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Evidence of 4-Cl-IAA and IAA Bound to Proteins in Pea Fruit and Seeds

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Abstract The auxins 4-chloroindole-3-acetic acid (4-Cl-IAA) and indole-3-acetic acid (IAA) occur naturally in pea vegetative and fruit tissues (*Pisum sativum* L.). Previous work has shown that 4-Cl-IAA can substitute for the seeds in the stimulation of pea pericarp growth, whereas IAA is ineffective. Both auxins are found as free acids and as low-molecular-weight conjugates from organic solvent-soluble extracts from pea fruit. Here we present evidence for an additional conjugated auxin species that was not soluble in organic solvent and yielded 4-Cl-IAA and IAA after strong alkaline hydrolysis, suggestive of auxin attachment to pea seed and pericarp proteins. The solvent-insoluble conjugated 4-Cl-IAA in young pericarp was on average 15-fold greater than solvent-soluble 4-Cl-IAA. The solvent-insoluble conjugated IAA was approximately half the levels reported for the solvent-soluble IAA fraction. To identify putative 4-Cl-IAA-bound proteins, polyclonal antibodies were raised to 4-Cl-IAA linked to bovine serum albumin protein (BSA). Immunoblots probed with anti-4-Cl-IAA-BSA antiserum detected three to four unique bands (32–40 kDa) in primarily maternal tissues, and a different set of protein bands were detected in mainly embryonic tissues (ca. 65–74 kDa in mature seed). 4-Cl-IAA and IAA were also identified from protein fractions separated by polyacrylamide gel electrophoresis using GC-MS. These data show that the majority of 4-Cl-IAA, the growth-active auxin in young pea pericarp, and significant levels of IAA are linked to protein fractions. Auxin-proteins may function in regulation of free bioactive 4-Cl-IAA and IAA levels, and/or 4-Cl-IAA or IAA may be targeted to specific proteins post-translationally to modify protein function or stability.

Keywords Auxin conjugation · Auxin-protein · 4-Chloroindole-3-acetic acid · Fragaria vesca · Pisum sativum · Fruit protein modification

Introduction

Naturally occurring auxins, including 4-chloroindole-3-acetic acid (4-Cl-IAA) and indole-3-acetic acid (IAA), are found in plants in both free and conjugated forms (Bandurski and Schulze 1977; Magnus and others 1997). Auxins have been implicated in a wide range of functions in plants, including cell division, cell elongation, vascular differentiation, root initiation, tropisms, and fruit development (Reinecke 1999; Davies 2004). Conjugation of auxin molecules has been implicated as part of a biochemical system for homeostatic control of auxin levels in higher plants (Bandurski and others 1995), and, consistent with this idea, the evolution of the ability of plants to form a complex vascular system appears to be correlated to the appearance of auxin conjugation (Cooke and others 2002). The majority of studies on auxin conjugates have focused on auxin linked to a single amino acid, or to a mono- or disaccharide

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(Bandurski and others 1995) isolated by solvent extraction of plant tissue. Isolation of a small organic solvent-extractable 3.6-kDa peptide from bean (*Phaseolus vulgaris*) conjugated with IAA demonstrated that IAA could be covalently bound to proteins as well (Bialek and Cohen 1986). However, in bean seeds, the amount of total auxin (IAA, free plus conjugated) obtained by direct hydrolysis of seed tissue was found to be much greater than the amount readily extractable with organic solvents (Bialek and Cohen 1989). These results led to identifying a larger-molecular-weight 35-kDa protein from bean seed, IAA-modified protein 1 (IAP1), which was covalently bound to IAA (Walz and others 2002) with approximately half of the IAP1 protein in seeds modified with IAA. The localization of *ipa1* mRNA transcripts in the developing seeds and utilization of the IAP1 protein during the germination process suggested that IAP1 may be a seed-storage protein (Walz and others 2002). Quantitative analysis of IAA from acetone powders or gel-purified protein and immunoblot analysis with anti-IAA antibodies have shown that IAA-bound proteins occur in other plants, including strawberry (*Fragaria vesca* spp.) fruit and *Arabidopsis* seed (Walz and others 2002; Park and others 2006). A 76-kDa protein was identified as the major IAA-linked protein from strawberry achenes and receptacles. MS/MS sequencing of its tryptic digest indicated that the peptide was likely an ATP synthase (Park and others 2006). The function of IAA bound to protein is not known but could include alteration of protein stability or activity, or storage for use as free IAA when required (Walz and others 2002; Park and others 2006; Seidel and others 2006).

4-Cl-IAA is a potent auxin that occurs naturally in at least nine species of the Vicieae tribe from the Fabaceae family, including pea (*Pisum sativum* L.) and *Vicia faba* (compare Reinecke 1999). 4-Cl-IAA occurs in many pea tissues, including immature seeds (Marumo and others 1968; Magnus and others 1997), the shoot, root, and cotyledons of 3-day-old etiolated seedlings (Schneider and others 1985), and fruit (pericarp; Magnus and others 1997) as identified by GC-MS. 4-Cl-IAA methyl-ester and aspartate-amide conjugates have also been unequivocally identified in pea seeds (Marumo and others 1968; Hattori and Marumo 1972), and 4-Cl-IAA conjugates have been quantified in young developing seeds and fruit (pericarp) following alkaline hydrolysis of organic solvent extracts (Magnus and others 1997). However, whether 4-Cl-IAA is linked to an organic solvent-insoluble fraction (protein) in pea or other plant species has previously remained unknown.

