Isolation and Characterization of a 60 kDa 2,4-D-Binding Protein from the Shoot Apices of Peach Trees \textit{(Prunus persica L.)}; It Is A Homologue of Protein Disulfide Isomerase

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To obtain a candidate auxin-binding protein (ABP), a soluble 60 kDa protein was isolated from an extract of shoot apices of peach trees \textit{(Prunus persica L.)} by affinity chromatography on a 2,4-dichlorophenoxyacetic acid (2,4-D)-linked Sepharose4B column. The 60 kDa polypeptide, designated Pp60, was purified as a single band on SDS-PAGE by column chromatography. Its dissociation constant (Kd) for $[^{14}C]2,4$-D was calculated to be $3.5\times10^{-5}$ M. The binding of Pp60 for $[^{14}C]2,4$-D was inhibited by naphthalene-1-acetic acid (NAA) and p-chlorophenoxyisobutyric acid (PCIB) as well as 2,4-D. Indole-3-acetic acid (IAA) had little effect on the binding. These results suggested that Pp60 is a protein that has an affinity for 2,4-D, NAA, and PCIB in vitro. The partial amino acid sequences of Pp60 showed high homology to those of protein disulfide isomerase (EC 5.3.4.1). Immunoblot analysis demonstrated that Pp60 exists ubiquitously in shoots and leaves. In fruit, expression of Pp60 is restricted at an early stage of development.

Key words: 2,4-Dichlorophenoxyacetic acid (2,4-D) — Protein disulfide isomerase (PDI) (EC 5.3.4.1) — \textit{Prunus persica L.} (peach).

Auxins play vital roles in the regulation of growth and development within higher plants (Evans 1974). Auxins are essential for various physiological phenomena such as elongation and enlargement of cells, apical dominance, differentiation of vascular elements, and root differentiation (Wareing and Phillips 1976, Sagee et al. 1990). In fruits, auxins have important roles in abscission, enlargement of cells, apical dominance, and induction of ethylene during development and maturation (El et al. 1993, Brown 1997, Miller and Walsh 1990).

The signals of auxins have been thought to be perceived by receptor proteins that specifically recognize auxins. To investigate the proteins that perceive auxins, many attempts have been made to isolate those that have auxin-binding activities in plants (Jones 1994) as candidate auxin receptors. ABP, isolated from \textit{Zea mays}, is the best-characterized auxin-binding protein (Lobler and Klambt 1985, Tillmann et al. 1989, Inohara et al. 1989, Hasse et al. 1989). Electrophysiological and immunological studies indicate that ABP1 acts as an auxin receptor (Barbier-Brygoo et al. 1989, Venis et al. 1992, Jones et al. 1998). Several soluble auxin-binding proteins with enzyme activities have been identified including \(\beta\)-glucosidase (Campos et al. 1992), 1,3-\(\beta\)-glucanase (Macdonald et al. 1991), glutathione S-transferase (Bilang et al. 1993, Zettl et al. 1994), glutathione-dependent formaldehyde dehydrogenase (Sugaya and Sakai 1996a), and indole acetaldehyde reductase (Sugaya and Sakai 1996b). However, the mechanisms of auxin action in plant cells are not yet well understood.

In a previous study, we isolated soluble ABPs (ABP19 and ABP20) from the shoot apices of peach trees \textit{(Prunus persica L. cv. Akatsuki)} grown in an orchard (Ohmiya et al. 1993), isolated genes encoding these polypeptides, and found that these polypeptides had a small region which shared 40% homology with BoxA, a putative auxin-binding site of maize ABP1 (Ohmiya et al. 1998). ABP19/20 were isolated with affinity chromatography on a 2,4-D-linked Sepharose4B column. In the process of purification, we also found 2,4-D binding activities in other protein fractions as well as in the one containing ABP19/20. As responses of plant cells for auxins depend on the nature of the cells, there should be various pathways for signal transduction of auxins and regulation of auxin actions. Many types of auxin-binding proteins, including auxin receptors, carriers of auxins, and proteins that associate with auxin for regulating auxin levels in the cell, must be investigated to clarify auxin actions. Therefore, we decided to isolate and characterize not only ABP19/20 but also the proteins with 2,4-D-binding properties in other fractions.

