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Disulfide formation in plant storage vacuoles permits assembly of a multimeric lectin

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Short title: Disulfide bond formation in plant storage vacuoles

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Synopsis:

The endoplasmic reticulum (ER) has long been considered the plant cell compartment within which protein disulfide bond formation occurs. Members of the ER-located protein disulfide isomerase (PDI) family are responsible for oxidising, reducing and isomerising disulfide bonds, as well as functioning as chaperones to newly synthesised proteins. Here we demonstrate that an abundant 7S lectin of the castor oil seed protein storage vacuole, *Ricinus communis* agglutinin 1 (RCA), is folded in the ER as disulfide bonded A-B dimers in both vegetative cells of tobacco leaf and in castor oil seed endosperm, but that these assemble into $(A\text{-}B)_2$ disulfide-bonded tetramers only after Golgi-mediated delivery to the storage vacuoles in the producing endosperm tissue. These observations reveal an alternative and novel site conducive for disulfide bond formation in plant cells.

Keywords: Disulfide, vacuole, endosperm, endoplasmic reticulum, ricin, tobacco.

Abbreviations: ER (endoplasmic reticulum), PDI (protein disulfide isomerise), PSV (protein storage vacuole), RCA (*Ricinus communis* agglutinin 1).

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Introduction:

During endosperm development in the castor oil seed of *Ricinus communis*, lipids and proteins accumulate in oil bodies and protein storage vacuoles (PSV), respectively. The most abundant of the storage proteins, which provide an early source of amino acids to fuel post-germinative growth, are the 11S (legumin-type) crystalloid proteins and 2S albumins. The remaining PSV proteins are the 7S globulin (vicilin-like) lectins, ricin (*Ricinus communis* agglutinin II; RCA II) and its tetrameric relative *Ricinus communis* agglutinin I (RCA I, henceforth referred to as RCA). It is known that the glycosylated 7S lectins and non-glycosylated 2S albumins are co-translationally imported into the ER and transported as precursors via the Golgi to the PSV [1, 2]. One of the two 7S lectins, the cytotoxin ricin, is a disulfide-linked dimer comprising a galactose-binding B chain with 4 intrachain disulfides (apparent molecular mass of 34 kDa), and a ribosome inactivating A chain (apparent molecular mass of 32 kDa). Ricin is initially folded and transported as a ~67 kDa precursor known as proricin, in which the A and B chains are coupled by a 12 residue linker peptide containing a vacuolar targeting signal [3, 4]. Upon delivery into the PSV the linker is processed, resulting in the release of mature ~66 kDa ricin. RCA is also made as a precursor, with an identical signal peptide and linker propeptide (Figure 1). The RCA A chain (~32 kDa) is 93% identical to ricin A chain, while the B chains of the two proteins are 84% identical, with RCA B chain (~36 kDa) possessing an additional glycan [5]. Two of the 18 differences between the respective A chains of RCA and ricin involve the substitution of cysteine residues into RCA, one of which (Cys 156) is reported to form a disulfide link with an adjacent RCA molecule to generate a mature ~136 kDa lectin with the subunit arrangement B-A-A-B [6-8]. It has been assumed that all 11 disulfide bonds in mature RCA are formed in the ER to link the two proRCA precursors together, as depicted in Figure 1. During seed maturation, the two lectins accumulate to ~10% particulate protein.

Here, using a biochemical approach, we have examined the assembly of RCA in tobacco cells and in developing castor oil seed endosperm tissue. Surprisingly, we observed that neither covalent nor non-covalent association of two proRCA molecules occurred within the ER lumen. From analysis of lectin biosynthesis in maturing castor oil seeds, it was shown that the final disulfide bond between the RCA A subunits occurred after deposition and processing in the vacuole, such that a mixed population of ~136 kDa and ~68 kDa RCA accumulated during the later stages of endosperm maturation, before the onset of seed dessication. These observations show that disulfide bond formation can occur at sites other than the ER in the endomembrane system of plant cells.

Experimental:

Materials

Polyclonal rabbit anti-ricin antiserum was supplied by Vector Laboratories, while an RCA-specific peptide antibody was prepared by CovalAb, both of Peterborough, Cambridgeshire, UK. Complete™ protease inhibitor cocktail tablets were provided by Roche Applied Sciences, Mannheim, Germany, and the QuickChange™ *in vitro* mutagenesis system by Stratagene, La Jolla, California, USA. [^{35}S]-Promix (a mixture of [^{35}S]-cysteine and [^{35}S]-methionine), Amplify™ and Percoll™ were from GE Healthcare, Amersham, Buckinghamshire, UK, while all other reagents were from Sigma Aldrich, Poole, Dorset, UK.

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Recombinant DNA

All DNA constructs were generated in the CaMV 35S-promoter-driven expression vector pDHA [9]. Expression constructs encoding preproricin (ppRT) and preproricin with a mutated vacuolar sorting signal ($ppRT_{I271G}$) have been described previously [3, 10]. A pDHA construct containing the *Ricinus communis* agglutinin precursor cDNA, preproRCA (ppRCA), was utilised [11]. The equivalent mutation within the vacuolar sorting signal of RCA ($ppRCA_{I270G}$) was generated using the QuickChange™ *in vitro* mutagenesis system, using the mutagenic primer 5'-CGTCACAGTTTTCTTTGCTTGGAAGGCCAGTGGTGCC-3' (and its reverse complement, not shown). The site of codon mutation is underlined.

Transient transfection and *in vivo* radiolabelling of tobacco leaf protoplasts

Protoplasts were prepared from axenic leaves (4 to7 cm long) of *Nicotiana tabacum* cv. Petit Havana SR1 [12], and subjected to polyethylene glycol-mediated transfection with plasmids as previously described [13]. Cells were radiolabelled with [^{35}S]-Promix and chased for the times indicated in the figures, as previously described [10]. In some experiments, before radioactive labelling, protoplasts were incubated for 1 hour at 25°C in K3 medium (3.78 g/l Gamborgs B5 basal medium with minimal organics, 750 mg/l $CaCl_2.2H_2O$, 250 mg/l NH_4NO_3, 136.2 g/l sucrose, 250 mg/l xylose, 1 mg/l 6-benzylaminopurine, 1 mg/l α-naphthalenacetic acid) supplemented with 36 µM brefeldin A (7mM stock in 100% ethanol). At the desired time points, 3 volumes of cold W5 medium (9 g/l NaCl, 0.37 g/l KCl, 18.37 g/l $CaCl_2.2H_2O$, 0.9 g/l glucose) were added and protoplasts were pelleted by centrifugation at 60 × *g* for 10 minutes at 4°C. Separated cell and media samples were frozen on dry ice and stored at –80°C, unless further manipulations were to be performed.