Pea has been used as a reference system to understand the role of 4-Cl-IAA in plant tissues (Reinecke 1999) and to understand how a plant uses two auxins to affect plant growth and development. Exogenous 4-Cl-IAA stimulates pea internode section growth, root initiation, ethylene evolution in shoot cuttings, and pericarp elongation (Katekar and Geissler 1983; Ahmad and others 1987; compare Reinecke and others 1995). A compound reported as putative methyl-ester of 4-Cl-IAA reaches a maximum during rapid pea seed fill (Gandar 1960), and 4-Cl-IAA's presence and stimulatory effect on growth in young pericarps (Reinecke and others 1995; Magnus and others 1997) suggest a role for 4-Cl-IAA in pea fruit development. In pea, normal pericarp growth requires the presence of seeds, and the removal of seeds 2–3 days after anthesis (DAA) results in the slowing of pericarp growth and subsequent abscission (Eeuwens and Schwabe 1975; Ozga and others 1992). Developing pea seeds and pericarps contain gibberellins (GAs) (García-Martínez and others 1991; Rodrigo and others 1997) and auxins (4-Cl-IAA and IAA; Marumo and others 1968; Magnus and others 1997), and application of 4-Cl-IAA (Reinecke and others 1995) and GA (GA_1 or GA_3; Eeuwens and Schwabe 1975; Ozga and Reinecke 1999) to deseeded pericarp (2 DAA) can substitute for seeds and stimulate pericarp growth. However, IAA was ineffective in promoting pericarp growth (Reinecke and others 1995). Both 4-Cl-IAA and IAA stimulate ethylene synthesis in pericarps, but only 4-Cl-IAA reduces the negative response of ethylene on pericarp growth (Johnstone and others 2005). Furthermore, metabolism and expression studies using the pea split-pericarp assay (compounds are applied to split and deseeded pericarp) have shown that the presence of seeds or 4-Cl-IAA (but not IAA) is required to maintain GA metabolism (van Huizen and others 1995; Ozga and others 2009), stimulate GA biosynthesis gene expression (*PsGA20ox1* coding for GA 20-oxidase and *PsGA3ox1* coding for GA 3-oxidase; van Huizen and others 1997; Ozga and others 2003, 2009), and reduce specific GA catabolism gene expression (*PsGA2ox1* coding for GA 2-oxidase) in young pericarps (Ozga and others 2009). These results support involvement of 4-Cl-IAA in the regulation of GA biosynthesis and suggest unique modes of auxin (4-Cl-IAA vs. IAA) action based on alternative molecular recognition mechanisms and signal transduction, although differing auxin metabolism or cellular compartmentalization may also play a role in pea fruit growth.

To further understand the occurrence and function of the auxins 4-Cl-IAA and IAA in fruit and seed development in pea, an additional class of auxin bound to organic solvent-insoluble fractions (protein) was assessed in these tissues. We now report that pea fruit and seeds contain 4-Cl-IAA and IAA conjugated to organic solvent-insoluble protein fractions in significant levels to affect the auxin economy in these plant tissues. Furthermore, antibodies specific for 4-Cl-IAA- and IAA-proteins were used to aid in the determination of specific auxin-bound proteins in these tissues.

Materials and Methods

Plant Material and Treatments

Mature air-dried seeds of *Pisum sativum* L., line I_3 (Alaska-type) were used for mature seed extracts. For pericarp tissue and immature seeds, pea plants (line I_3; Alaska-type) were grown under a 16 h/8 h light/dark photoperiod (19°C/17°C) with an average photon flux density of 402 μE m^{-2} s^{-1} (van Huizen and others 1995). Fruits (pericarps) at 1–2 days after anthesis (DAA) that were 12–20 mm long (seeds removed), and immature seeds 10 and 20 DAA were harvested into liquid N_2 and stored at −80°C until extraction. Strawberry (*Fragaria vesca* spp. *vesca* f. *semperflorens* cv. Yellow Wonder) was cultivated as previously described (Park and others 2006) and fruits were frozen in liquid N_2 and stored at −80°C until analysis. *Arabidopsis thaliana* (ecotype Columbia) seeds were purchased from Lehle Seeds (Round Rock, TX).

Protein Extraction

Pericarps, seeds, or fruit were ground in liquid N_2 and extraction buffer (50 mM Tris–HCl, pH 8.0) containing protease inhibitors (one Complete EDTA-free Protease Inhibitor Cocktail Mini Tablet per 5 ml buffer; Roche, Mannheim, Germany). The protein extracts were centrifuged (17,900*g* for 20 min at 4°C), and the total protein content in the supernatant was measured using the Bradford assay (Bradford 1976). Two volumes of Laemmli buffer (Laemmli 1970) were added to the resulting supernatant. Extracts were then boiled for 5 min to solubilize the protein. Protein extracts were subsequently stored at −20°C until electrophoresis.

Electrophoresis

The protein extracts were loaded at 20 μg total protein per well for immunoblot analysis or 40 μg total protein per well for the auxin hydrolysis experiments. The protein extracts were separated on precast 10% Tris−HCl resolving, 4% stacking SDS polyacrylamide gels (SDS-PAGE; Bio-Rad Laboratories, Hercules, CA). Gels were run for approximately 40 min at 150 V (constant voltage) in a Tris−glycine (pH 8.4) running buffer.