In this report, we investigated the characteristics of one of the proteins and discuss its possible functions in
peach trees.

Materials and Methods

Plant materials—Shoot apices of peach trees (Prunus persica L. cv. Akatsuki) including developing leaves were harvested at the National Institute of Fruit Tree Science and stored at −80°C until use.

Preparation of ABP fraction—The following procedures were carried out at 4°C. 2,4-D-linked Sepharose 4B was prepared as described previously (Ohmiya et al. 1993). Shoot apices (30 g) of peach trees were homogenized by Polytron (Kinematica, Switzerland) with 200 ml of 50 mM Tris-HCl buffer (pH 7.6) containing 10 mM EDTA, 20 mM 2-mercaptoethanol, 1 mM phenylmethylsulfonylfluoride (PMSF), and 50 mM sodium ascorbate (extraction buffer). Fifteen grams of insoluble polyvinylpolypyrrolidone (PVPP) were added to the above extraction buffer before homogenization. The homogenate was centrifuged at 10,000 × g for 20 min, and the supernatant was filtered through two layers of Miracloth (Calbiochem, U.S.A.). The filtrate was centrifuged at 22,000 × g for 30 min, and the supernatant was applied to a Sephadex G-25 column (9.5 cm i.d. × 35 cm) that had been equilibrated with 10 mM 2-mercaptoethanol) containing 20 mM KCl. The void fraction was collected and applied to a CM-Toyopearl column (Tosoh, Japan). The protein preparation and 0.91 kBq of 2,4-D dichlorophenoxyacetic acid (2GBq mmol−1) in 250 μl of 20 mM MES-KOH buffer (pH 6.5) were incubated at 25°C for 20 min. After the incubation, 1 ml of MES buffer (pH 6.5) was added to the reaction mixture for the binding assay. Inhibition values were expressed as the extent of relative inhibition in the presence of 1 mM 2,4-D.

Preparation of an antiserum against Pp60—The eluate of the 2,4-D affinity column was subjected to SDS-PAGE. The band containing Pp60 was cut and injected into a rabbit with Freund’s complete adjuvant at two week intervals for two months.

Immunoblot analysis—Shoot apices, leaves, and fruits were harvested and homogenized with extraction buffer and quartz sand, mortar, and pestle at 4°C. The homogenates were centrifuged, and the supernatants were applied on SDS-PAGE. After electrophoresis, proteins on acrylamide gel were blotted on a PVDF membrane. The membrane was incubated with the antiserum against Pp60, followed by incubation with alkaline phosphatase conjugated anti-rabbit IgG serum (Boehringer, Japan) as a secondary antiserum. Detection of alkaline phosphatase activity was performed with 4-nitro blue tetrazolium chloride and 5-Bromo-4-chloro-3-indoly-phosphate as substrates.

Determination of partial amino acid sequences—Protein was separated by SDS-PAGE, electroblotted on PVDF membranes, and subjected to N-terminal amino acid sequencing by a protein sequencer (Beckman, Japan). When N-terminal amino acid residues were blocked, the protein was digested with Staphylococcus aureus V8 protease (Wako, Osaka, Japan) in gel according to the method of Cleveland et al. (1977).

Results and Discussion

Using the method of Ohmiya et al. (1993), we obtained a soluble extract and subjected it to column chromatography on CM-Toyopearl. A previous report (Ohmiya et al. 1993) had indicated that the protein fraction that was retained on the CM-Toyopearl column contained ABP19/20 as major polypeptides with auxin-binding activity. It also showed that about 60% of auxin-binding activity in the soluble extract from shoot apices had passed through the CM-Toyopearl column.

