also in good agreement with the putative cytoplasmic location of the protein encoded by the RTM1 gene of arabidopsis [13] because the control of the long-distance movement of tobacco etch virus is most likely a cytoplasmic event.

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