Production of 4-Cl-IAA-BSA Antiserum

4-Cl-IAA was coupled to bovine serum albumin (BSA, RIA grade; Sigma, St. Louis, MO) without a spacer by an active ester method similar to that used in the conjugation of GA_{20}-BSA (Yamaguchi and others 1987). The coupling was monitored by MALDI-TOF (Bruker Daltonics Inc., Billerica, MA). Sinapinic acid was used as the matrix and BSA was used for calibration. The change in molecular mass of BSA was consistent with the addition of approximately five 4-Cl-IAA residues linked to each BSA molecule, within the range recommended for antigens (Landsteiner 1990).

4-Cl-IAA-BSA emulsified with complete Freund's adjuvant was injected subcutaneously into two New Zealand white rabbits, and 4-Cl-IAA-BSA emulsified with incomplete Freund's adjuvant was subsequently injected at 2-week intervals three additional times. The primary immunization was with 1 mg of 4-Cl-IAA-BSA and 500 μg for boost injections. Immunization and sera collection were done by ResGen (Huntsville, AL). Based on their titer against the 4-Cl-IAA-alkaline phosphatase enzyme tracer, one antiserum (called anti-4-Cl-IAA antibody here) was selected. Production of the anti-IAA-glycine-BSA antibody used in the present work (called anti-IAA antibody here) was previously described for use in detection of IAA proteins by Park and others (2006).

Conjugation of 4-Cl-IAA and Alkaline Phosphatase

The 4-Cl-IAA-linked enzyme tracer for ELISA was prepared by coupling 4-Cl-IAA active ester (0.5 mg) to alkaline phosphatase (1 mg, Roche, Mannheim, Germany) in a mixture of dimethylformamide and water (1:1, v/v) overnight at 4°C (Yamaguchi and others 1987). The reaction mixture was dialyzed for 12 h twice in 10% aqueous dimethylformamide, and then subsequently dialyzed for 12 h five times in distilled water. After dialysis, the tracer reaction mixture (1.7 ml) was diluted 1:1 (v:v) with glycerol (50 mM $ZnCl_2$ and 50 mM Tris–HCl, pH 8.0) and the enzyme tracer was stored at −20°C until use.

ELISA

Microplates (96-well Maxisorb; Nalgene Nunc International, Rochester, NY) were incubated with 100 μl per well of 10 μg ml^{-1} goat anti-rabbit IgG Fc fragment (EMD Biosciences, Inc., La Jolla, CA) in a 100-mM sodium carbonate buffer (pH 9.6) at 4°C overnight. The wells were then washed with distilled water three times and incubated with 100 μl of the 1:1200 diluted anti-4-Cl-IAA antibody or with 100 μl of the diluted 4-Cl-IAA preimmune serum in Tween 20 Tris-buffered saline (TTBS) at 37°C for 3 h. The wells were washed again with distilled water three times and incubated with 200 μl of 1% gelatin. This was followed by incubation with 20 μl of an auxin or indole analog (Trp, IAA, 4-Cl-IAA, IBA, or phenylacetic acid) solution (10 nmol to 10 fmol) and 80 μl of the tracer

(1:800 diluted alkaline-phosphatase-conjugated 4-Cl-IAA in 1% gelatin) at room temperature for 2 h. Maximum tracer binding (B_0) and unspecific binding (UB) were determined according to the method reported by Atzorn and Weiler (1983), except that for UB 10 nmol of 4-Cl-IAA-Me was added. The wells were then washed three times with TTBS and two times with Tris-buffered saline (TBS) and incubated with 200 µl of 1 mg ml^{-1} *p*-nitrophenylphosphate in a 100-mM sodium carbonate buffer (pH 9.6) at 37°C for 2 h. The absorbance was read at 405 nm using a microplate reader (Emax; Molecular Devices, Sunnyvale, CA). The standard curves of auxins and indole analogs were obtained by logit-transformation (Weiler and others 1981). Cross-reactivity was determined as described in Weiler and Zenk (1976).

Immunoblot Analysis

The protein gels were blotted for 60 min at 0.89 mA cm^{-2} onto nitrocellulose (Bio-Rad Laboratories) membranes. All subsequent steps were done at room temperature except with primary antisera. The membranes were incubated with blocking buffer (5% [w/v] dried skim milk in TBS) for 1 h followed by incubation with rabbit anti-4-Cl-IAA antibody, anti-IAA antibody, or preimmune serum at 1:1000 dilution with 5% (w/v) dried skim milk in TTBS overnight at 4°C. The membranes were then washed with TTBS five times for 5 min. The membranes were subsequently incubated with goat anti-rabbit secondary antibody conjugated to alkaline phosphatase for 90 min (Bio-Rad Laboratories; 1:2000 dilution with 5% dried skim milk in TBS). The membranes were then washed with TTBS five times for 5 min followed by TBS two times for 2 min. Blots were developed using a nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate colorimetric method (Bio-Rad Laboratories). To reduce variability for comparing immunoblots, only one gel run with identical samples is represented in a figure. The resulting immunoblot was subsequently split in half (for example, Fig. 1a, b) prior to treatment with different antibodies (preimmune or 4-Cl-IAA antibody).