Immunoprecipitation from tobacco protoplast protein extracts

Frozen samples were homogenised by adding 2 volumes of cold protoplast homogenisation buffer (150 mM Tris-HCl pH 7.5, 150 mM NaCl, 1.5 mM EDTA, 1.5% (w/v) Triton X-100) supplemented immediately before use with Complete™ protease inhibitor cocktail. Homogenates were used for immunoprecipitation with polyclonal rabbit anti-ricin antiserum, which recognises both proricin and proRCA. Protein A-Sepharose beads were washed three times with NET-gel buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.1% (w/v) Nonidet P-40, 0.25% (w/v) gelatin, 0.02% (w/v) NaN_3), and immunoselected polypeptides were analysed by 15% reducing or non-reducing SDS-PAGE. Gels were fixed, treated with Amplify™, and radioactive polypeptides revealed by fluorography.

Transient transfection, *in vivo* radiolabelling and immunoprecipitation from *Arabidopsis* suspension culture protoplasts

A suspension culture of *Arabidopsis thaliana* cells, ecotype Columbia T87 [14], was obtained from the Max-Planck Institut für Molekulare Pflanzenphysiologie, Potsdam, Germany. This was maintained axenically at 25°C in JPL *Arabidopsis* cell culture medium [15] with constant shaking at 150 rpm and a 16 hour light / 8 hour dark cycle. Cells were subcultured every 7 days. Protoplasts were prepared essentially as previously described [16], where cells were washed twice in MCP buffer (500 mM sorbitol, 1 mM $CaCl_2.2H_2O$, 10 mM MES pH 5.6) prior to enzyme digestion, before purification of intact cells on a Percoll™ step-gradient overlaid with betaine solution (500 mM betaine, 1 mM $CaCl_2.2H_2O$, 1 mM MES pH 6.0). Polyethylene glycol-

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mediated transfection with plasmids was also performed as previously described [17]. Cells were radiolabelled, homogenised and proteins immunoprecipitated with anti-ricin antiserum as described above. Immunoselected polypeptides were analysed by 15% non-reducing SDS-PAGE and visualised by fluorography.

Velocity gradient fractionation

600 µl homogenised cell samples containing approximately 2 million protoplasts were loaded on top of 11 ml continuous 5% to 25% (w/w) linear sucrose gradients made in 20 mM Tris-HCl pH 8.0. Gradients were formed in Beckman 12 ml ultracentrifuge tubes using a Searle Densiflow IIC filler device connected to the pumps of an FPLC system (GE Healthcare, Amersham, Buckinghamshire, UK). These were centrifuged at 100,000 × *g* for 20 hours at 20°C in a Beckman SW40 rotor (Beckman Coulter, High Wycombe, Buckinghamshire, UK), before unloading manually from the top in fractions of 600 µl. Radiolabelled proteins were immunoselected from gradient fractions and resolved by non-reducing SDS-PAGE and fluorography, as above. Size markers resolved from a parallel gradient were bovine serum albumin (67 kDa), lactate dehydrogenase (140 kDa), catalase (232 kDa), ferritin (440 kDa) and thyroglobulin (660 kDa). This were visualised following reducing SDS-PAGE by staining with Coomassie Brilliant Blue.

In vivo radiolabelling of *Ricinus communis* endosperm tissue

Ricinus communis plants were grown from dry seeds in an equal mix of multi-purpose compost and vermiculite under greenhouse conditions at 15°C with a 16 hour light / 8 hour dark cycle. Prior to planting, seeds were imbibed in running water overnight. The development of *Ricinus communis* seeds is divided into seven stages (A to G) based on size, testa formation and state of hydration [18]. Endosperm tissue was excised from the ripening seeds during testa formation at various developmental stages, as indicated in the figures (typically 7 to 9 weeks after flowering, when the lectins and storage proteins are being rapidly synthesised [1]). Intact endosperm halves were treated with 25 µCi of [^{35}S]-Promix, which was added directly to the abaxial surface. After a pulse of 1 hour at room temperature, the [^{35}S]-Promix was washed off with sterile water, and 10 µl of 2.5 mM unlabelled cysteine and methionine was added to each half. At each time point, six endosperm halves were frozen and ground to a fine powder in liquid N_2. The frozen powder was mixed with 1.0 ml of PC buffer (1% (w/v) Nonidet P-40, 0.2 M galactose), supplemented immediately before use with Complete™ protease inhibitor cocktail. Samples were then stored at –80°C.

Immunoprecipitation from *Ricinus communis* endosperm tissue

Samples were defrosted, and membranes were removed from the suspension by centrifugation at 100,000 × *g* for 30 minutes. Duplicate 25 µl aliquots of the supernatant were added to 1.0 ml of cold 10% (w/v) trichloroacetic acid. The precipitated protein was collected by filtration onto a Whatman GF/A filter disc, washed with 10% trichloroacetic acid, and its radioactivity content determined by scintillation counting after immersing the disc in 5.0 ml of scintillant. A volume of supernatant containing 10^6 counts per minute was taken at each stage for immunoprecipitation. The crude endosperm homogenates were dispersed in an equal volume of IP buffer (1% (w/v) Nonidet P-40, 10 mM Tris-HCl pH 7.5, 150 mM NaCl, 2 mM EDTA, 0.4 M galactose) supplemented immediately before use with Complete™ protease inhibitor cocktail. Immunoprecipitation was carried out with polyclonal rabbit anti-ricin antiserum as above, although Sepharose beads were this time washed three times with 0.2%

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Nonidet P-40, twice with 0.2% Nonidet P-40, 0.5 M NaCl, and once with 10 mM Tris-HCl pH 7.5. Immunoselected polypeptides were analysed by 15% reducing or non-reducing SDS-PAGE and visualised by fluorography.

Isopycnic organelle gradient fractionation

Ricinus communis endosperm tissue isolated from maturing castor oil seeds at developmental stage F/G was homogenised in a Petri dish on ice by continuous chopping for 15 minutes using a hand-held razor blade, after addition of ice-cold grinding buffer (150 mM Tris-HCl pH 7.5, 10 mM KCl, 1 mM $MgCl_2$, 1 mM EDTA, 100 mM lactose, 12% (w/w) sucrose) supplemented immediately before use with Complete™ protease inhibitor cocktail, using 6 ml of buffer per gram of endosperm tissue .The homogenate was centrifuged at 1,000 × *g* for 10 minutes at 4°C. 600 µl of supernatant was loaded on top of a 9 ml 30% to 60% (w/w) linear sucrose gradient, formed as above, overlaid with 2 ml of 20% (w/w) sucrose. These were centrifuged at 150,000 × *g* for 2 hours at 4°C in a Beckman SW40 rotor (Beckman Coulter, High Wycombe, Buckinghamshire, UK), before unloading manually from the top in fractions of 600 µl. Non-reducing Western blot analysis of 60 µl aliquots of these fractions was performed as previously described, using either an anti-RCA peptide antibody (described below) or anti-BiP antiserum [13].

