Polyphenoloxidase Silencing Affects Latex Coagulation in *Taraxacum* Species

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Latex is the milky sap that is found in many different plants. It is produced by specialized cells known as laticifers and can comprise a mixture of proteins, carbohydrates, oils, secondary metabolites, and rubber that may help to prevent herbivory and protect wound sites against infection. The wound-induced browning of latex suggests that it contains one or more phenoloxidizing enzymes. Here, we present a comprehensive analysis of the major latex proteins from two dandelion species, *Taraxacum officinale* and *Taraxacum kok-saghyz*, and enzymatic studies showing that polyphenoloxidase (PPO) is responsible for latex browning. Electrophoretic analysis and amino-terminal sequencing of the most abundant proteins in the aqueous latex fraction revealed the presence of three PPO-related proteins generated by the proteolytic cleavage of a single precursor (pre-PPO). The laticifer-specific pre-PPO protein contains a transit peptide that can target reporter proteins into chloroplasts when constitutively expressed in dandelion protoplasts, perhaps indicating the presence of structures similar to plastids in laticifers, which lack genuine chloroplasts. Silencing the PPO gene by constitutive RNA interference in transgenic plants reduced PPO activity by more than 90% compared to controls. Latex fluidity analysis in silenced plants showed a strong correlation between residual PPO activity and the coagulation rate, indicating that laticifer-specific PPO plays a major role in latex coagulation and wound sealing in dandelions. In contrast, very little PPO activity is found in the latex of the rubber tree *Hevea brasiliensis*, suggesting functional divergence of latex proteins during plant evolution.

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good source of high-molecular-weight rubber (Mooibroek and Cornish, 2000; Bushman et al., 2006) and was investigated as an alternative to *H. brasiliensis* during World War II, when rubber supplies to Europe and the United States were interrupted. Unfortunately, the extraction of rubber from Russian dandelion latex is laborious and expensive because of rapid coagulation, and further development was abandoned when *Hevea* rubber became available. Coagulation of *H. brasiliensis* latex is caused by the major latex proteins (MLPs), which include hevein, the hevein receptor, and chitinase (Gidrol et al., 1994; Chrestin et al., 1997). A similar role has been proposed for the polyphenoloxidases (PPOs) present in the latex of certain *Hevea* spp. (Hanower and Brzozowska, 1977), although our data indicate that this is not the case.

PPOs are found throughout the plant kingdom (Mayer and Harel, 1979; Vaughn and Duke, 1984; Mayer, 1987; Vaughn et al., 1988; Sherman et al., 1991), and they probably play a role in defense against pathogens and herbivores (Vörös et al., 1957; Felton et al., 1989; Duffey and Felton, 1991; Constabel and Ryan, 1998; Stout et al., 1999; Gatehouse, 2002). They are plastid-localized copper metalloenzymes that catalyze the oxidation of *o*-diphenols to *o*-diquinones (diphenolase activity; EC 1.10.3.2) and, in some species, also the *o*-hydroxylation of monophenols (monophenolase activity; EC 1.14.18.1; Vaughn et al., 1988; Mayer, 2006). Quinones are highly reactive electrophiles responsible for much of the oxidative browning in fruits and vegetables after wounding (Yoruk and Marshall, 2003). The wound-inducible expression of PPOs has been reported in apple (*Malus domestica*; Boss et al., 1995), tomato (*Solanum lycopersicum*; Constabel et al., 1995; Thipyapong and Steffens 1997), potato (*Solanum tuberosum*; Thipyapong et al., 1995), and hybrid poplar (*Populus* spp.; Constabel et al., 2000). In addition, the down-regulation of PPO activity by antisense RNA in tomato confers hypersusceptibility to pathogens (Thipyapong et al., 2004), whereas PPO over-expression confers enhanced resistance to bacterial diseases (Li and Steffens, 2002). It has also been suggested that PPOs evolved to protect plants against photochemical oxidation, since most PPOs characterized thus far appear to be localized in the plastids of photosynthetic cells (Sherman et al., 1995).