For the immunoblot competition study, 32 mM IAA-alanine (IAA-ala) or IAA-glycine (IAA-gly) (Research Organics, Cleveland, OH) in 5% (v/v) aqueous MeOH was added to the incubation solution prior to adding the anti-IAA antibody during the immunoblot assay. MeOH (5% v/v) was added to the incubation solution for the control immunoblot assay. IAA-ala is similar in charge and size to IAA-gly used as the ligand to bind to BSA in the antibody production, and preliminary studies showed that they gave essentially the same response.

Analysis of Auxin Conjugation to Pea Protein

To determine if 4-Cl-IAA and/or IAA were bound to a protein fraction, mature seeds and 2-DAA pericarps were ground to a fine powder in liquid N_2. An acetone powder was prepared by extensively washing the ground powder with cold 80% aqueous acetone (250 ml g^{-1} tissue) to remove free IAA or 4-Cl-IAA and low-molecular-weight organic solvent-soluble auxin conjugates not bound to protein. The 80%-acetone-washed powder was subjected to 7 N NaOH hydrolysis in the presence of 500 ng [$^{13}C_6$]IAA and 100 ng [2,5,6,7-$^2H_4$]4-Cl-IAA in Teflon containers under a stream of water-saturated, O_2-free nitrogen at 100°C for 3 h (Bialek and Cohen 1989). After adjusting the pH to 2.5, the extract was purified on a C_{18} solid-phase column (Prep-Sep C_{18} 300 mg; Fisher Scientific, Pittsburgh, PA) as described by Chen and others (1988) and methylated using diazomethane.

In addition, SDS-PAGE gels (40 µg of 2-DAA pericarp protein extract per lane) were sectioned after electrophoresis into zone 1 (approximate protein size range of zone 1 was 206-80 kDa; 3 gels or 27 lanes) and band 1 (centered at 39.6 kDa, 5 mm wide; 6 gels or 54 lanes) and band 2 (centered at 32.4 kDa, 5 mm wide; 6 gels or 54 lanes). Zone 1 and bands 1 and 2 were separately subjected to base hydrolysis. Gel sections were placed into Teflon containers and then subjected to 7 N NaOH hydrolysis in the presence of [$^{13}C_6$]IAA and [2,5,6,7-$^2H_4$]4-Cl-IAA (200 ng each for zone 1 and 100 ng each for bands 1 and 2) under a stream of water-saturated nitrogen at 100°C for 3 h. After adjusting the pH to 2.5, the extract was purified on a C_{18} solid-phase column (as described above) and methylated using diazomethane.

Mass spectral analyses of methylated samples were performed using an Agilent model 6890 gas chromatograph interfaced to a Mass Selective Detector (5973 MSD, Agilent, Palo Alto, CA). The samples were brought up in ethyl acetate and injected into an Agilent HP-5MS capillary column (30 m × 0.25 mm × 0.25 µm film thickness) through a heated injection port (250°C) operated in the splitless mode with helium as the carrier gas at a flow rate of 1 ml min^{-1}. Upon injection, the column temperature was held at 70°C for 2 min, then increased at 15°C min^{-1} to 280°C, and then held at 280°C for 5 min, for a total run time of 21 min. Molecular and base peak ions were monitored for the methyl esters of endogenous IAA (*m/z* 130 and 189), endogenous 4-Cl-IAA (*m/z* 164 and 223), and the [$^{13}C_6$]IAA (*m/z* 136 and 195) and [$^2H_4$]4-Cl-IAA (*m/z* 168 and 227) internal standards. The amount of IAA and 4-Cl-IAA released by alkaline hydrolysis of the protein fraction was calculated by the isotope dilution equation (Magnus and others 1997). The low levels of 4-Cl-IAA from