Purification of ricin and *Ricinus communis* agglutinin

Ricin and RCA were purified as previously described [19, 20], with slight modifications. 10 g of endosperm tissue at different developmental stages was frozen in liquid N_2 and ground to a fine powder. This was further ground for 5 minutes in 100 ml PBS containing 0.2 M NaCl. After 3 hours at 4°C the extract was filtered and centrifuged twice at 10,000 × *g* for 30 minutes. The resulting supernatant was adjusted to 60% saturation with ammonium sulphate. This was left stirring overnight at 4°C, before centrifugation as above. The resulting pellet was dissolved in ~10ml PBS containing 0.2 M NaCl, and the ammonium sulphate removed by dialysis. The freshly prepared soluble protein was then immediately loaded onto a 50 ml column of propionic acid-treated Sepharose 6LB, pre-equilibrated with PBS, and unbound proteins were removed by washing with 300 ml of PBS, with 10 ml fractions being collected. Bound ricin was then eluted with 300 ml PBS containing 5 mM N-acetylgalactosamine, before PBS containing 100 mM galactose was used to elute bound RCA. Fractions were assayed for protein content by spectrophotometric measurement at 280 nm, and aliquots of suitable fractions were analysed by non-reducing SDS-PAGE and Coomassie Brilliant Blue staining or silver staining, and Western blotting with an anti-RCA peptide antibody. The peptide antibody was raised in a rabbit against a surface-exposed 11 amino acid sequence of *Ricinus communis* agglutinin B chain (single letter code ISKSSPRQQVV), of which 8 amino acids are different to the equivalent position in ricin B chain.

Results:

Mature *Ricinus communis* agglutinin 1 (RCA) is reported to be a B-A-A-B tetramer containing an interchain disulfide bond between each subunit (Figure 1 and [7]). The primary sequence of RCA shows it to contain a signal peptide for targeting to the endoplasmic reticulum, followed by A chain and B chain sequences that are separated by a 12 amino acid linker (Figure 1 and [11]). An identical linker peptide was originally observed in the primary translation product of the ricin precursor, and this was shown

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to contain a vacuolar targeting signal [3, 4]. Previous studies with ricin have also revealed that events during its synthesis and trafficking can be mimicked when it is ectopically expressed in tobacco leaf protoplasts [10]. Using this system to investigate the assembly of RCA, we observed from pulse-labelling that preproRCA is made as a ~69 kDa precursor which disappears as it is converted to mature A and B subunits during a chase with cold amino acids (Figure 2A). Such biochemical processing of the related proricin molecule has been recognised as a hallmark of vacuolar deposition, since this is the location of the relevant proteases (Figure 2A and [1, 21, 22]). However, when identical samples were examined on non-reducing gels, it was evident that disulfide-bonded proRCA dimers of an expected molecular mass of ~136 kDa did not form (Figure 2B, asterisk). Very similar results were obtained using protoplasts from Arabidopsis cell suspension cultures (Figure 2C). The multiple bands observed when these proteins are resolved on non-reducing gels are typical of proteins constrained by a variable number of disulfide bonds. The slight decrease in overall sizes of the precursor bands during the early chase points most likely reflects glycan modifications (Figure 2B, 2C and [23, 24]) as the proteins traffic through the Golgi in a brefeldin A-sensitive manner (Figure 3A), as has been previously reported for proricin [10]. The marked size reduction observed after 3 hours of chase is indicative of proteolytic removal of the linker upon arrival at the vacuole [10]. If the RCA linker is mutated within its putative vacuolar targeting sequence (I270G) then, by default, it becomes secreted with time and avoids vacuolar processing (Figure 3B).

Since there is no evidence for the formation of covalent disulfide-linked dimers in tobacco protoplasts, we checked whether proRCA assembled non-covalently into a ~136 kDa multimer. This was achieved by separating the newly made proteins on non-denaturing velocity gradients following a 1 hour pulse and 0 or 3 hour chase. Fractions were then immunoprecipitated and samples resolved by non-reducing SDS-PAGE (Figure 4). The position of RCA was compared to that of protein markers run on a parallel gradient (Figure 4, top panel). It is evident that proRCA and processed RCA co-localise around the peak fractions of bovine serum albumin (67 kDa; labelled I) and not around lactate dehydrogenase (140 kDa; labelled II).

Since we could observe no formation of tetrameric RCA in tobacco or *Arabidopsis* cells, we next analysed its endogenous synthesis during the maturation of *Ricinus communis* seed endosperm ([18]; stages depicted in Figure 5A). Since ricin and RCA are co-synthesised in *Ricinus communis* seeds, and since they are such closely related polypeptides, polyclonal antibodies raised against either protein interact with both antigens. We therefore prepared an RCA-specific peptide (Figure 5B) in which 8 out of 11 residues of RCA B chain differed from the equivalent peptide in RTB. Antibodies raised to this peptide were able to differentiate between the two non-reduced lectins when denatured, as in a Western blot (Figures 5C and 5D), but were found to not to interact with either protein upon immunoprecipitation (data not shown). We then purified and separated the two *Ricinus* lectins from young and mature seed tissue. This was achieved by eluting the lectins from propionic acid-washed Sepharose 6LB, using *N*-acetylgalactosamine to elute ricin followed by galactose to elute RCA. This is possible due to the different sugar binding specificities of the B chains of ricin and RCA [19, 25, 26]. An elution profile is shown for stage F/G tissue, where two clear peaks are evident (Figure 5C). The first, eluting with *N*-acetylgalactosamine, was shown by Coomassie blue staining of a non-reducing gel, and by the absence of any interaction with the RCA-specific peptide antibody, to be ricin (Figure 5C, left-hand

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gels). The second peak, eluting from the column with galactose, was shown to contain two forms of RCA, a ~68 kDa form and a ~136 kDa form (Figure 5C, right-hand gels). In younger endosperm tissue (stage B/C), overall lectin yields were much lower, and so silver staining was used instead of Coomassie to visualise the purified protein, due to its additional sensitivity. It is clear from both silver stained gels and from Western blots that very little tetrameric RCA is evident in this younger tissue (Figure 5D, right-hand gels). To further explore the formation of the fully assembled RCA, tissue extracts from a wider range of tissue stages were analysed by Western blotting with the RCA-specific antibody. It is clear that predominantly dimeric RCA (~68 kDa) accumulates early in seed development, while the 136 kDa tetrameric form is observed only at later stages of development (Figure 5E, top panel). However, some RCA remains as A-B dimers throughout. That the tetrameric form is truly a disulfide-bonded species is shown in the reducing gel of these samples (Figure 5E, lower panel).

To examine exactly where in the cell tetramer assembly occurs, we fractionated late stage developing endosperm, separating the organelles by sucrose density gradient ultracentrifugation (as described in, for example, [27, 28]). In such gradients, ER-derived membranes are recovered just below the interface between 20% and 30% sucrose, as revealed here by the location of the ER resident chaperone BiP (Figure 6A, lower panel), while disrupted PSVs release their contents to be recovered at the top of the gradient [21]. In the RCA blot, the RCA-specific antibody that does not cross react with ricin was used. It is clear that the material in the first few fractions contains both ~68 and ~136 kDa forms of RCA. However, the ~136 kDa form of RCA is not detectable in the fractions commensurate with ER (ascertained from the localisation of BiP and from the known sucrose density of endosperm-derived ER microsomes (Figure 6A and [28])). That a disulfide-bonded ~136 kDa RCA did not form within the ER was confirmed by pulse-chase and immunoprecipitation using late stage developing endosperm tissue (stage F/G). Only during the chase can it be seen that larger B-A-A-B forms (~136 kDa), which are the hallmark of fully assembled RCA, become evident (Figure 6B, lanes 2 and 3). These forms disappear when resolved on a reducing gel (Figure 6B, lanes 5 and 6), confirming that upon delivery into PSV, the RCA precursors are processed and assembled in a disulfide-bonded manner. The precise fraction that is converted from ~68 kDa to the larger disulfide-bonded forms is not known, since the antibody used for immunoprecipitation here is, necessarily, the polyclonal *Ricinus* lectin antibody that also detects ~66 kDa ricin.