In the present study, the fraction that passed through

![Fig. 1 Elution profiles for chromatography on DEAE-Toyopearl.](https://academic.oup.com/pcp/article-abstract/41/4/503/1816040/fig1.png)

**Fig. 1** Elution profiles for chromatography on DEAE-Toyopearl. •: protein concentrations (μg ml−1), ●: [14C]-2,4-D (dpm) binding by 180 μl of each fraction. Bar indicates the fractions that were pooled for further purification.
PDI as a 2,4-D-binding protein from peach trees

the CM-Toyopearl column was subjected to further purification. The fractions with binding activities to [14C]-2,4-D were separated by DEAE-Toyopearl (Fig. 1) and Sephacryl S-300 (Fig. 2). The fractions that had the binding activities (Fig. 2) were then applied on 2,4-D-linked Sepharose 4B. SDS-PAGE analysis showed that three major polypeptides existed in the fraction. The molecular masses of these polypeptides were estimated by SDS-PAGE (Fig. 3) to be 36, 37 and 60 kDa, designated as Pp (Prunus persica) 36, Pp37 and Pp60, respectively. They were also estimated at 75, 75 and 80 kDa by gel permeation chromatography in native form (data not shown). These results suggest that both Pp36 and Pp37 exist as dimers and that Pp60 exists as a monomer in an aqueous neutral buffer.

Pp60 was stained by the Schiff-reagent and retained on the ConA column (data not shown), suggesting that it is glycosylated. Proteins retained on the column were eluted with a solution containing α-D-methyl mannoside, and Pp60 was obtained as a single polypeptide (Fig. 3). In contrast, although we tried hydroxy apatite and ion-exchange chromatographies several times, it was difficult to separate Pp36 and Pp37 in their native state. Thus, the fraction containing Pp60 was subjected to further analysis.

To characterize the affinity properties of Pp60 for 2,4-D, a Scatchard analysis was carried out (Fig. 4). The apparent dissociation constant (Kd) of Pp60 was 3.5 × 10⁻⁵ M. Considering that the Kd value of ABP1 has been reported as 5.7 × 10⁻⁸ M for NAA (Lobler and Klambt 1985) and that of ABP19/20 as 4.1 × 10⁻⁵ M for 2,4-D, the Kd values indicated that the affinity of Pp60 for 2,4-D was not very high. However, as the optimum concentrations of auxins for physiological effect in the cell depend on the tissue and as the amounts of auxins in the cell differs among tissues (Wareing and Phillips 1976), many proteins with a wide variety of Kd values should exist. The binding of [14C]-2,4-D to Pp60 (Table 1) was inhibited by NAA (66%) and PCIB (97%). On the other hand, IAA weakly

Table 1 Relative effects of auxins and derivatives on [14C]-2,4-D binding by Pp60

<table>
<thead>
<tr>
<th>Compound (100 μM)</th>
<th>Inhibition (%)</th>
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<tbody>
<tr>
<td>2,4-D</td>
<td>73</td>
</tr>
<tr>
<td>IAA</td>
<td>8</td>
</tr>
<tr>
<td>NAA</td>
<td>66</td>
</tr>
<tr>
<td>PCIB</td>
<td>97</td>
</tr>
<tr>
<td>Benzolic acid</td>
<td>10</td>
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</tbody>
</table>

a 100% inhibition: extent of relative inhibition in the presence of 1 mM 2,4-D (% inhibition in absolute terms).
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Fig. 5 Comparison of partial amino acid sequences of Pp60 (frag.1, frag.2 and frag.3) with the amino acid sequences of protein disulfide isomerase from alfalfa (Shorrosh and Dixon 1991). Fragment 1 was the N-terminal sequence of Pp60. Fragment 3 contains an amino acid sequence that is known to be the active site for PDI (Shorrosh and Dixon 1991). Identical amino acids are indicated by asterisks.

