The Cleavable N-terminal Domain of Plant Endopolygalacturonases from Clade B May Be Involved in a Regulated Secretion Mechanism*

Polygalacturonases represent the most abundant carbohydrate hydrolase family in the Arabidopsis thaliana genome, and they are thought to be involved in nearly all of the developmental processes requiring cell wall modifications during the life cycle of the plant. By phylogenetic analysis, plant polygalacturonases fall into at least three groups, one of which is distinguished from the others by the presence of an additional N-terminal domain. We have used RDPG1, the polygalacturonase involved in pod dehiscence in oilseed rape (Brassica napus), as a model to investigate the function of this domain. We have confirmed that this domain is absent in the mature protein by determination of the N-terminal sequence of mature RDPG1 purified from oilseed rape pod. We have furthermore investigated the accumulation and subcellular localization of the precursor containing the N-terminal domain and of the mature protein throughout the development and maturation of the pod. Using recombinant expression in Pichia pastoris, we have produced the RDPG1 precursor, and we present evidence that the N-terminal domain of plant polygalacturonases is not involved in folding or inactivation of the precursor but may play a role in the intracellular transport of this protein family via a novel regulated secretion pathway.

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Polygalacturonases (PGs)1 (EC 3.2.1.15) are one of the most important classes of enzymes associated with pectin degradation and cell wall rearrangements. Pectins are a major component of the cell wall of dicotyledonous plants, where they make up about 30% of the primary wall and most of the middle lamella (1). Diverse roles have been proposed for pectins, including the regulation of porosity and hydration and mechan-
guish between clades A and B based on mode of action or tissue specificity. However, one feature that sets clade B, and possibly clade E, apart from the other clades is the presence of an additional 40–50-amino acid-long N-terminal domain immediately following the signal peptide (3, 5, 13, 15).

Propeptides, which are most often located at the N terminus of the mature protein domain, are a common feature of many protein precursors. However, their function has been extensively studied only in protease and peptide hormone precursors, in which the removal of the propeptide is associated with activation of the enzyme or hormone moeity (16). In addition to their role in zymogen activation, propeptides may carry sorting signals for the correct targeting of their cognate proteins to their final subcellular location (17). More recently, propeptides were found necessary for the correct folding of bacterial and yeast secretory proteases in vivo as well as in vitro in denaturation-renaturation experiments (18). By analogy, it has commonly been assumed that the predicted propeptide of PGs from clade B is involved in keeping the enzyme inactive until its activity is required or in directing the protein to a specific location within the cell wall, but these hypotheses have never been tested (3, 13, 19). Thus, elucidating the function of this N-terminal domain may help to understand the physiological relevance behind the distinction between clades A and B. The rdpg1 gene, encoding the oilseed rape (Brassica napus) pod dehiscence-related PG (B. napus) was amplified by PCR from the full-length cDNA (18). By analogy, it has commonly been assumed that the predicted propeptide of PGs from clade B is involved in keeping the enzyme inactive until its activity is required or in directing the protein to a specific location within the cell wall, but these hypotheses have never been tested (3, 13, 19). Thus, elucidating the function of this N-terminal domain may help to understand the physiological relevance behind the distinction between clades A and B. The rdpg1 gene, encoding the oilseed rape (Brassica napus) pod dehiscence-related PG (B. napus) was amplified by PCR from the full-length cDNA (18).

We have therefore revisited and confirmed the presence of a cleavable N-terminal domain, which is absent in the mature protein for PGs from clade B using RDPG1 as a model, and we have furthermore investigated the site and time of cleavage of this N-terminal domain from the precursor protein in the plant. Recombinant expression of the RDPG1 precursor in Pichia pastoris provided us with a unique source of the precursor and enabled us to address the function of the N-terminal domain in folding and in activation of RDPG1. Finally, we present data pointing at a function for the N-terminal domain of PGs from clade B in the intracellular transport of its enzyme moiety via a novel and regulated secretion pathway. The elusive and transitory nature of most precursors makes their study difficult, and to our knowledge, this is the first time that the function of the propeptide-like domain of a protein different from a hormone or a protease has been studied.