Discussion:

We reasoned that the assembly of RCA1 would require the formation of disulfide bonds (11 in total) within and between two RCA precursors during their oxidative folding in the ER lumen, in a manner analogous to the assembly of other multimeric, disulfide-linked proteins. However on analysing newly synthesised RCA expressed ectopically in tobacco and *Arabidopsis* protoplasts or endogenously in developing castor oil seed endosperm, we found no evidence for disulfide-associated precursors in the ER. Indeed, in immature endosperm there was very little, if any, disulfide-linked association of the RCA heterodimers. Here we show that not only is the final stage in assembly of tetrameric RCA (the disulfide-linked association of two RCA A chains) observed exclusively in the later stages of seed development, but that it also occurs outside the environment of the endoplasmic reticulum, most likely within the PSV.

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Disulfide bonds between the thiol groups of cysteine residues help stabilise the folded structure of proteins, and their formation is usually catalysed by oxidised members of the protein disulfide isomerase (PDI) family in the oxidising environment of the ER. PDIs can act as chaperones, but are primarily thioredoxin-like oxidoreductases that form and reshuffle disulfide bonds. Continued PDI action relies on an electron transfer cascade to recycle it from its reduced to its oxidised form. This is accomplished by Ero1 (endoplasmic reticulum oxidoreductin 1), which in turn transfers electrons to molecular oxygen with the generation of H_2O_2 [29, 30]. The latter is potentially very damaging, but through feedback regulation to reduce the oxidase activity of Ero1 [31-33], and possibly through detoxification by an ER resident peroxidoredoxin [34], oxidative damage caused by H_2O_2 can be prevented. In plant systems, Ero1 homologues have been identified in *Arabidopsis* [35], while a role for Ero1 in disulfide formation in the endosperm of rice has recently been demonstrated [36].

PDIs have been localised in subcellular compartments other than the ER [37], including chloroplasts [38-40]. Their functions in the more reducing conditions that prevail outside the ER are often unclear, but they are likely to involve the reduction of disulfides rather than their formation [41]. For example in animal cells, the reducing activity of cell surface PDI has been shown to play a role in nitric oxide signalling pathways [42, 43], reduction and release of surface receptors [44] and cell adhesion [45]. Various hypotheses have been put forward to assign a function to PDI members reported in the nucleus and cytosol, but often these require further confirmation [37]. An alternative oxidising compartment where disulfide bond formation is known to occur concomitant with protein folding is within the intermembrane space of mitochondria, where a disulfide relay system has recently been identified and characterised (reviewed in [46]).

In *Arabidopsis*, 22 PDI-like proteins have been identified [47], although only 12 of these possess a classical PDI structure [48]. However, while it is clear that ER residents such as BiP and PDI can be transported to lytic vacuoles for degradation [49, 50] and that oxidised proteins are sent to the vacuole for degradation by autophagy [51], there is no evidence for PDI activity in vacuoles. Recently, the *Arabidopsis* PDI5 isoform was found to localise to both the ER and vacuoles in developing seed tissues. Through disulfide exchange, this protein interacts with and inhibits cysteine proteases on their journey to vacuoles [52]. Such chaperoning precedes the initiation of programmed cell death. There is therefore no available evidence for the presence of a disulfide relay, or supporting enzymatic activity of PDI, in plant vacuoles, leaving it unclear as to how the final disulfide that connects each RCA heterodimer, to complete the assembly of tetrameric RCA in castor oil seed PSV, is formed. Whether this event involves simply air oxidation, and whether it is promoted by protein accumulation, remains to be established. It should be noted that the final disulfide in this lectin occurs after processing of the RCA precursor by the vacuolar processing enzyme (VPE) [22, 53]. This type of proteolysis is common in the maturation of seed storage proteins [54-57]. Proteolytic removal of the linker (Figure 1) cannot occur outside the PSV in castor oil seeds, since newly synthesised VPE is transported in latent form and is activated only upon vacuolar delivery [53]. A preliminary crystallographic structure of RCA reveals a disulfide bridge between Cys 156 of two adjacent A chains [7]. We speculate that processing of the proRCA linker may subtly alter the conformation of the resulting A-B heterodimer such that neighbouring dimers, accumulating and concentrating over time,

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have their respective Cys 156 residues optimally juxtaposed to favour spontaneous disulfide formation.

If RCA1 has two quaternary structural forms (dimeric and tetrameric), why does the literature refer to this protein as a tetrameric lectin? Commercially purified RCA is supplied as a homogenous disulfide bonded B-A-A-B protein. However, as we demonstrate here, RCA in the endosperm of mature castor oil seeds is actually present as a mixture of ~68 kDa A-B and ~136 kDa B-A-A-B forms. Indeed, in early developing endosperm, only the ~68 kDa A-B dimer is present. One likely explanation for the description of RCA as a tetrameric lectin is that in dry seeds (from which the lectin is typically purified), the larger ~136 kDa species predominates. When purifying RCA, castor oil seed extracts are subjected to affinity chromatography and gel filtration. The less abundant smaller species, being the size of mature ricin and being indistinguishable from it with polyclonal antisera, could readily be mistaken for ricin contamination and therefore discarded.

In summary, we show that the ER of plant cells is not the exclusive location for disulfide bond formation. In the assembly of tetrameric RCA1, the final disulfide between two RCA A subunits occurs within the PSV. While the mechanism remains to be elucidated, it is clear that conditions within the seed PSV are conducive for such a post-translational modification.

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Figure legends:

Figure 1. Schematic representation of RCA and its putative tetrameric forms.
The free cysteine in the RCA monomer (SH) and all predicted disulfide bonds (S-S) are indicated. The fork symbols indicate *N*-glycosylation sites. SP, signal peptide; linker, 12-residue propeptide containing a vacuolar sorting signal.

Figure 2. ProRCA reaches the vacuole as an A-B heterodimer in tobacco protoplasts, but does not form disulfide-bonded tetramers. Tobacco mesophyll (**A** and **B**) or *Arabidopsis* suspension culture (**C**) protoplasts transfected with pDHA (vector) alone or plasmids encoding ER-targeted preproricin or preproRCA were radiolabelled with [^{35}S]-amino acids for 1 hour and chased in the presence of unlabelled amino acids for the indicated times, before immunoprecipitation from cell homogenates with anti-ricin antiserum, and analysis by either reducing (**A**) or non-reducing (**B** and **C**) SDS-PAGE and visualisation by fluorography. In all cases, numbers on the left indicate molecular mass markers in kilodaltons.