The rapid wound-induced browning of dandelion latex suggests the presence of significant PPO activity in the laticifers. Here, we show that the PPO is the major component of the latex proteome in *Taraxacum* spp. and that the down-regulation of PPO activity by RNA interference (RNAi) in transgenic *T. officinale* and *T. kok-saghyz* plants inhibits browning and coagulation. This suggests that PPO may be a key factor controlling the coagulation of dandelion latex and thus its protective role. This contrasts to the situation in *H. brasiliensis*, where we show that PPO appears to have a negligible effect on latex coagulation.

### RESULTS

Identification and Molecular Characterization of Enzymes Involved in the Rapid Browning of Dandelion Latex after Wounding

Phenol-oxidizing enzymes such as PPOs, peroxidases, and laccases often cause wound-induced browning in the plant kingdom. It is often difficult to separate the activities of these enzymes due to overlapping substrate specificities. In order to investigate the cause of rapid latex browning in dandelion (as shown for *T. kok-saghyz* latex in Fig. 1, A and B), *T. officinale* and *T. kok-saghyz* petioles were dissected to

![Figure 1](https://www.plantphysiol.org/content/151/3/335/F1.large.jpg)

**Figure 1.** Analysis of latex browning in *Taraxacum* spp. A and B, Photographs of expelling latex from *T. kok-saghyz* petioles immediately (A) and 30 min (B) after wounding. First browning of latex was observed directly after abscission of petioles (red arrow). C, Determination of PPO activity in the aqueous latex phase of *T. officinale* and *T. kok-saghyz* in the absence (−) and presence (+) of tropolone using L-DOPA as substrate. Enzymatic activity is presented in μkat mg⁻¹ protein. D and E, PPO activity was also analyzed in L-DOPA-treated cross sections of *T. officinale* petioles preincubated with (E) or without (D) tropolone. PPO activity appears to be laticifer specific (black arrowheads). La, Laticifer; Ph, phloem; Xy, xylem. Bars = 100 μm.
encourage the expulsion of latex, which was collected and centrifuged to obtain the aqueous phase for subsequent enzyme activity analysis (for details, see "Materials and Methods"). In the presence of 3,4-dihydroxy-L-phenylalanine (L-DOPA), a PPO substrate that is also oxidized by laccases (Eisenman et al., 2007) and peroxidases (Albrecht and Kohlenbach, 1990), we observed total latex enzymatic activities of approximately 0.218 \( \mu \text{kat mg}^{-1} \) (T. officinale) and approximately 0.237 \( \mu \text{kat mg}^{-1} \) (T. kok-saghyz). Addition of the potent PPO inhibitor tropolone (Fuerst et al., 2006) caused a significant reduction in the total enzymatic activity to approximately 0.004 \( \mu \text{kat mg}^{-1} \) for T. officinale and approximately 0.002 \( \mu \text{kat mg}^{-1} \) for T. kok-saghyz (Fig. 1C). The same result was evident for L-DOPA-stained laticifers in thin cross sections of T. officinale petioles in the absence (Fig. 1D) or presence (Fig. 1E) of tropolone. Here, clear signals were observed only in laticifers that had not been treated with the PPO inhibitor (Fig. 1D, arrowhead). The very weak residual browning activity (Fig. 1E, arrowhead) in tropolone-treated laticifers can be explained either by the incomplete inhibition of the PPO reaction or by the action of other enzymes such as peroxidases, which are tropolone insensitive.

Taken together, the results above suggest that activity of one or more PPOs in the latex proteome is responsible for the rapid oxidation of L-DOPA. The protein composition of the latex aqueous phase from the dandelion petiole, therefore, was analyzed by SDS-PAGE. As shown in Figure 2A, T. kok-saghyz latex contained five dominant protein bands that were designated MLPa (approximately 16 kD), MLPb (approximately 41 kD), MLPc (approximately 43 kD), MLPd (approximately 55 kD), and MLPe (approximately 57 kD). Bands with molecular masses equivalent to those of MLPa, MLPb, and MLPe were also represented in the petiole latex of the closely related species T. officinale, hinting that the proteins might have conserved roles. N-terminal sequencing by Edman degradation provided confirmation by showing that corresponding bands had identical N-terminal peptides in both species: DELIPFADKV for MLPa and DPIMAPDILTQ for both MLPb and MLPe (Fig. 2A). However, additional non-PPO-related amino sequences were generated for MLPe, indicating that MLPe is composed of more than one protein (data not shown). The PPO protein in MLPe, therefore, was designated MLPe\(^{\text{PPO}}\). No sequences were obtained from MLPc and MLPd, indicating that the N-terminal residues were modified and unsuitable for Edman chemistry. It should be noted that the MLP pattern was identical in latices derived from stems, petioles, and roots (data not shown).