**EXPERIMENTAL PROCEDURES**

**Plant Material**—Culture of oilseed rape cultivars Topas and Fido and flower tagging to determine the days after anthesis (DAA) at each harvest were carried out as described by Petersen et al. (6). Whole plants were sprayed with 2-methyl-4-chlorophenoxyacetic acid (2,4-DPA) as described by Chauvaux et al. (20).

**Production of Polyclonal Antibodies to Pro-RDPG1 and to Its N-terminal Domain or Presumed Propeptide**—Pure pro-RDPG1 recombinantly expressed in P. pastoris was used as antigen to produce antiserum OA01 to pro-RDPG1. The presumed propeptide of RDPG1 was expressed in E. coli as a fusion protein with His$_6$-dihydrofolate reductase (Complete™, Roche Molecular Biochemicals) and centrifugation (12,000 × g for 20 min). The resulting salt-free supernatant contained the readily soluble protein. The pellet was re-suspended in 20 ml of water and centrifuged at 10,000 × g for 30 min. The supernatant was discarded and the pellet was resuspended in 10 ml of 1 mM MOPS, 1 mM LiCl, pH 7.0, and stirred for 30 min on ice. The sample was centrifuged as described above. The resulting pellet was discarded, whereas the resulting LiCl supernatant represented proteins that are strongly bound to the cell wall through ionic interactions. For immunopurification of RDPG1, the salt-free supernatant or the 2-fold diluted LiCl supernatant was mixed with 1 ml of immunoaffinity matrix and incubated overnight at 4 °C, under rotation, for binding of RDPG1 to the antibody. The gel was then poured into a disposable column and allowed to settle. Washing was carried out with 10 ml of phosphate-buffered saline. After a pre-elution wash with 10 ml of 10 mM sodium phosphate, pH 7.0, RDPG1 was eluted with 100 mM glycine, pH 2.0, and collected in 1 ml fractions into tubes containing 50 μl of 1 mM sodium phosphate, pH 8.0.

**Recombinant Expression of Pro-RDPG1 and Åpro-RDPG1 in P. pastoris**—The nucleotides 156–1388 and 285–1388 regions of the cDNA clone RDPG1 (6), corresponding to the presumed proenzyme (L24–S66) and mature (S67–P333) forms of RDPG1, respectively, were amplified by PCR using the forward primers 5′-AAAGATGCTTCTTTGAGTAGGTTAGGACATGCTTAC-3′ and 5′-ATCAGCTGTTTCCTTTGAGTAGGTTAGGACATGCTTAC-3′ and the reverse primer 5′-GCTCTAGATCATATTAGGCGGATTAG3′. These primers had been designed to introduce an EcoRI or Pst I restriction site at the 5′-end of the PCR product and a XbaI site at its 3′-end (underlined in the sequence), as well as two stop codons at the 3′-end (in italics in the sequence). The EcoRI/XbaI- or PstI/XbaI-digested PCR product, corresponding to nucleotides 156–1388 or 285–1388 regions, respectively, was cloned into the P. pastoris expression vector pPICZαB (Invitrogen). The RDPG1 cDNA fragments were inserted in frame at the 3′-end of the Saccharomyces cerevisiae α-factor secretion signal sequence to ensure secretion of the recombinant proteins and a stop codon had been included before the myc epitope His$_6$ tag. The resulting recombinant plasmids proRDPG1pICZαB and Åpro-RDPG1pICZαB were transformed into E. coli XL1-Blue, checked by sequencing, linearized with SacI, and subsequently transformed in the P. pastoris X-33 strain by electroporation according to the manufacturer's instructions (Invitrogen). Transformants were selected on plates with YPD medium and Zeocin (100 μg/ml). For each construct, approximately 50 transformants were screened for high secretion of the recombinant protein in 10 ml cultures under expression inducing conditions for up to 96 h. Aliquots of the culture medium (150 μl) were taken out every 24 h and analyzed by immuno-dot-blot. The transformants with the highest level of secreted recombinant protein (pro45 and Å11 for pro-RDPG1 and Åpro-RDPG1, respectively) were chosen for large-scale expression.