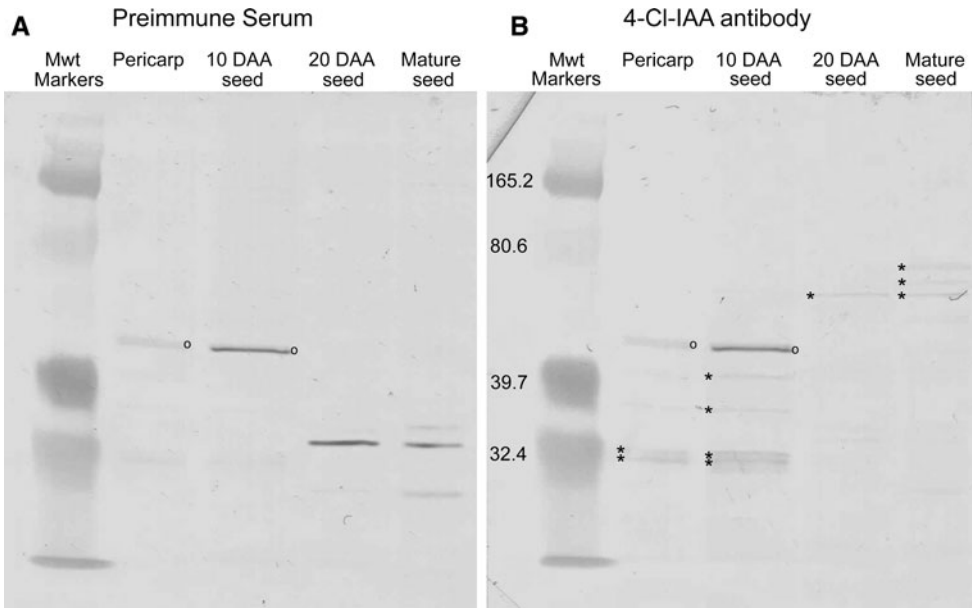

Fig. 1 Immunoblot analysis of pea pericarp and seed proteins bound by preimmune serum and the anti-4-Cl-IAA antibody. Antibodies bound to proteins were detected by a goat anti-rabbit antibody-alkaline phosphatase assay. The molecular weights of the proteins were estimated against the migration of molecular weight markers. Only a protein band of approximately 47 kDa for pericarp and 10-DAA seed protein (o) was observed with the preimmune serum (**a**) and the anti-4-Cl-IAA antibody (**b**). The anti-4-Cl-IAA antibody (**b**) recognized two major protein bands at 32 and 33 kDa for pericarp tissue and 10-DAA seeds, two minor bands at 37 and 40 kDa for 10-DAA seeds, one band at 65 kDa for 20-DAA seeds, and three bands at 65, 68, and 74 kDa for mature seeds (bands marked with *)

the PAGE-purified fractions precluded full spectral characterization.

Results and Discussion

Quantitative Analysis of 4-Cl-IAA and IAA from Solvent-Insoluble Extracts

To determine if 4-Cl-IAA and/or IAA were bound to protein, cold acetone-insoluble total protein extracts from young pericarps and mature seeds of pea were subjected to 7 N base hydrolysis in the presence of isotopically labeled internal standards of IAA and 4-Cl-IAA. The level of 4-Cl-IAA in the protein fraction of young pericarp tissue was 46–109 ng gFW^{-1} (Table 1), on average 15-fold greater than the levels reported for the organic solvent-soluble fractions (Magnus and others 1997), which is indicative of its potential to be an important part of the auxin economy of 4-Cl-IAA in this tissue. In contrast, the solvent-insoluble conjugated IAA accounted for 32–35 ng gFW^{-1} of pericarp tissue, approximately half the levels reported for the organic-soluble fractions which are the major IAA fractions of young pericarp tissue (Magnus and others 1997). Thus, conjugation of 4-Cl-IAA and IAA to proteins in pea may play a role in regulation of free bioactive auxin levels, and/or 4-Cl-IAA/IAA may be targeted to specific proteins, post-translationally modifying the protein function or turnover as previously suggested for IAA-proteins (Park and others 2006; Seidel and others 2006).

Mature pea seeds also contained solvent-insoluble conjugates of 4-Cl-IAA and IAA at 170 and 99 ng gDW^{-1}, respectively (average; Table 1). Organic solvent-insoluble IAA and 4-Cl-IAA may be a source of auxin during seed germination because free auxin is much lower at 17.4 and 3.7 ng gDW^{-1} for 4-Cl-IAA and IAA in mature embryos without testa, respectively (Ayele 2006). Immature seeds are likely actively synthesizing auxins (Gandar 1960; Eeuwens and Schwabe 1975; Ribnicky and others 2002), and 6-DAA seeds have higher levels of free IAA and 4-Cl-IAA (1495 and 231 ng gFW^{-1}, respectively; Magnus and others 1995) than mature seeds. Excised immature pea seeds treated with [^{14}C]IAA have the capacity to label a soluble protein fraction (not pelleted by ultracentrifugation) with [^{14}C]IAA, presumably by amide linkage with IAA because the labeled protein is resistant (92%) to 1 N NaOH (Jakubowska and Kowalczyk 2004). Interestingly, the pellet from ultracentrifugation was also labeled with IAA, but only 58% was presumably amide-linked and the remaining 42% was ester-linked (1 N NaOH hydrolyzable). The [^{14}C]IAA feeding study raises the question of whether a portion of auxin may be naturally linked to protein or other macromolecules by ester as well as amide linkage.

Table 1 GC-MS-SIM quantitation of 4-Cl-IAA and IAA in base-hydrolyzed protein extracts of *Pisum sativum* pea seed and pericarp tissue[a]

Tissue	Experiment	IAA		4-Cl-IAA	
		ng g FW^{-1}	ng g DW^{-1}	ng g FW^{-1}	ng g DW^{-1}
1-2 DAA pericarp	1	32.0	320.4	108.7	1086.7
	2	34.6	345.6	45.5	455.1
Mature seed	1	71.8	75.6	188.9	198.9
	2	115.3	121.4	134.0	141.1

[a] Total protein fractions of seed and pericarp tissues were extracted using an acetone washing procedure to remove free IAA or 4-Cl-IAA and low-molecular-weight organic solvent-soluble auxin conjugates. The resulting fractions were alkaline hydrolyzed in the presence of isotopically labeled IAA and 4-Cl-IAA for GC-MS-SIM estimation of the levels of IAA and 4-Cl-IAA linked to protein