Based on the above peptide sequence information, we cloned the corresponding full-length genomic sequences by inverse PCR and the cDNA sequences by 5' and 3' RACE. Comparison of these sequences revealed the presence of a 1.8-kb open reading frame in both species, uninterrupted by introns, each encoding an approximately 67-kD protein similar to PPOs. The resulting cDNA clones were designated ToPPO-1 (for T. officinale PPO1; accession no. EU154993) and TkPPO-1 (for T. kok-saghyz PPO1; accession no. pending; Supplemental Fig. S1A). MLPa, MLPb, and MLPe\(^{\text{PPO}}\) are generated by the proteolytic cleavage of ToPPO-1 or TkPPO-1 (pre-PPOs), with MLPb comprising the tyrosinase domain, MLPa comprising the C-terminal portion of the protein, and MLPe\(^{\text{PPO}}\) comprising both (Fig. 2B). Further analysis identified an additional 10-kD N-terminal bipartite transit peptide for plastid targeting (Fig. 2B). The function of the transit peptide was confirmed in the case of ToPPO-1 by joining the corresponding DNA sequence to the DsRed reporter gene.

**Figure 2.** Molecular characterization of the MLPs of T. officinale (T.o.) and T. kok-saghyz (T.k.). A, SDS-polyacrylamide gel stained with Coomassie Brilliant Blue, showing three (T.o.; MLPa, MLPb, and MLPe\(^{\text{PPO}}\)) and five (T.k.; MLPa to MLPe/e PPO) dominant bands. N-terminal sequences for MLPa, MLPb, and MLPe\(^{\text{PPO}}\) are shown at right. Molecular masses are shown in kD. B, Schematic representation of the dandelion PPO-1 and its proteolytic products. Cleavage of the pre-PPO-1 protein (top) at the sites indicated with arrows produces small amounts of a nearly full-length product (MLPe\(^{\text{PPO}}\)) lacking the transit peptide (tp; green), together with larger amounts of the tyrosinase domain (MLPb; brown) and a C-terminal domain (MLPa; white). The first three amino acids from the individual cleavage products are indicated.
and expressing the fusion proteins under the control of the strong constitutive cauliflower mosaic virus (CaMV) 35S promoter (Guilley et al., 1982) in *T. officinale* and *T. kok-saghyz* protoplasts (Fig. 3). The ToPPO-1 bipartite transit peptide efficiently targeted the DsRed reporter protein into the plastids, as indicated by the overall orange-red fluorescence of these organelles (Fig. 3, A–D and I–L). In contrast, DsRed fluorescence was not detected in the chloroplasts of protoplasts transfected with the same construct lacking the bipartite transit peptide sequence. Here (Fig. 3, E–H and M–P), and as expected, DsRed fluorescence was restricted to the cytoplasm of the protoplasts. Chloroplast autofluorescence is represented by green pseudocolor in Figure 3.

Next, we analyzed the ToPPO-1 and TkPPO-1 mRNA levels (Fig. 4A). Reverse transcription (RT)-PCR analysis with total RNAs isolated from *T. officinale* and *T. kok-saghyz* latex (Fig. 4A, lanes 2 and 4) showed that both genes were expressed strongly. Corresponding weak bands were observed in the lanes representing leaf sections lacking major vascular bundles (Fig. 4A, lanes 1 and 3), probably reflecting the presence of a small number of laticifers in leaf tissue.