**Purification of Pro-RDPG1 and Åpro-RDPG1 Recombinantly Expressed in P. pastoris**—For large scale expression of pro-RDPG1 and Åpro-RDPG1, the P. pastoris transformant A11 or pro45 was cultivated in the BMGY/BMMY according to the manufacturer's instructions (Invitrogen). After 72 h of culture in BMMY, cells were harvested by centrifugation (3000 g for 10 min) and discarded. The supernatant (2 liters) was filtered through Whatman folded filters and a protease inhibitor mixture (Complete™, Roche Molecular Biochemicals) was added. All of the following steps were performed at 4 °C. The supernatant was ultrafiltrated to a final volume of 100 ml using a Minitan unit equipped with using a nickel-nitritolitrionic acid agarose column were performed according to the manufacturer's directions (Qiagen). The purified His$_6$-dihydrofolate reductase-presumed propeptide fusion protein was dialyzed against a 50 mM potassium phosphate buffer, pH 7.5, containing 100 mM KCl, 2 mM urea, and glutathione (2 mM GSH and 0.2 mM GSSG). The protein was immunized in rabbits intramuscularly in the neck region at 4-week intervals with 70 μg of antigen emulsified with Ribi adjuvant R-730 (RIBI ImmunoChem Research Inc.). Preimmune serum was collected before the first injection, and antisera were collected 12 days after each of four injections.

**Immunopurification of RDPG1 from Oilseed Rape Dehiscence Zones** (Dehiscence a new regulated secretion pathway). The elusive and transitory nature of most precursors makes their study difficult, and to our knowledge, this is the first time that the function of the propeptide-like domain of a protein different from a hormone or a protease has been studied.
a high flux Biomax polysulphone membrane with a cut-off of 10 kDa (both from Millipore). The resulting crude extract was applied to a SP-Sepharose Fast Flow XK50/100 column (Amersham Pharmacia Biotech) equilibrated with 100 mM sodium acetate, pH 5.5, containing 0.45 M NaCl. After a washing step using the same buffer, pro-RDPG1 or RDPG1 was eluted with 100 mM sodium acetate, pH 5.5, containing 0.2 M NaCl. The fractions containing enzyme activity were pooled; dialyzed twice for 12 h at room temperature the samples were renatured in the same buffer containing 6.5 M guanidinium chloride and 10 mM DTT. After incubation for 30 min at room temperature, the samples were then placed on ice, and endopolygalacturonase activity was determined on a Thermostated Dako, diluted 1:2000, was carried out in Tris-buffered saline/5% horse serum/2% Tween 20.

**Results**

**Immunopurification of RDPG1 from Mature Pod DZ and Determination of Its N-terminal Sequence**—Native RDPG1 was purified to homogeneity from mature pod dehiscence zones (approximately 50 DAA) using immunoaffinity chromatography on an RDPG1-antiserum column in order to determine its precise N terminus and thereby verify the existence of a cleavable, propeptide-like N-terminal domain. Purification of RDPG1 to homogeneity, as assessed by SDS-PAGE, could thus be achieved in a single step. From 5 g of oilseed rape DZ (fresh weight), 0.25 mg of pure RDPG1 was obtained. SDS-PAGE revealed the presence of two bands in the immunopurified RDPG1: a major band of 49 kDa and a minor band of 54 kDa. Western blots probed with an antiserum raised against the N-terminal domain of the mature RDPG1 showed that the major band corresponded to mature RDPG1, whereas the minor band contained pro-RDPG1 (Fig. 2A). N-terminal sequencing of the protein in the major band identified Glu-65 as the exact N terminus of the mature RDPG1. This confirms that RDPG1 is synthesized as a precursor containing a cleavable, propeptide-like N-terminal domain and furthermore reveals
the precise length of this N-terminal domain and the exact N terminus of the mature RDPG1. Thus the RDPG1 precursor is composed of a predicted 23-amino-acid-long signal peptide (Met-1–Ala-23) (24), a 41-amino-acid-long cleavable N-terminal domain (Leu-24–Thr-64), and a 367-amino-acid-long catalytic domain starting at Glu-65. Furthermore, N-terminal sequencing of the purified protein revealed microheterogeneity with three minor forms starting at Ser-58, Asp-60, and Ser-66, respectively (Fig. 1B).