Antibody Characterization

The specificity of the anti-4-Cl-IAA antibody to indole analogs and naturally occurring auxin analogs in pea was assessed by coupling 4-Cl-IAA to alkaline phosphatase for use as enzyme tracers for ELISA. Based on the ELISA data, the anti-4-Cl-IAA antibody had higher specificity at the four position of the indole ring than the anti-IAA antibody [the relative immunoreactivity of the anti-4-Cl-IAA antibody to IAA methyl ester (IAA-ME) was 6.56 (Table 2), and that of the anti-IAA antibody to 4-Cl-IAA-ME was 14.36 (Park and others 2006)]. Because the anti-4-Cl-IAA antibody has some IAA-ME cross-reactivity and the anti-IAA antibody has some 4-Cl-IAA-ME cross-reactivity, each antibody may detect each other's auxin-linked peptides, but with less efficiency. The immunoreactivity of the anti-4-Cl-IAA antibody was low for 4-Cl-IAAsp and very low for free 4-Cl-IAA, IBA-ME, and D-Trp-ME, and it did not react to free IAA or phenylacetic acid-ME. The anti-4-Cl-IAA and anti-IAA antibodies should not immunoreact with tryptophan in proteins or free 4-Cl-IAA or IAA (Fig. 1, Table 2; Park and others 2006). The low cross-reactivity of anti-4-Cl-IAA and anti-IAA antibodies to low-molecular-weight 4-Cl-IAA and IAA aspartate conjugates (Table 2; Park and others 2006) is likely caused by the polarity of the auxin-aspartate conjugates or hairpin conformation (Antolic and others 2001). The anti-IAA antibody has been previously reported to immunoreact with IAA-gly to the greatest extent, followed by IAA-ME (19.47), IBA-ME (17.44), and 4-Cl-IAA-ME (14.36), and to immunoreact weakly with IAAsp, 4-Cl-IAAsp, PAA-Me, and Trp in decreasing order (Park and others 2006).

Immunodetection of Auxin-Bound Proteins

The immunoblots visualized a group of proteins that were specifically immunoreactive to the anti-4-Cl-IAA antibody (response was absent or of very low intensity in immunoblots probed with the associated preimmune serum; Fig. 1).

Table 2 Cross-reactivities of anti-4-Cl-IAA-BSA antibody to various auxins and indole analogs as determined by ELISA[a]

Anti-4-Cl-IAA antibody	
Chemical	Cross-reactivity (%)
4-Chloro-indole-3-acetic acid-ME	100
Indole-3-acetic acid-ME	6.56
4-Chloro-indole-3-acetic acid	0.43
D-Tryptophan-ME	0.02
Indole-3-acetic acid	0
4-Cl-indole-3-acetyl-D/L-aspartate	1.84
Phenylacetic acid-ME	0
Indole-3-butyric acid-ME	0.33

ME methyl ester

[a] A rabbit anti-4-Cl-IAA antibody-goat anti-rabbit antibody-alkaline phosphatase tracer assay was used to assess relative binding specificity. A range of indole and auxin concentrations were assayed in the ELISA and used to calculate 50% inhibition of enzyme response (reduced binding) of the 4-Cl-IAA-alkaline phosphatase tracer

In young pea pericarps, which are maternally derived tissue, immunoblots probed with anti-4-Cl-IAA antibody detected two unique bands at approximately 32 and 33 kDa. In 10-DAA seeds, which consist mainly of maternally derived testa tissue, protein bands similar to those visualized in the pericarp samples were detected with two major bands at 32 and 33 kDa and two minor bands at 37 and 40 kDa. A band at 47 kDa was also present on the 10-DAA seed protein immunoblot, but because the same intense band was observed on the preimmune serum immunoblot, the 47-kDa band was not considered to be modified with 4-Cl-IAA. Different protein bands were detected when the immunoblots of mainly embryonic tissues (20-DAA and mature seed) were probed with the anti-4-Cl-IAA antibody. In 20-DAA seeds (when the developing embryo fills the seed coat) and in mature seeds, one to three protein bands were detected at about 65, 68, and 74 kDa. These data suggest that the type and occurrence of 4-Cl-IAA-bound proteins are tissue and developmentally specific.

Pea pericarp tissue had four major bands on immunoblots probed with the anti-IAA antibody at 65, 48, 37, and 36 kDa and two minor bands at 41 and 38 kDa (Figs. 2, 3). Pea seeds gave a simpler profile, with only one or two protein bands detected by the anti-IAA antibody. One unique band at 65 kDa was detected in all pea tissues tested (young pericarps, 10- and 20-DAA developing seeds, and mature seeds; Fig. 2b). Although of weaker intensity, this protein band was also detected on immunoblots probed with anti-4-Cl-IAA antibody for 20-DAA developing seeds and mature seeds (Fig. 2a). Immunoblots of pericarp and 10-DAA seed protein also gave a band at 37 kDa when probed with either the anti-IAA (more intense) or the anti-4-Cl-IAA (less intense) antibody. Because the 65- and 37-kDa bands were stronger on the immunoblots probed with anti-IAA antibody, these bands may represent proteins modified with IAA. Finding whether it is possible for IAA and 4-Cl-IAA to be linked to the same protein awaits isolation of individual pea auxin-modified proteins and MS identification of the ligands attached to specific proteins. The preimmune serum for the anti-IAA antibody detected only a few weak bands that did not comigrate with the anti-IAA-antibody-detected proteins (data not shown), except for possible interference with the 37-kDa protein for 10-DAA seeds.