Figure 3. ProRCA is transported to the vacuole in a sequence-specific manner via the Golgi complex. A: Tobacco protoplasts transfected with the indicated constructs were radiolabelled with [^{35}S]-amino acids for 1 hour and chased in the presence of unlabelled amino acids for the indicated times, before immunoprecipitation from cell homogenates with anti-ricin antiserum, and analysis by reducing SDS-PAGE and fluorography. Where indicated, protoplasts were pre-incubated for 1 hour with 36µM BFA before radiolabelling. **B:** Tobacco protoplasts were transfected and treated as in A. Radiolabelled polypeptides were immunoprecipitated from separated cell (top panel) and medium (lower panel) homogenates and analysed by non-reducing SDS-PAGE and fluorography. In all cases, numbers on the left indicate molecular mass markers in kilodaltons.

Figure 4. ProRCA does not form non-covalent tertramers in tobacco protoplasts. Top panel: The marker proteins bovine serum albumin (67 kDa), lactate dehydrogenase (140 kDa), catalase (232 kDa), ferritin (440 kDa) and thyroglobulin (660 kDa) were subjected to velocity gradient centrifugation on a 5 to 25% (w/w) linear sucrose gradient. Fractions were resolved on reducing SDS-PAGE and revealed by Coomassie staining. The range of each protein across the gradient is indicated by a horizontal line and the numbers I, II, III, IV and V, respectively. Centre and lower panels: Tobacco protoplasts transfected with plasmid encoding ER-targeted preproRCA were radiolabelled with [^{35}S]-amino acids for 1 hour and chased in the presence of unlabelled amino acids for 0 or 3 hours, as indicated. Total cell homogenates were subjected to velocity gradient centrifugation on a 5 to 25% (w/w) linear sucrose gradient. Fractions were immunoprecipitated with anti-ricin antiserum, analysed by non-reducing SDS-PAGE, and visualised by fluorography. In all cases, numbers on the left indicate molecular mass markers in kilodaltons.

Figure 5. RCA can form teramers in maturing *Ricinus communis* seed endosperm. A: The different developmental stages of a maturing castor oil seed [17]. **B:** The three-dimensional crystal structure of ricin holotoxin, with the region of maximum sequence divergence (an exposed surface loop incorporating amino acids 66 to 76 of the B chain) circled (left). The three letter amino acid codes of these residues (and two flanking residues either side) are shown (top right), along with the equivalent residues in RCA

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B chain (lower right). Differing amino acid residues are underlined. An RCA-specific peptide antibody was raised against these 11 residues of RCA B chain, 8 of which are different to ricin B chain. **C:** Elution profiles of ricin and RCA purified from castor oil seeds at maturation stage F/G. Points at which *N*-acetylgalactosamine or galactose were added to elute ricin and RCA, respectively, are indicated by arrows. Aliquots of crude ammonium sulphate precipitated castor bean extract (left hand lane), or relevant fractions from the purification, were analysed by non-reducing SDS-PAGE and Coomassie staining (top panel), and immunobloting with anti-RCA peptide antibodies described in B (lower panel). **D:** Same as (**C**), except that ricin and RCA were purified from castor oil seeds at stage B/C of development. **E:** Endosperm halves from castor oil seeds harvested at the indicated stages of maturation were homogenised, resolved by non-reducing or reducing SDS-PAGE as indicated, and immunoblotted. In all cases, numbers on the left indicate molecular mass markers in kilodaltons.

Figure 6. Tetrameric RCA is found only in the vacuoles of mature castor bean endosperm. A: Endosperm halves from castor oil seeds at developmental stage F/G were homogenised and subjected to isopycnic centrifugation on a 9 ml linear 30% to 60% sucrose gradient overlaid with 2 ml of 20% sucrose. Fractions were resolved on non-reducing SDS-PAGE and immunoblotted with either anti-RCA peptide antibodies or anti-BiP antiserum. **B:** Endosperm halves from castor oil seeds at developmental stage F/G were radiolabelled with [^{35}S]-amino acids for 1 hour and chased in the presence of unlabelled amino acids for the indicated periods of time, before immunoprecipitation from cell homogenates with anti-ricin antiserum and analysis by reducing or non-reducing SDS-PAGE and fluorography. In all cases, numbers on the left indicate molecular mass markers in kilodaltons.

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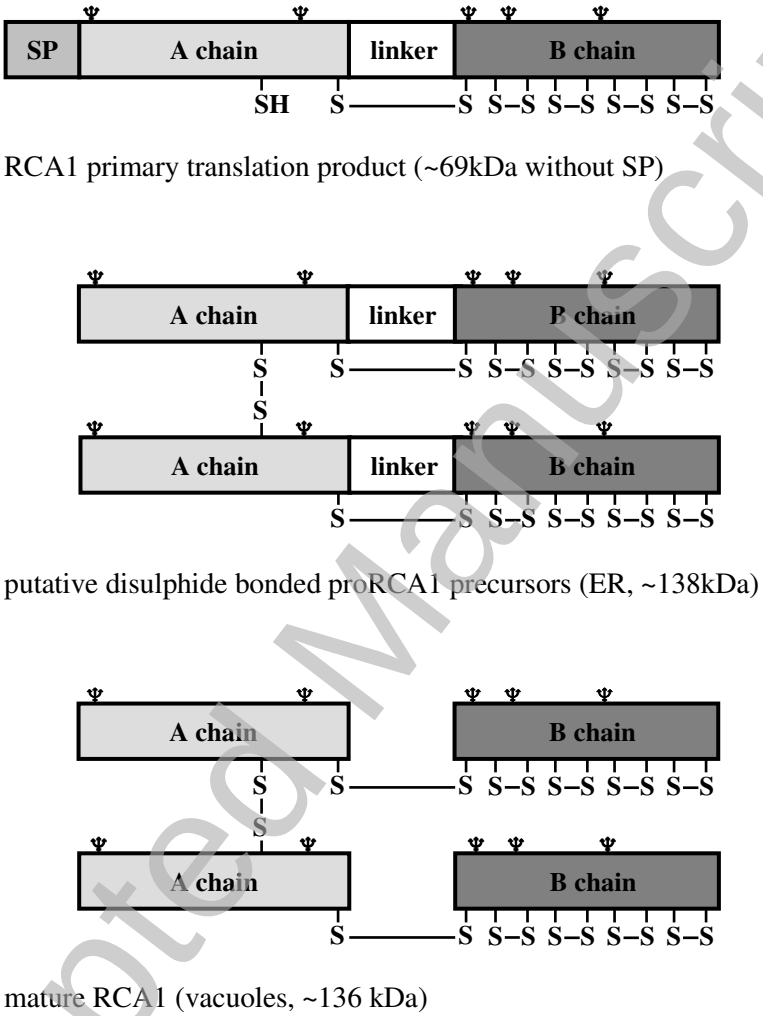