The expression of ToPPO-1 was studied further in transgenic *T. officinale* plants by expressing GUS under the control of the ToPPO-1 promoter (for sequence, see Supplemental Fig. S1B). Nine independent transgenic lines shown to contain the pToPPO-prom-1/GUS construct (data not shown) were designated ToPPO-prom1/1 to ToPPO-prom1/9. The expression pattern driven by the ToPPO-1 promoter is shown in Figure 4, B to F. In cross sections of petioles stained with the substrate 5-bromo-4-chloro-3-indolyl-β-glucuronic acid (Fig. 4B), GUS activity was restricted to cells located close to the vascular bundles (Fig. 1D). However, precise localization was not possible due to the rapid diffusion of the substrate in the expelling latex after cutting. Therefore, GUS activity was analyzed in longitudinal sections that contained unwounded laticifers. As shown in Figure 4C, reporter enzyme activity was restricted to cells showing the typical structure of articulated anastomosing laticifers. The same cell organization (Fig. 4D) can also be visualized by staining dandelion laticifers with Sudan III (Au et al., 2009). As expected from this result, GUS activity, indicated by the blue color, could be detected only in latex from the transgenic line (Fig. 4E, ToPPO-prom1/1) but not in the wild-type control (Fig. 4E, WT). In addition, immunological studies using a GUS-specific polyclonal antiserum revealed the presence of the GUS enzyme in ToPPO-prom1/1 latex (Fig. 4F). Taken together, these

![Figure 3](image-url)
results showed that the latex protein component in dandelion is represented predominantly by a laticifer-specific PPO enzyme. We next considered whether this protein was responsible for the rapid browning and subsequent coagulation of dandelion latex after wounding.

Down-Regulation of PPO Expression in Transgenic Dandelion Plants and Its Impact on Latex Coagulation

The potential relationship between PPO activity and latex coagulation in *T. kok-saghyz* and *T. officinale* was investigated by RNAi. Therefore, a specific RNAi construct was designed to target the PPO tyrosinase domain and was expressed under the control of the CaMV 35S promoter. Twenty-five independent *T. officinale* and *T. kok-saghyz* transgenic lines were generated by *Agrobacterium tumefaciens*-mediated leaf disc transformation. Transgene integration and transcription were confirmed by PCR and RT-PCR, respectively. As expected, the efficiency of *ToPPO-1* and *TkPPO-1* knockdown varied among the independent transformants, ranging from almost complete silencing to nearly wild-type PPO levels (data not shown).

The protein composition of latex from transgenic plants showing the strongest knockdown effects (*T. officinale* and *T. kok-saghyz*) was analyzed by Western blotting. The Western blot results indicated that the protein component responsible for latex coagulation was significantly reduced in the transgenic lines compared to the wild-type control. This finding provides strong evidence for the role of PPO in the coagulation process of dandelion latex.
officinale lines ToLP1 and ToLP2 and T. kok-saghyz lines TkLP1 and TkLP2) are shown in Figure 5, A and B (see also Supplemental Fig. S2). Latex from all the transgenic plants showed a significant reduction in the abundance of MLPa (black arrows) and MLPb (red arrows), but the amount of MLPePPO appeared to be the same in wild-type and transgenic plants (green arrows), reflecting the fact that MLPe is composed of several proteins. A unique additional protein band was detected in line ToLP1 (but not ToLP2), and N-terminal sequencing by Edman degradation identified this as a pectin methyl esterase (Fig. 5A, asterisk).

Since the MLPb and MLPePPO proteins include the PPO catalytic domain (Fig. 2B) but only the smaller MLPb appears to be depleted in transgenic plants, we decided to test the latex extracts using a seminative gel electrophoresis method that allows the in-gel detection of PPO activity (see “Materials and Methods”). It should be noted that proteins migrate slightly faster under these conditions. As expected according to their relative abundance, most PPO activity in wild-type plants was associated with MLPb (Fig. 5, C and D, red arrows) and a smaller amount with MLPePPO (Fig. 5, C and D, green arrows). In all four of the transgenic lines, the signals for both MLPb and MLPePPO were reduced, confirming the knockdown of MLPePPO, even though this was not clear from standard SDS-PAGE experiments (Fig. 5, A and B). No PPO activity was associated with the novel protein band identified in line ToLP1. PPO activity in each of the transgenic lines was determined more precisely using an in vitro assay, confirming the strong reduction of enzyme activity in lines ToLP1, ToLP2, TkLP1, and TkLP2 (Fig. 5, E and F). To determine whether latex from the transgenic lines coagulated more slowly than wild-type latex, the leaves, stems, and roots from lines ToLP1, ToLP2, TkLP1, and TkLP2 were dissected and the flow of latex was evaluated in comparison with wild-type plants using a capillary-based test system, a technique routinely used to investigate blood coagulation (see “Materials and Methods”). The capillary suction distances (csd) were consistent over three measurements, and the comparative values recorded for wild-type and RNAi-knockdown lines were almost completely abolished. No corresponding activity signal could be detected for MLPa, since this derivate does not contain a tyrosinase domain. E and F, Determination of the enzymatic activity signal could be detects for MLPa, since this derivate does not contain a tyrosinase domain. E and F, Determination of the enzymatic activity in wild-type and transgenic RNAi-knockdown lines. The enzymatic activity is presented as µkat mg⁻¹ protein. The values presented are means ± se of at least three biological samples in each case. Significant differences were found between transgenic and wild-type plants (calculated using Student’s t test; P = 0.01).