Investigation of a Putative Role of the Cleavable N-terminal Domain of RDPG1 in Protein Folding in Vivo by Comparison of Recombinant Expression of Pro-RDPG1 and ΔPro-RDPG1 in P. pastoris—In order to investigate whether the N-terminal domain of RDPG1 is necessary for the correct folding of its cognate protein in vivo, we compared the expression of pro-RDPG1 and Δpro-RDPG1 (the N-terminal domain truncated form of pro-RDPG1, corresponding to the mature RDPG1) recombinantly in P. pastoris. For this, a truncated cDNA lacking the signal peptide coding sequence (bases 1–155) or lacking the signal peptide and the N-terminal domain coding sequence (bases 1–284) was cloned into the P. pastoris vector pPICZα in frame with a DNA sequence encoding a yeast signal peptide to direct the recombinant protein to the endoplasmic reticulum and through the secretory pathway. P. pastoris was transformed with the resulting plasmids (Fig. 1, C and D). Both constructs were able to direct secretion of comparable levels of pro-RDPG1 and Δpro-RDPG1, respectively, into the culture medium, indicating that cleavable N-terminal domain is not required for correct folding of RDPG1 in P. pastoris, as unfolded proteins are not allowed to be secreted but are degraded inside the cell. However, P. pastoris could not posttranslationally process the precursor protein into the mature endopolygalacturonase, as shown by SDS-PAGE (Fig. 2B) and by N-terminal sequencing (Fig. 1C). This indicates that the protease(s) responsible for this maturation process in the plant is absent in P. pastoris. The normally transitory nature of precursors is a great obstacle to their isolation and study and the inability of P. pastoris to process pro-RDPG1 into RDPG1 provided us with a unique source of the pro-RDPG1 precursor for further investigations of the function of its cleavable N-terminal domain.

Purification of Recombinant Pro-RDPG1 and ΔPro-RDPG1 and Investigation of a Putative Role of Cleavable N-terminal Domain in Enzyme Activation—In order to compare their specific activities, recombinant pro-RDPG1 and Δpro-RDPG1 were purified from the P. pastoris culture broth before their activity was measured. The purification to homogeneity of pro-RDPG1 and Δpro-RDPG1 was achieved with ultrafiltration followed by two ion exchange chromatography steps (Table II). For Δpro-RDPG1, the last step was a combined anion and cation exchange chromatographic separation, in which the enzyme passed through the anion exchanger and bound to the cation exchanger. From 2 liters of culture broth, 8–10 mg of 500–600-fold purified pro-RDPG1 and 3–4 mg of 600–800-fold purified Δpro-RDPG1 were obtained. In the last separation step Δpro-RDPG1 eluted in a single peak, corresponding to a single band in SDS-PAGE. In contrast, pro-RDPG1 eluted in four peaks. SDS-PAGE revealed that each peak contained a single protein, but of varying molecular masses, and immunoblotting confirmed that all four proteins originated from pro-RDPG1, because they were all recognized by an antiserum to RDPG1. N-terminal sequencing of each isoform showed that this heterogeneity was due to random, partial proteolysis within the cleavable N-terminal domain of RDPG1 is shown in gray. Large arrows, major N termini; small arrows, minor N termini.
isoforms starting at Ser-26 (full-length pro-RDPG1 and most abundant form), Asp-42, Lys-57, and Ser-58, respectively (Fig. 1C). Full-length pro-RDPG1 was used for all further analyses. Specific activities of pure pro-RDPG1 and Δpro-RDPG1, determined using various pectic substrates, were very similar, showing that the precursor is fully active. Moreover, the specific activities of pro-RDPG1 and Δpro-RDPG1 were comparable to that of the native, mature enzyme immunopurified from oilseed rape pod DZ, indicating that the enzymatic properties of the recombinant proteins obtained from P. pastoris were similar, if not identical, to those of the native protein, thus confirming that these recombinant proteins are good models to study pro-RDPG1 and RDPG1. Specific activities using our standard pectin substrate were 410, 447.5, and 372 units/mg for pro-RDPG1, Δpro-RDPG1, and native RDPG1, respectively. Thus, the cleavable N-terminal domain of RDPG1 does not play a role in enzyme activation, in contrast to nearly all propeptides in enzymatic precursors in which this has been investigated.