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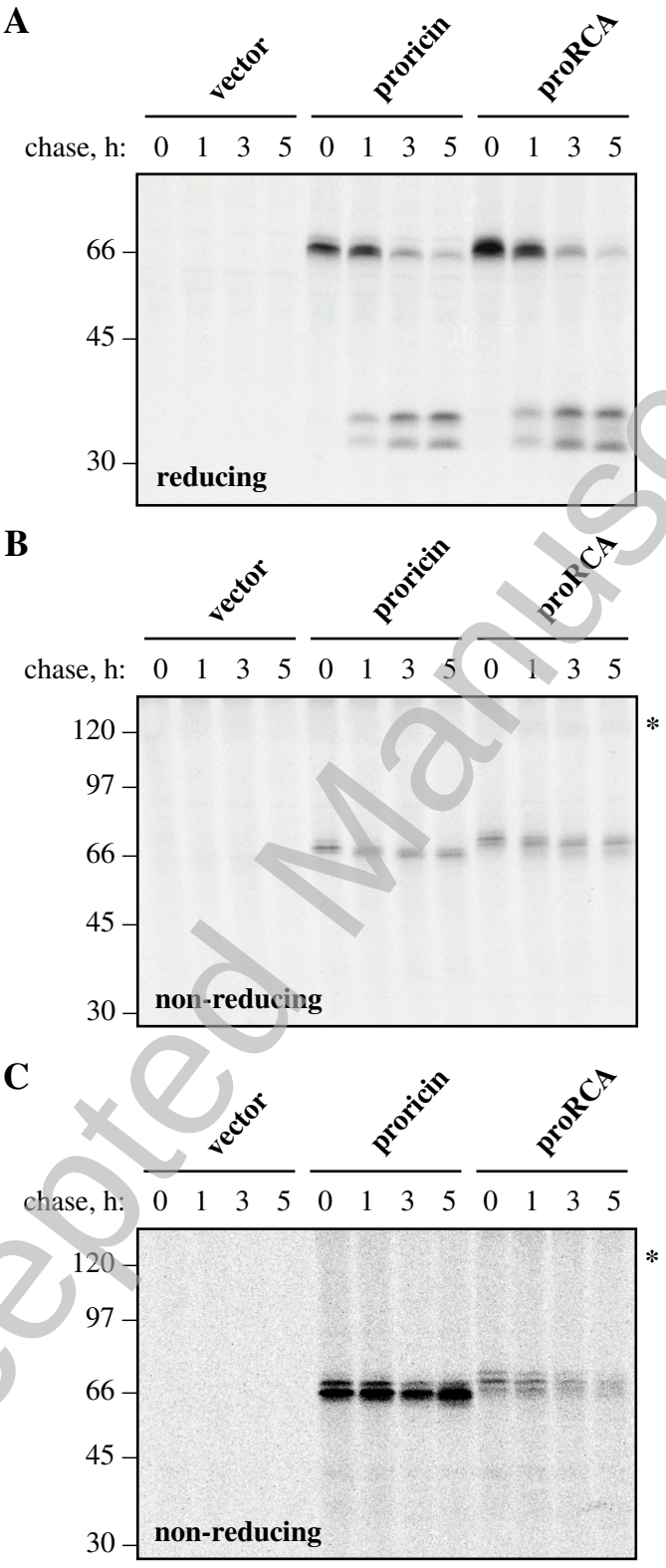

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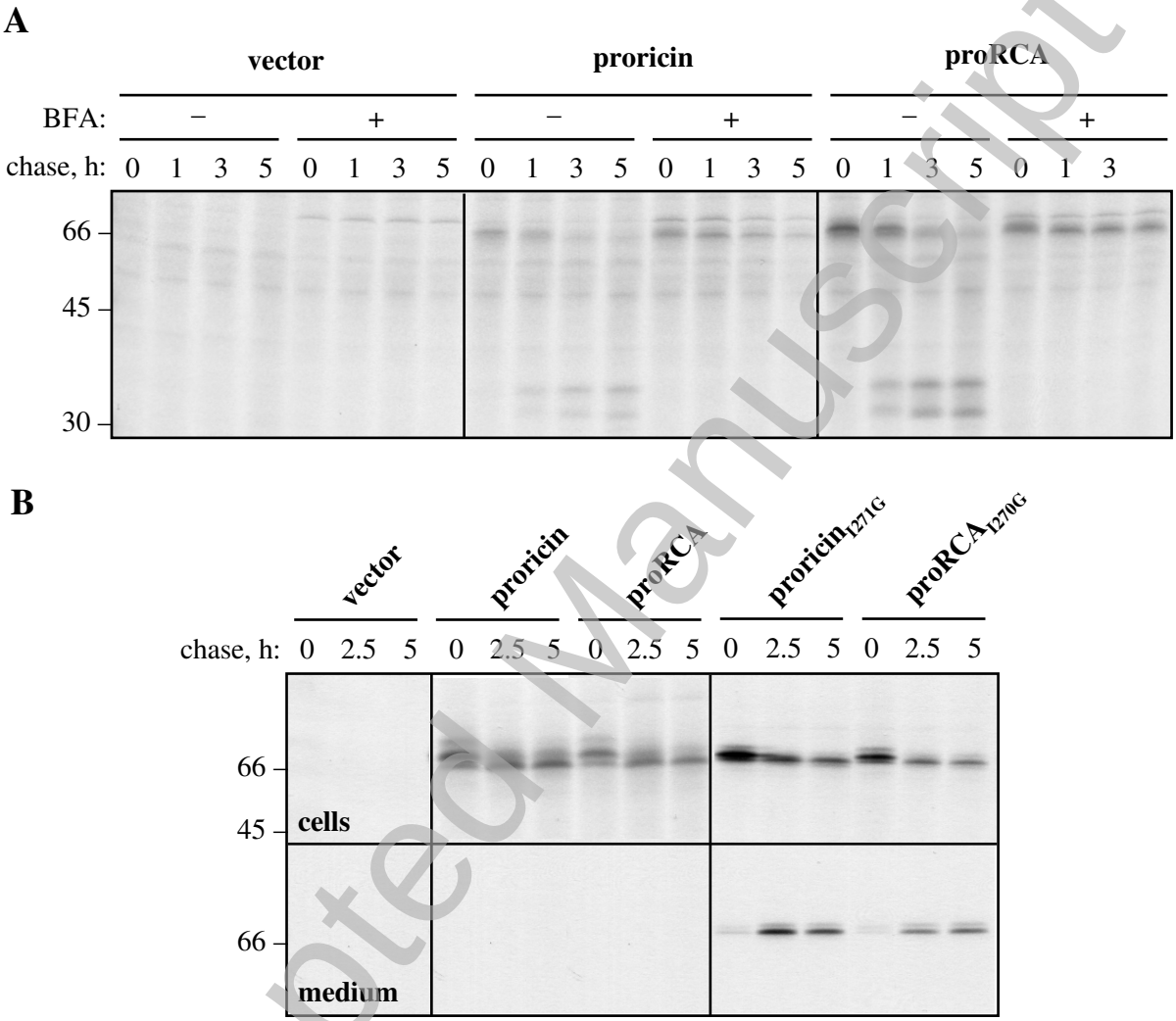