Figure 5. Analysis of PPO abundance and activity in wild-type (wt) and RNAi-knockdown T. officinale (To) and T. kok-saghyz (Tk) plants. A and B, SDS-PAGE showing major latex proteins in wild-type and transgenic plants expressing an RNAi construct targeting the PPO tyrosinase region (ToLP1, ToLP2, TkLP1, and TkLP2). The positions of MLPa (black arrows), MLPb (red arrows), and MLPePPO (green arrows) are indicated. The asterisk in A shows a novel and uncharacterized major protein in line ToLP1. C and D, In-gel assay showing proteins with PPO activity. The MLPb (red arrows) and MLPePPO (green arrows) activities in the RNAi plants are almost completely abolished. The asterisk in A shows a novel and uncharacterized major protein in line ToLP1. E and F, Determination of the enzymatic activity signal could be detected for MLPa, since this derivate does not contain a tyrosinase domain. E and F, Determination of the enzymatic activity in wild-type and transgenic RNAi-knockdown lines. The enzymatic activity is presented as µkat mg⁻¹ protein. The values presented are means ± se of at least three biological samples in each case. Significant differences were found between transgenic and wild-type plants (calculated using Student’s t test; P = 0.01).

In order to verify whether there is a more general correlation between latex coagulation and PPO activ-
ity, we studied PPO activity and latex fluidity in the following laticiferous plants from three families: H. brasiliensis (Euphorbiaceae), Ficus elastica (Moraceae), and Sonchus asper as an additional Asteraceae member. The latices of T. officinale and T. kok-saghyz served as controls. Photographs of greenhouse-cultivated plants (for cultivation conditions, see “Materials and Methods”) and of latex expelled from them after injury are shown in Figure 7A. To analyze latex PPO activity in these plants, latex aqueous phase samples containing 2 μg of total protein were spotted onto a nitrocellulose membrane and incubated with L-DOPA. In parallel, PPO activity was determined by spectrophotometry. The outcome of both experiments is shown in Figure 7B. The strongest enzymatic activities were found in the Asteraceae, with values of 0.61 μkat mg⁻¹ protein for S. asper, 0.17 μkat mg⁻¹ protein for T. officinale, and 0.20 μkat mg⁻¹ protein for T. kok-saghyz. In contrast, PPO activity in H. brasiliensis latex was very weak (0.01 μkat mg⁻¹ protein), and no activity was observed in the latex of F. elastica.

We next measured the latex fluidity in a glass capillary as described above. In this setup (Fig. 7C), S. asper latex had the highest PPO activity and coagulated directly after loading (7.2 mm csd; lane 1), followed by T. kok-saghyz (24 mm csd; lane 3) and T. officinale (29 mm csd; lane 2), which had very similar values, then H. brasiliensis (35.5 mm csd; lane 4) and F. elastica (113.5 mm csd; lane 5). The rate of latex coagulation correlated strongly with the level of laticifer-specific PPO activity in F. elastica and all the Asteraceae plants we studied but not in H. brasiliensis, where rapid latex coagulation occurred despite the weak PPO activity. It should be noted that latex with high levels of PPO activity also turned brown in the capillary experiments, as shown for the two dandelion species (Fig. 7C, bottom left).