To determine if an IAA analog could compete with binding of the anti-IAA antibody to possible pea IAA-proteins and result in reduced staining of bands on the immunoblot, IAA-ala or IAA-gly was added to the incubation solution during the immunodetection procedure. A reduction in staining intensity occurred on the immunoblot with IAA-ala compared to the control immunoblot, supporting that the observed 65-, 37-, and 36-kDa protein bands were linked to IAA (Fig. 3, see legend; similar results were also obtained using IAA-gly). Interestingly, a 71-kDa protein band detected for strawberry fruit with the anti-IAA antibody (Fig. 3) was also reduced by IAA-ala treatment. A 76-kDa protein band (likely the same protein band observed here) was detected in immunoblots of strawberry fruit using the anti-IAA antibody, and the protein was purified and partially sequenced (Park and others 2006). *Arabidopsis* also had a band at 36 kDa that was reduced by IAA-ala treatment. Walz and others (2002), using an antibody to the 3.6-kDa bean IAA peptide, reported that the major cross-reacting protein from *Arabidopsis* was 35 kDa. The *Arabidopsis* 35-kDa peptide was partially purified and shown to release IAA following 7 N alkaline hydrolysis. In addition, competition experiments using 4-Cl-IAA-ME were attempted; however, the solubility of 4-Cl-IAA-ME proved to be a limiting factor. Because 4-Cl-IAA is significantly less polar than IAA (Magnus and others 1997), we found we could not dissolve a sufficient amount of 4-Cl-IAA-ME into the aqueous-based incubation solution to effectively compete with the 4-Cl-IAA protein interaction.

The presence of 4-Cl-IAA- and IAA-modified proteins in pea pericarp tissue was further confirmed by GC-MS

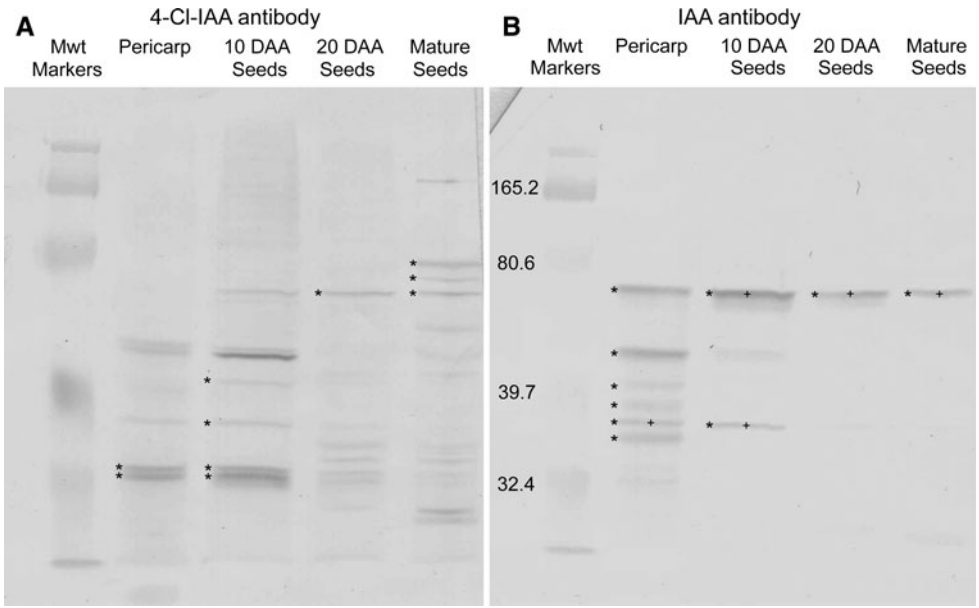

Fig. 2 Immunoblot analysis of pea pericarp and seed proteins bound by anti-4-Cl-IAA and anti-IAA antibodies. Protein bands were detected and major protein bands were observed with the anti-4-Cl-IAA antibody (**a**) as described in the legend for Fig. 1. The anti-IAA antibody (**b**) recognized four major protein bands in pericarp tissue at 36, 37, 48, and 65 kDa and two minor bands at 38 and 41 kDa. The anti-IAA antibody (**b**) recognized a band at 65 kDa in the 10- and 20-DAA seeds and in mature seeds. A band was also observed at 37 kDa in the 10-DAA seeds. All bands observed with the anti-4-Cl-IAA (**a**) and the anti-IAA antibodies (**b**) are marked with an asterisk (*). Bands in gel B marked with + were observed with both anti-4-Cl-IAA and anti-IAA antibodies