Affected the binding (8%). These properties indicate that Pp60 has affinities specifically to 2,4-D, NAA and PCIB, but a low affinity to IAA in vitro. As many factors may affect auxin-binding in plant cells, further analysis of binding properties and binding conditions is required.

Partial amino acid sequences of Pp60 were determined and compared to sequences in the Swiss-Prot data bank. The N-terminal sequence (frag.1 in Fig. 5) and internal sequences (frag.2 and frag.3 in Fig. 5) of Pp60 showed homology to protein-disulfide isomerase (PDI) from plants.

In order to clarify the localization of Pp60, we raised polyclonal antibodies against the protein. Immunoblot analysis revealed that Pp60 existed ubiquitously in shoots and leaves (Fig. 6). In fruit, Pp60 was specifically expressed at an early stage of fruit development (Fig. 7). It is known that cell division actively occurs at this stage in peach fruit

Fig. 7 Immunoblot analysis of expression of Pp60 during fruit development using antiserum against Pp60. Soluble proteins (0.5 µg) were subjected to SDS-PAGE.

(Masia et al. 1992).

In a previous study (Ohmiya et al. 1998), we isolated genes encoding ABP19/20 and found that they were highly homologous to proteins related to the germin family. The deduced amino acid sequences of ABP19/20 have a small region structurally similar to BoxA, a putative auxin-binding site of maize ABP1 (Ohmiya et al. 1998). Partial amino acid sequences of Pp60 showed that these polypeptides had no similarities to ABP19/20. In addition, the binding property of Pp60 was different from those of ABP19/20. ABP19/20 had high affinity for 2,4-D, but IAA, NAA and PCIB did not inhibit the 2,4-D binding very much (Ohmiya et al. 1983). These results suggest that Pp60 may have a different role from that of ABP19/20 in peach trees. The hypothesis is also supported by immunological studies showing different expression patterns of Pp60 and ABP19/20 in peach trees (unpublished data). As Pp60 has a low affinity for IAA, Pp60 could have functions other than those involved in auxin actions.

N-terminal (frag.1) and internal (frag.2 and frag.3) sequences of Pp60 are highly homologous to those of PDI from plants such as alfalfa (Fig. 5). Two of the amino acid residues in frag.1 (indicated as X in Fig. 5) are likely to be
cysteine residues, although that has not been determined. If so, the conserved sequence (-WCGHCK-), which is known to be an active site for PDI (Shorrosh and Dixon 1991), may exist in Pp60.

PDI catalyzes the formation, reduction, and isomerization of disulfide bonds in proteins. It is also known to act as the β-subunit of prolyl-4-hydroxylase, glycosylation site-binding protein, thyroid hormone-binding protein, and a component of the microsomal lipid transfer protein complex (Freedman 1989, John et al. 1989, Ferrari and Soling 1999). In plants, however, the function of PDI has not yet been clarified. PDI may act as a carrier like the lipid transfer protein, a protein for storage like thyroid hormone-binding protein, or mediator for biochemical reactions by binding compounds such as 2,4-D and NAA.

Recently, the function of PDI as a catalyst for protein folding with molecular chaperones in animals has been reported (Gilbert 1997), and the function of this protein in plants has been demonstrated (Boston et al. 1996). Li and Larkins (1996) proposed that maize PDI may act as a molecular chaperone as in animals. They also indicated that the maize PDI gene is highly expressed during endosperm development, which coincides with the development of endoplasmic reticulum. Since development of the endoplasmic reticulum is very important for the growth and maturation of the fruit, PDI would have crucial roles in fruit development. PDI has been known to be one of the abundant proteins of ER and have C-terminal K/HDEL motifs (Laboissiere et al. 1995). ABP1, the best characterized auxin-binding protein from Zea mays and its families also have KDEL motifs and are located at the ER (Venis et al. 1992, Jones et al. 1998). Considering the function of PDI as a molecular chaperone, some interaction of Pp60 and other proteins such as ABP1 may exist in the ER-lumen and be involved in fruit development.