**DISCUSSION**

Latex is a milky sap produced by a large number of diverse plants. It typically comprises a mixture of proteins, carbohydrates, oils, secondary metabolites, and gums that may protect injured plants from infection and herbivory. The rapid browning of dandelion latex after wounding argues for the presence of one or more phenol-oxidizing enzymes, specifically PPOs, peroxidases, and/or laccases. Our data indicate that PPOs are responsible for the browning of dandelion latex, since the browning process can be almost completely prevented both in vitro and in vivo following treatment with tropolone, a PPO inhibitor (Fig. 1). The residual browning activity in these experiments might reflect either the incomplete inhibition of PPO activity or the activity of peroxidases and/or laccases. However, genomic and proteomic approaches have thus far failed to identify these latter enzymes in T. officinale and T. kok-saghyz latex (D. Wahler, unpublished results), whereas we found that PPO is a major constituent of the aqueous latex fraction. Therefore, we propose that residual browning activity reflects incomplete inhibition of PPO by tropolone.

*T. kok-saghyz* latex contained five dominant protein bands, three of which were also observed in *T. officinale*. N-terminal sequencing by Edman degradation

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**Table 1. Amount of latex harvested after wounding, as well as rubber quantity and quality from wild-type T. kok-saghyz plants and transgenic lines TkLP1 and TkLP2.**

Each value represents the mean ± sd from at least three biological samples. Significant differences were found between transgenic and wild-type plants for the amount of expelled latex (calculated using Student’s t test; *P* = 0.01). M, Molecular mass; Rc, rubber content.

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<th>Plant</th>
<th>Amount of Latex Harvested After Wounding mg</th>
<th>Rubber Quantity Rc mg mL⁻¹ latex</th>
<th>Rubber Quality mW D × 10⁶</th>
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<td>Wild type</td>
<td>0.5</td>
<td>139.93</td>
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<td>1.9</td>
<td>155.13</td>
<td>5.7</td>
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<td>TkLP2</td>
<td>2.3</td>
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showed that the matching bands shared the same N-terminal peptides, strongly indicating that they are structurally and functionally conserved. The peptide sequences were used to isolate corresponding PPO genes and cDNA sequences, which were named ToPPO-1 and TkPPO-1. The genes encode proteins with two copper-binding motifs found in many other PPOs. Further analysis confirmed that three of the proteins, MLPa, MLPb, and MLPe\textsuperscript{PPO}, are generated by proteolytic cleavage of ToPPO-1/TkPPO-1, with MLPb comprising the tyrosinase domain, MLPa comprising the C-terminal domain, and MLPe\textsuperscript{PPO} comprising both (Fig. 2). Proteolytic processing of many other PPOs has been described, such as the PPO enzymes found in broad bean leaf (*Vicia faba*; Robinson and Dry, 1992), grape (*Vitis vinifera*; Rathjen and Dry, 1992), and coffee (*Coffea arabica*; Mazzafera and Robinson, 2000). More recently, Flurkey and Inlow (2008) analyzed

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<th>Asteraceae</th>
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<td><em>Taraxacum officinale</em></td>
<td><em>Taraxacum kok-saghyz</em></td>
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<td><em>Hevea brasiliensis</em></td>
<td><em>Ficus elastica</em></td>
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**Figure 7.** Latex and PPO activity in different laticiferous plants. A, Photographs of *S. asper*, *T. officinale*, *T. kok-saghyz*, *H. brasiliensis*, and *F. elastica* and of droplets of latex expelled from each plant. Differences in latex fluidity and coagulation are apparent from the size and flow of the droplets. B, Top, PPO activity visualized by dot-blot L-DOPA staining for all the plants listed above (2 \mu g of total protein from the latex aqueous phase was spotted onto a nitrocellulose membrane and placed on 1% agarose gels containing 20 \mu M L-DOPA). Bottom, Enzymatic activity is presented in \mu kat mg\textsuperscript{-1} protein. C, Latex from *S. asper* (1), *T. officinale* (2), *T. kok-saghyz* (3), *H. brasiliensis* (4), and *F. elastica* (5); all the plants listed above were harvested after wounding by drawing into 10-\mu L microglass capillaries. The inset (lower left) shows latex browning in the capillary system for *T. officinale* (2) and *T. kok-saghyz* (3). D, After no further movement of the latex, the distance from the end of the capillary to the stopping point was determined in mm csd. All values are presented as means ± SD of at least six biological samples in each case. No significant differences existed in enzyme activity (B) and csd (D) between *T. officinale* and *T. kok-saghyz*, as calculated using Student’s t test (*P* = 0.01).
specific proteolysis target sequences in fungal and plant PPOs and showed that N-terminal proteolysis sites were located immediately upstream of a conserved Arg residue, whereas C-terminal proteolysis sites were located downstream of a conserved Tyr residue. The same amino acids are present in ToPPO-1 and TkPPO-1 (Supplemental Fig. S1A), so a similar proteolysis mechanism is probably involved.