**Investigation of a Putative Role of the Cleavable N-terminal Domain of RDPG1 in Protein Folding Using in Vitro Denaturation-Renaturation Experiments**—The denaturation and renaturation of pro-RDPG1 and Δpro-RDPG1 was investigated by incubation of the proteins with 6.5 M guanidinium chloride followed either by buffer exchange to a negligible guanidinium chloride concentration or by rapid dilution to 0.1 M guanidinium chloride. Reduction of the disulfide bridges was done by addition of 10 mM DTT in the denaturation buffer. In order to confirm that pro-RDPG1 and Δpro-RDPG1 had been denatured upon incubation with guanidinium, with or without DTT, their intrinsic fluorescence spectra were recorded. For both proteins, a reduction in $E_{max}$ and a shift in $\lambda_{max}$ toward higher wavelength were observed in the presence of guanidinium, thus confirming that the proteins were denatured. The $\lambda$-shift was even higher in samples containing both guanidinium and DTT, suggesting, as expected, that the proteins are more extensively unfolded in the presence of DTT than in guanidinium alone (Table III). Recovered enzymatic activity was used as a measure of the extent of renaturation. In all cases, the maximum level of recovered activity was achieved after 15 min of renaturation and was stable for at least 2 h (data not shown). In experiments without disulfide bridge reduction, pro-RDPG1 and Δpro-RDPG1 recovered up to 92.6 and 74.6% of their initial activity, respectively (Fig. 3). This confirms that the cleavable N-terminal domain of RDPG1 is not required for folding. For both proteins, buffer exchange on a Nap-5 column yielded a higher renaturation level than rapid dilution, probably because the chromatographic support provides a refolding scaffold to the protein. In all cases, the levels of recovered activity were somewhat higher for pro-RDPG1 than for Δpro-RDPG1, but this marginal difference most likely reflects that these proteins have different requirements for optimal renaturing conditions, and the conditions used here may have been more favorable for pro-RDPG1 than for Δpro-RDPG1. The level of renaturation after denaturation in the presence of DTT was considerably lower than in the absence of DTT for both pro-RDPG1 and Δpro-RDPG1 (Fig. 3). This suggests that at least some disulfide bridges have been eliminated by the DTT treatment and that their correct reformation is essential for activity. Formation of disulfide bridges is known to be a rate-limiting step in folding, and correct reoxidation of up to all 12 cysteine residues of pro-RDPG1 and Δpro-RDPG1 may thus require a longer renaturation time than in our experimental conditions, as well as the presence of a catalyst, such as protein disulfide isomerase (25).

### Table II

| Enzyme   | Step                  | Total protein | Total activity | Specific activity | Yield | Purification |
|----------|-----------------------|---------------|----------------|-------------------|-------|--------------|
|          |                       | mg            | units          | units/mg          | %     | fold         |
| pro-RDPG1| Crude extract         | 3400.0        | 2750           | 0.8               | 100   | 1            |
|          | Ultrafiltration       | 1200.0        | 3600           | 3.0               | 131   | 4            |
|          | SP-Sepharose          | 30.0          | 7688           | 256.3             | 280   | 320          |
|          | Dialysis C            | 21.8          | 4646           | 213.1             | 149   | 266          |
|          | Mono-S                | 1.0           | 4100           | 410.0             | 149   | 512          |
| Δpro-RDPG1| Crude extract        | 1350.0        | 947            | 0.7               | 100   | 1            |
|          | Ultrafiltration       | 600.0         | 2608           | 4.3               | 275   | 6            |
|          | SP-Sepharose          | 12.2          | 3630           | 297.5             | 383   | 425          |
|          | Dialysis C            | 11.4          | 1702           | 149.5             | 180   | 213          |
|          | Mono-Q-Mono-S         | 3.7           | 1656           | 447.5             | 175   | 639          |

* Arbitrary PG activity units as defined in Ref. 22.
* Specific activity after dialysis was underestimated due to the low activity of the enzymes at low ionic strength.