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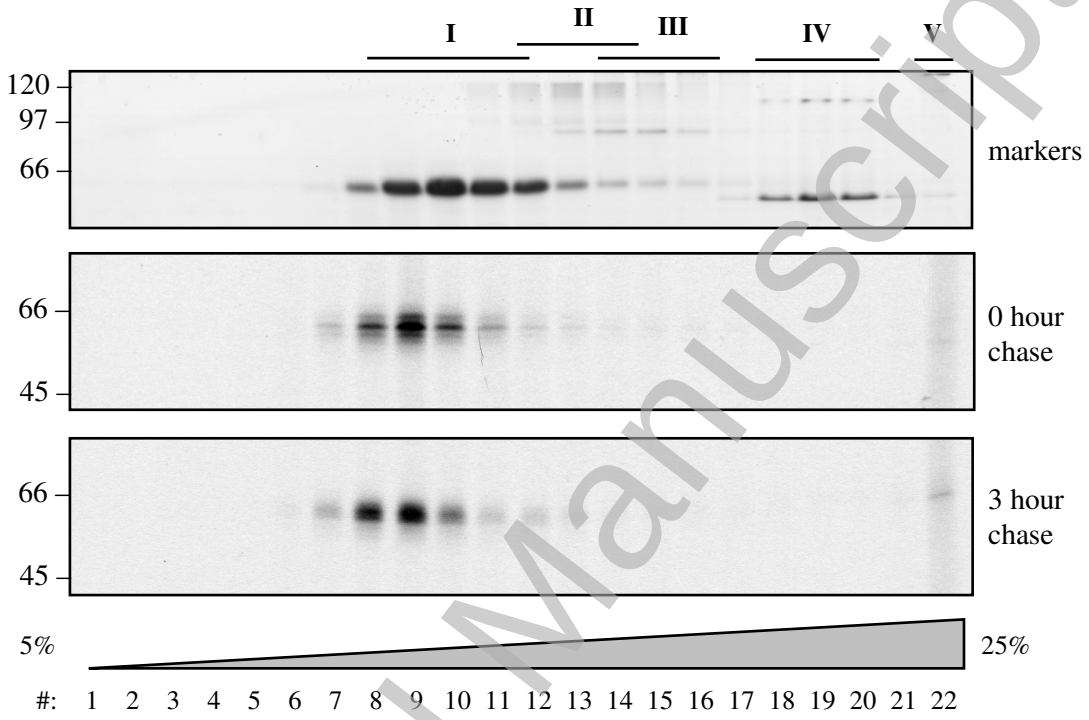

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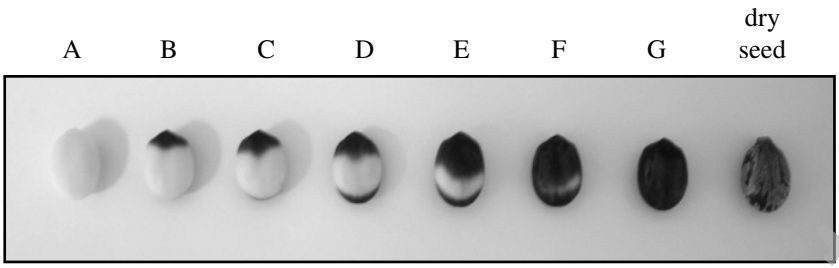
A



B

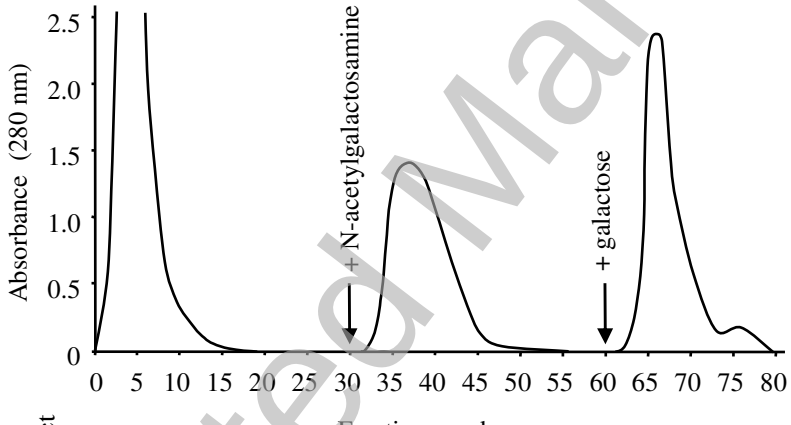
Ricin B chain

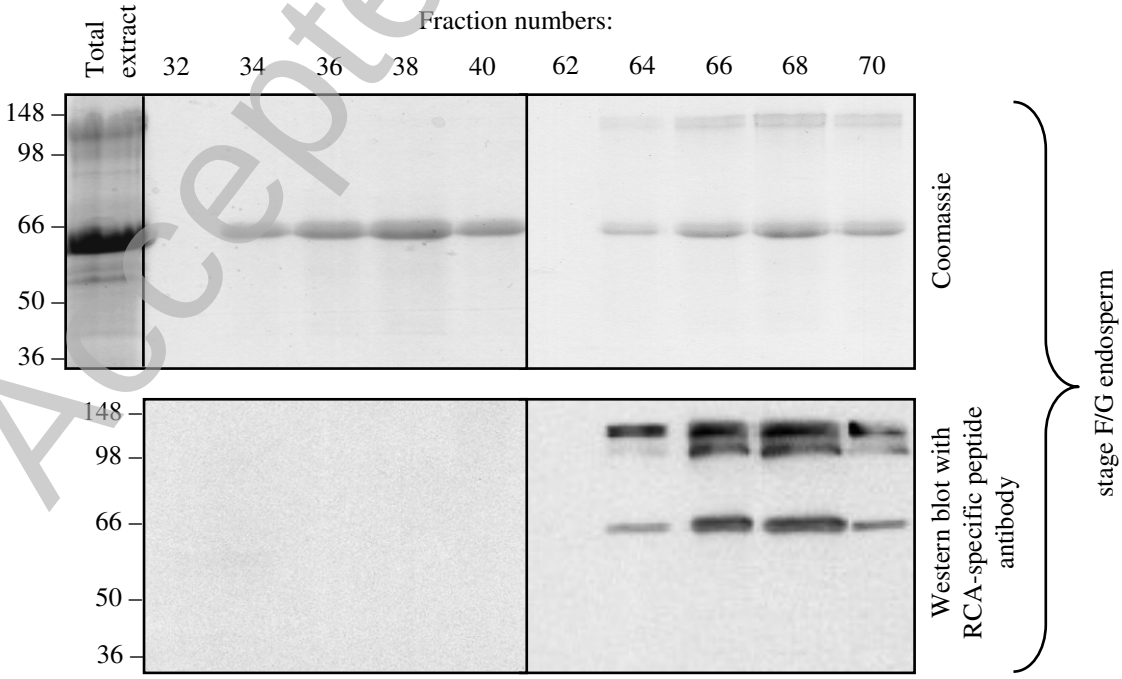
^{64}---Leu Thr Ile Ser Lys Ser Ser Pro Arg Gln Gln Val Val Ile Tyr---^{78}