Kim and Mayfield (1997) revealed that PDI modulates the binding of chloroplast polyadenylate-binding protein to the 5'-untranslated region of the psbA mRNA by reversibly changing the redox status. They suggested that a PDI could have an important role in light-regulated translation of chloroplast mRNAs. Immunoblot analysis showed that the amount of Pp60 was highest at an early stage (stage I) (Faust 1989) of fruit development. Fruit at stage I contains large numbers of chloroplasts in which transcription and translation of genes for photosynthesis take place; photosynthesis should be important for fruit development (Masia et al. 1992). Therefore, Pp60 may have an important role in regulation of fruit development in peaches.

Morre et al. (1995a) demonstrated that auxins stimulate both NADH oxidase activity and elongation of soybeans, and suggested that the thiol group has an essential role in this stimulation. They also showed that auxins modulate the PDI-like activity which catalyzes thiol-disulfide exchange in isolated plasma membranes from soybeans (Morre et al. 1995b). Chueh et al. (1997) indicated that auxin treatment increases thiol content and decreases disulfide content of isolated plasma membranes in the presence of thiol reagents. These results suggest that the stimulation of thiol-disulfide exchange activity by auxins may be essential for hypocotyl elongation.

In the presence of thiol reagents, the concentration of 2,4-D for maximum effect on thiol-disulfide activity was $1 \times 10^{-3}$ M (Chueh et al. 1997). This concentration is consistent with the Kd value of 2,4-D binding by Pp60, a protein structurally similar to PDI. These results suggest that Pp60 is a protein related to PDI and that 2,4-D affects the thiol disulfide exchange activity by directly binding to the protein.

Glutathione S-transferase (GST) has been isolated as an auxin-binding protein (Bilang et al. 1993, Zettl et al. 1994). GST catalyzes the conjugation of glutathione tripeptide into a large variety of electrophilic and hydrophobic compounds. GST is also an enzyme that interacts with the thiol group and is thought to be involved in the homeostasis of thiol content in cells as well (Uhlig and Wendel 1992). Glutathione-dependent formaldehyde dehydrogenase, which is another type of ABP from mung bean hypocotyls (Sugaya and Sakai 1996a), required the thiol group for its enzyme activity. Internal sequences of Pp36 and Pp37 have similarities to the amino acid sequences of adenosine kinase (McNally et al. 1997) and phosphoribulokinase (Milanez and Mural 1988), respectively (data not shown). PDI and phosphoribulokinase are enzymes whose activities are regulated by thiol-disulfide exchange as mediated by thioredoxin (Brandes et al. 1996). Adenosine kinase also needs the thiol group for its activity (Bagui 1996). These properties raise the question of whether or not the binding of auxin to Pp60 needs a thiol group or not. Considering these facts, it is possible that auxins may be involved in thiol-disulfide exchange of various proteins including ABPs and Pp60 and may modulate activities of enzymes existing in the cells that could be important for the cell enlargement process. In order to clarify these hypotheses, further analysis would be needed to investigate the effects of auxins and thiols on enzyme activity of Pp60 and the physiological changes in plant cells caused by these effects. It is also necessary to clarify the primary structures and enzymatic properties of Pp60 isolated in the present paper. These studies will help in further understanding the functions of the protein.

We thank Drs. Nagao Matsuta, Hiroyuki Iketani and Toshiya Yamamoto of the National Institute of Fruit Tree Science, for their advices during this investigation.

This paper is contribution 1164, from the National Institute of Fruit Tree Science. This work was supported by a Domestic Research Fellowship of the Japan Science and Technology Corporation (JST).


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(Received September 9, 1999; Accepted February 4, 2000)