### Table III

| Sample             | $\lambda_{max}$ | $E_{max}$ | $\Delta$ shift | Relative activity (U/ml, percent of control) |
|--------------------|-----------------|----------|----------------|--------------------------------------------|
| Pro-RDPG1          | 338.7           | 468.58   |                | pro-RDPG1                                  |
| Pro-RDPG1 + GuC    | 340.5           | 304.79   | 1.8            | 100                                        |
| Pro-RDPG1 + Gu/DTT | 343.5           | 280.84   | 4.8            | 50                                         |
| ΔPro-RDPG1         | 337.5           | 542.01   |                | pro-RDPG1                                  |
| ΔPro-RDPG1 + Gu    | 343.5           | 348.28   | 6.0            | 200                                        |
| ΔPro-RDPG1 + Gu/DTT| 346.0           | 345.76   | 8.5            | 100                                        |

* GuC, guanidinium chloride.

**FIG. 3.** Compared renaturation of pro-RDPG1 and Δpro-RDPG1. Open bars, denaturation with guanidinium chloride and renaturation with buffer exchange; gray bars, denaturation with guanidinium chloride and rapid dilution; black bars, denaturation with guanidinium chloride and DTT and renaturation with rapid dilution.
Can be segmented into three phases. The first phase (0–70 DAA) is characterized by elongation of the pod to its full size. At the end of this phase, degradation of the pectin in the wall of edge cells and replum cells occurs and seed growth takes place. At the end of this phase, senescence takes place, and extensive degradation of the pectin in the middle lamella of the cell wall leads to cell separation and dehiscence. Furthermore, they showed that treatment of the pods with the auxin analogue 4-CPA results in a 10-day delay of cell separation in the DZ, leading to dehiscence. These results indicate that a low level of auxin in the DZ is necessary for dehiscence to take place. In order to investigate whether the release of RDPG1 to the cell wall required a low level of auxin in the DZ, spraying oilseed rape plants with 4-CPA was performed. Subcellular localization of RDPG1 was analyzed by electron microscopy as above. Upon spraying with 4-CPA at 39 DAA, the appearance of RDPG1 in the cell wall was delayed from 47 to 52 DAA. At the same time, the intracellular accumulation of RDPG1 was significantly higher than in nonsprayed, control plants, indicating that a low level of auxin in the DZ is necessary for dehiscence to take place.

The N-terminal Domain of Clade B Plant Polygalacturonases

In the DZ of oilseed rape, the pectin-rich middle lamella, where it accumulated throughout senescence (Fig. 5C). The intracellular RDPG1 labeling appeared to be restricted to defined areas, reminiscent of subcellular organelles, possibly the endoplasmic reticulum, thus suggesting that RDPG1 is stored retained inside the cell for a period of time prior to its secretion into the wall. No labeling was observed using the N-terminal domain antisera in the DZ of green or senescent pods, confirming that pro-RDPG1 does not accumulate in the DZ.

Chauvaux et al. (20) have observed that there is a decrease in auxin content specifically in the DZ of oilseed rape pods prior to degradation of pectin in the middle lamella of the cell wall leading to dehiscence. Furthermore they showed that treatment of the pods with the auxin analogue 4-CPA results in a 10-day delay of cell separation in the DZ. These results indicate that a low level of auxin in the DZ is necessary for dehiscence to take place. In order to investigate whether the release of RDPG1 to the cell wall required a low level of auxin in the DZ, we sprayed oilseed rape plants with 4-CPA. Subcellular localization of RDPG1 was analyzed by electron microscopy as above. Upon spraying with 4-CPA at 39 DAA, the appearance of RDPG1 in the cell wall was delayed from 47 to 52 DAA. At the same time, the intracellular accumulation of RDPG1 was significantly higher than in nonsprayed, control plants, indicating that a low level of auxin in the DZ is necessary for dehiscence to take place.

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Fig. 4. Time course accumulation of RDPG1 in oilseed rape pod dehiscence zone. Western blots of total protein extracts from oilseed rape pod DZ were probed with a RDPG1 antiserum. Each lane was loaded with a total protein amount equivalent to one-quarter of a DZ. The labeled polypeptide had a mobility corresponding to 49 kDa.