RCA B chain

^{64}---Leu Thr Thr Tyr Gly Tyr Ser Pro Gly Val Tyr Val Met Ile Tyr---^{78}

C





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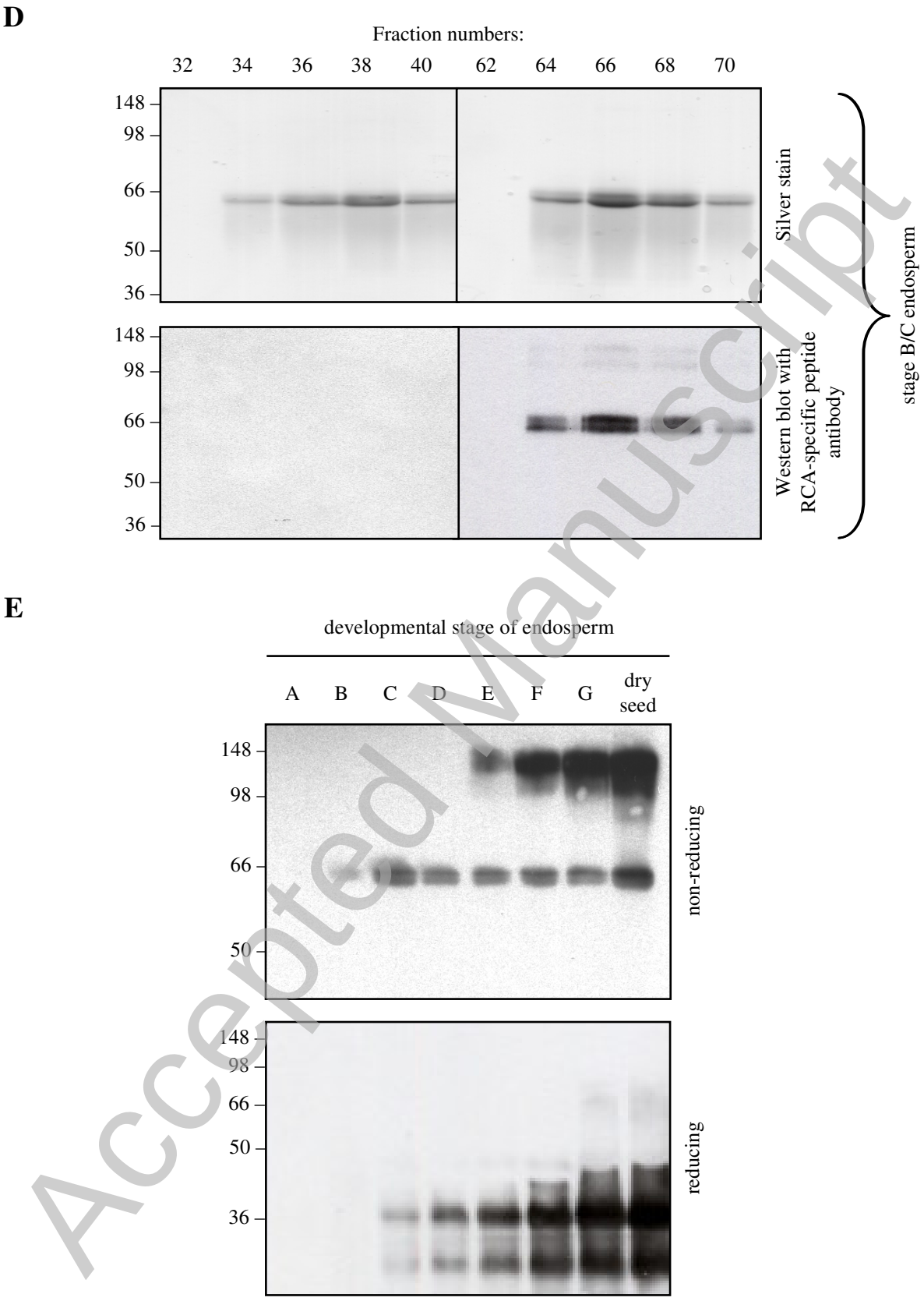

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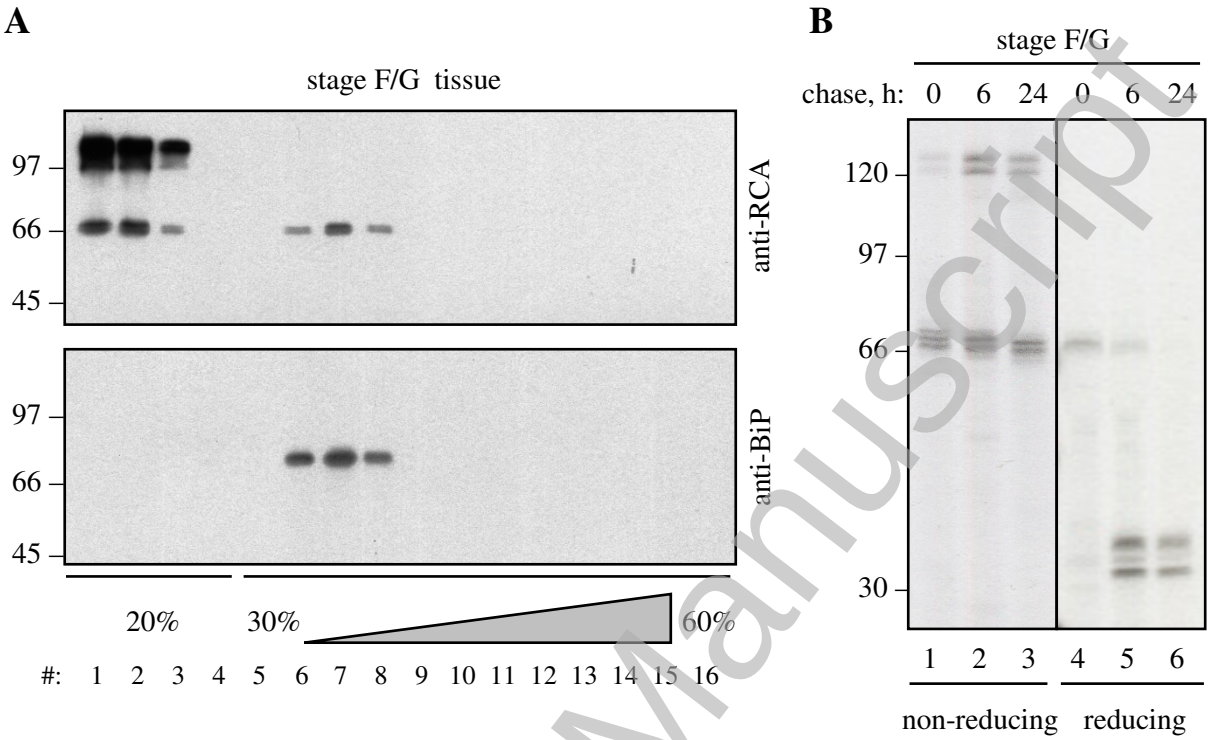

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