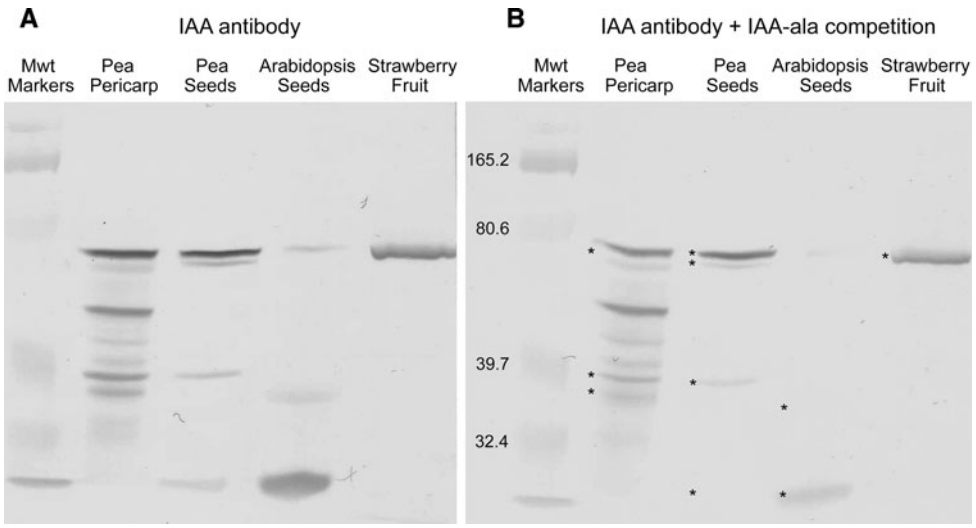

Fig. 3 Competition assay for proteins bound by the anti-IAA antibody on immunoblots. Protein bands were detected as described in the legend of Fig. 1. Pea pericarp and seed, *Arabidopsis* seed, and strawberry fruit proteins were bound by the anti-IAA antibody (**a**). Protein bands that gave a reduced signal after IAA-ala competition (**b**) are marked with an asterisk (*). Pea tissues gave several bands that were reduced by IAA-ala treatment. One band at approximately 71 kDa in strawberry fruit and two bands in *Arabidopsis* seed (at 36 kDa and a low-molecular-weight band) were reduced by IAA-ala treatment

quantification of 4-Cl-IAA and IAA from base hydrolysis of pericarp protein separated by polyacrylamide gel electrophoresis (Table 3). 4-Cl-IAA was determined to be present at 0.87 ng in gel band 2, which includes the protein bands observed at approximately 32 and 33 kDa in the anti-4-Cl-IAA antibody immunoblots (Fig. 1b). Gel band 1 contained 0.46 ng of IAA and this gel region includes the protein bands at 41, 38, and 37 kDa, as observed in the anti-IAA antibody immunoblots (Fig. 2b). The 35-kDa IAA-modified protein (IAP1) identified from bean, molecular weight determined by MALDI-TOF, runs anomalously at 42 kDa on SDS-PAGE (Walz and others 2002). Whether pea contains a homolog of IAP1 in gel band 1 awaits further purification of the gel band 1 region and its microsequencing. IAA was also detected in gel band 2 at 0.55 ng, but no prominent protein bands were observed in this region in the anti-IAA antibody immunoblots (Fig. 2b). Interestingly, the highest amount of 4-Cl-IAA and IAA was detected in gel zone 1 (protein size range was approximately 206-80 kDa), although bands in this region were not observed in the immunoblots (pericarp in Fig. 2a, b). The pericarp protein(s) conjugated to IAA and 4-Cl-IAA in gel zone 1 may have lower cross-reactivity to the anti-IAA and anti-4-Cl-IAA antibodies due to secondary structure effects; this points to a clear limitation in reliance solely of immunological methods of detection.

Knowledge about the presence of 4-Cl-IAA- and IAA-modified proteins in fruit and seeds of pea has the potential to greatly improve our understanding of the economy, activity, and functions of these auxins in plant systems. Currently, our knowledge of auxin-modified proteins in plants is limited to a few reports. The IAA-modified protein identified from bean (*Phaseolus vulgaris* L.) is a late-embryo abundant protein presumably involved in storage with no other known function (Walz and others 2002). Recently, the *PvIAP1* gene with the *PvIAP1* promoter was expressed ectopically in *Arabidopsis* and *Medicago*. The IAP1 protein accumulated in the transgenic seeds of both species; however, the PvIAP protein isolated from the transgenic seeds was not post-transcriptionally modified with IAA (Walz and others 2008). Proteins that are covalently modified by IAA molecules may be species specific, at least proteins destined for seed storage. Whether the IAA and 4-Cl-IAA protein bands observed from mature pea seeds are seed storage proteins awaits further analysis. In strawberry achenes and receptacles (*Fragaria vesca* spp.), a 76-kDa protein was identified as the major IAA-linked protein. Interestingly, MS/MS sequencing of its tryptic

Table 3 GC-MS-SIM quantitation of 4-Cl-IAA and IAA from protein separated by electrophoresis from 2-DAA *Pisum sativum* pericarp tissue

	IAA	4-Cl-IAA
Zone 1[a]	1.39 ng[b]	4.34 ng
Band 1	0.46 ng	nd
Band 2	0.55 ng	0.87 ng

nd not detected

[a] Sections of SDS-PAGE gel subjected to base hydrolysis: approximate protein size range: zone 1 = 206-80 kDa; band 1 = 39.6 kDa; band 2 = 32.4 kDa

[b] ng per gel-hydrolyzed sample

digest indicated that the peptide was likely an ATP synthase (Park and others 2006), and thus the data suggested that IAA could be used to modify proteins with catalytic function. If auxin is acting as a molecule to affect the protein's stability or activity, using 4-Cl-IAA versus IAA would give the protein a larger and more lipophilic prosthetic group (Reinecke and others 1999). Pea fruit growth is an ideal system by which to explore the function of auxin-bound proteins because both 4-Cl-IAA and IAA are naturally occurring but have dramatically different biological activities with respect to pericarp growth. Furthermore, our data suggest that the type and occurrence of 4-Cl-IAA- and IAA-bound proteins in pea are tissue and developmentally specific. Unique peptide bands linked to 4-Cl-IAA and to IAA were found in maternal and embryo tissues of pea. Whether 4-Cl-IAA is linked directly to peptides or IAA is linked to peptides and the IAA is subsequently chlorinated remains unknown. Future work will focus on purification and identification of proteins that were detected by the anti-4-Cl-IAA and anti-IAA antiserums in pea tissues to further our understanding of the roles of auxin-modified proteins in plant development.

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