Fig. 5. Electron microscopy of cross-sections of the DZ of oilseed rape pod and immunogold labeling of RDPG1 and its substrate in DZ depending on the age and the treatment of the pod. CPL, cytoplasm; CW, cell wall; ML, middle lamella. A, cross-section of an oilseed rape pod. B–D, RDPG1 immunogold labeling. At 35 DAA, RDPG1 is intracellular only and cell wall is intact (B) (bar = 7 μm); at 49 DAA, RDPG1 labels the cell wall between separated cells (C) (bar = 117 μm); and at 47 DAA in plants sprayed with 4-CPA at 39 DAA, RDPG1 labeling is intracellular and cell wall is intact (D) (bar = 1.4 μm). E and F, JIM5 immunogold labeling of low methyl esterified pectic homogalacturonan, the substrate of RDPG1. At 47 DAA, immediately prior to cell separation, JIM5 labeling shows greatly reduced amounts of pectin (E) (bar = 121 μm), whereas at 49 DAA in plants sprayed with 4-CPA, cell wall remains intact and JIM5 labeling shows great abundance of intact pectin (F) (bar = 47 μm).
cating that secretion of RDPG1 to the cell wall was blocked by the presence of 4-CPA and RDPG1 thus accumulated intracellularly (Fig. 5, C and D). Hence the ratio of intracellular RDPG1 to cell wall RDPG1 was altered in 47–49-DAA DZ in 4-CPA sprayed plants as compared with nonsprayed, control plants. Concomitantly with the delay of the appearance of RDPG1 in the cell wall, a delay in pectin degradation was observed as evidenced by the immunolabeling using the JIM5 monoclonal antibody (23), specific for pectic homogalacturonan with low degree of methyl esterification, the substrate of RDPG1. Subsequently, a delay in cell separation was also observed (data not shown), establishing a clear dependence of dehiscence on the release of RDPG1 to the cell wall.

**DISCUSSION**

The postulate that the additional 40–50-amino acid-long N-terminal domain of PGs from clade B is a propeptide was based on one example only, that of the tomato fruit PG. Comparison of the protein sequence deduced from full-length cDNA clones with the N-terminal sequence of the PG purified from tomato fruit revealed that the first 47 amino acids following the signal peptide were absent in the mature protein. This was further confirmed in *in vitro* translation experiments in the presence of microsomes (19, 29). In the present study, we have established that this is also true for the oilseed rape pod polygalacturonase RDPG1, another member of this protein subfamily. In identifying the additional N-terminal domain of another PG from clade B as a cleavable domain, which is absent in the mature protein, we have confirmed experimentally the universal presence of a cleavable N-terminal domain in all PGs from clade B. The cleavable N-terminal domains of PGs from clade B do not share significant sequence homology but have a high content of charged amino acids. Moreover, the cleavable N-terminal domain of RDPG1 exhibits susceptibility to random proteolysis, as shown by the occurrence of isoforms with different N termini, all residing within this domain, upon recombinant expression of pro-RDPG1 in *P. pastoris*. This indicates that the cleavable N-terminal domain is not fully and compactly folded. All of these are general structural features of propeptides (30, 31). The microheterogeneity found at the N terminus of the native RDPG1, purified from pod DZ, could be an indication that the processing of pro-RDPG1 into RDPG1 is a multistep process involving several proteases, *e.g.* an endoprotease and one or several aminopeptidases. Moreover, one or more proteases required for the cleavage of pro-RDPG1 are absent in the yeast *P. pastoris*, suggesting that this processing is unique to plants.

Our study reveals that the cleavable N-terminal domain of PGs from clade B is not involved in keeping its cognate enzyme inactive. This result is important, because it reveals that the often encountered hypothesis that the cleavable N-terminal domain of PGs from clade B is involved in preventing premature activation of the enzyme is wrong (3, 13, 19). We also show that the cleavable N-terminal domain of PGs from clade B is not required for folding or dimerization. Our results on folding from *in vitro* experiments and from *in vitro* denaturation-renaturation experiments correlate very well and support each other. However, this does not exclude the possibility that the N-terminal domain might have an influence on the kinetics of the folding of PGs from clade B in vivo. In conclusion, although the cleavable N-terminal domain of PGs from clade B presents the structural characteristics of a propeptide, it does not have the classical functions of a propeptide, in keeping its cognate enzyme as an inactivezymogen and/or in assisting protein folding. Indeed, in virtually every case in which this has been investigated, zymogen propeptides have been shown to keep their cognate enzyme inactive. Therefore, the cleavable N-terminal domain of PGs from clade B cannot be regarded as a true propeptide.

Our time course study of the accumulation of RDPG1 in the pod DZ using immunoblots and gold immunolocalization shows that the protein accumulates over a long period of time in the pod DZ in accordance with the *rdpg1* promoter activity analysis (32). The precursor form is absent from the bulk of RDPG1 in oilseed rape pod DZ. Only following a high enrichment of protein extracts in pro-RDPG1 and RDPG1 by immunoaffinity chromatography could pro-RDPG1 be detected in the oilseed rape DZ (Fig. 2A). This shows that pro-RDPG1 is a transitory form and that the N-terminal domain is cleaved early after biosynthesis of pro-RDPG1. Strikingly, our electron micrograph results furthermore indicate that RDPG1 is temporarily stored inside the cell for about 3 weeks after its biosynthesis and before its release to the cell wall. These data are corroborated by the observation that the auxin analogue 4-CPA retarded or even prohibited the release of RDPG1 to the cell wall whereas it did not seem to affect its biosynthesis. Because RDPG1 is responsible for the degradation of pectin in the middle lamella, leading to dehiscence (4, 6, 32), the abundant JIM5 immunogold labeling of the pectin in the middle lamella upon spraying with 4-CPA, provides an additional proof of the delay in secretion of RDPG1 upon spraying with 4-CPA, thus confirming the results of our RDPG1 immunogold labeling. Altogether, this suggests that the biosynthesis of RDPG1 and its secretion to the cell wall do not occur simultaneously but are two distinct processes, exhibiting different hormonal dependence, and that the secretion of RDPG1 to the cell wall does not occur via the default pathway but is a regulated event.

The presence of PG mRNAs in planta long before pectin undergoes enzymatic breakdown has been observed in oilseed rape pod dehiscence zone (this study), in tomato, and in *Charentais melon* (33–35). This raises the question of how premature action of PGs on their substrates is prevented and various hypotheses have previously been proposed. In tomato fruit, it has been proposed that PG could associate with the β-subunit, a small protein that could restrict PG activity by binding either to the enzyme or to its substrate (36). Recently *Brummel et al.* (37) have obtained data indicating that the tomato fruit expansin Exp1 may control access of PG to its substrate via pectin relaxation. In addition to such mechanisms regulating substrate accessibility within the cell wall, the sequestration of PG in a subcellular compartment until the triggering of its secretion to the wall could provide another efficient mechanism to prevent it from degrading pectin prematurely.

In plant cells, secretion to the cell wall is the default pathway for all proteins entering the endoplasmic reticulum. Transport of secretory proteins to another destination, *e.g.* the vacuole, requires a sorting signal within the protein sequence. This signal can be located on a cleavable N- or C-terminal peptide or within the mature protein (38–41). Therefore, our present data lead us to propose that RDPG1 is transported to the cell wall through a novel regulated secretion pathway. More precisely, we propose that RDPG1 is sorted away from the default secretion pathway and is stored intracellularly until its secretion to the cell wall is triggered by a signal that is yet to be uncovered and that may be regulated by the level of plant hormones and by the developmental stage of the tissue. Such a secretion mechanism could allow a fast and highly dynamic cell wall response, with the enzyme being physically isolated from its substrate until needed but instantly provided when needed. We further propose that the sorting signal for this novel pathway resides within the cleavable N-terminal domain. Sequence comparison of the cleavable N-terminal domain of 14 PGs from clade
B has identified the presence of a four-amino acid consensus sequence on 11 of the 14 sequences. This consensus sequence, (D/E)(X/G/P)(G/F), where X is not Gly, therefore represents a good candidate for a sorting signal. If the sorting signal lies within the cleavable N-terminal domain, it is reasonable to imagine that this novel secretion pathway is valid for all PGs from clade B. In turn, the physiological basis for the distinction between clade B and the other clades could be their secretion mechanism, and thus PGs from clade B could represent a class of PGs involved in rapid cell wall changes.

The expression in transgenic plants of a reporter protein (e.g. GUS or GFP) alone or fused with the cleavable N-terminal domain of RDPG1 and driven by the RDPG1 promoter and signal peptide and comparison of the fate of the reporter protein expressed with or without the cleavable N-terminal domain will help to challenge the hypothesis that the cleavable N-terminal domain of PGs from clade B is involved in the sorting of these proteins toward a novel regulated secretion pathway.

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