Transgenic *T. officinale* plants expressing GUS under the control of the *ToPPO-1* promoter showed that *PPO-1* expression is restricted to laticifers, which are characterized by an articulated anastomosing cell structure (Esau, 1965) similar to those found in opium poppy (*Papaver somniferum*; Thureson-Klein, 1970). However, in cross sections of transgenic petioles, clear histochemical localization of GUS activity to the laticiferous system was not possible because of the rapid diffusion of the metabolized GUS substrate after wounding. Therefore, additional studies were performed, including RT-PCR experiments, GUS staining of intact laticifers in longitudinal petiole sections, and direct detection of GUS activity in latex. Both *PPO-1* mRNA (Fig. 4A) and GUS activity in transgenic plants (Fig. 4, B, C, E, and F) were exclusively detected in the latex, indicating strict laticifer-specific activity of the corresponding promoter elements. In contrast, the transcription of three key genes of the benzylisookinoline alkaloid biosynthetic pathway in opium poppy was shown to be restricted to the parietal region of sieve elements adjacent or proximal to laticifers, even though the products of this pathway accumulated specifically in the laticifers (Bird et al., 2003). A similar situation cannot be excluded for *ToPPO-1* and *TkPPO-1* mRNAs, so further experiments involving immunofluorescence labeling and/or in situ hybridization are needed to gain further insight into dandelion *PPO-1* gene expression.

PPOs typically carry an N-terminal transit peptide that is cleaved from the precursor proteins to yield a mature protein (Sommer et al., 1994). This transit peptide is bipartite, for initial translocation to the chloroplast and subsequent transfer to the thylakoid lumen, indicating a two-step maturation process (Soll and Schleiff, 2004). The immature forms of *ToPPO-1* and *TkPPO-1* also carry an N-terminal plastid transit peptide (Fig. 2B) that directed a reporter protein into plastids when expressed in *T. officinale* and *T. kok-saghyz* protoplasts (Fig. 3). This was possible even though localization experiments indicated that *ToPPO-1* is expressed specifically in laticifers, which lack conventional plastids. The presence of plastid-targeting peptides on a laticifer-specific protein suggests that there may be structures similar to plastids in laticifers, a hypothesis supported by electron microscopy data revealing spherical complexes, 3 to 6 μm in diameter, within *H. brasiliensis* laticifers. These so-called Frey-Wyssling complexes are highly modified plastids enclosed within a double membrane that accumulate carotenes and PPOs (Frey-Wyssling, 1929; Dickenson, 1969; Archer, 1980). Anticipating that Frey-Wyssling complexes may also exist within dandelion laticifers, we speculate that the mature and active forms of ToPPO-1 and TkPPO-1 would be stored in this cellular compartment and would be released after wounding by a yet unknown mechanism. However, localization experiments are needed to verify this hypothesis.

PPOs are known to possess diphenolase activity, which catalyzes the oxidation of o-diphenols to o-quinones (Sánchez-Ferrer et al., 1995). Quinones are highly reactive molecules that are responsible for wound-induced browning in many plants. Quinones could react with a variety of substances in the cytosol of dandelion laticifers (e.g. proteins and oligosaccharides) to form a complex brown polymer. The direct availability of a functional mature PPO (by direct release from Frey-Wyssling complexes) would facilitate immediate wound sealing after injury (e.g. by herbivores) and therefore minimize the risk of secondary infection with facultative pathogens. We proposed that PPO activity is responsible for coagulation and browning in dandelion latex, and the RNAi knockdown of this enzyme strongly supports this hypothesis by prolonging the liquid phase. PPO activity in *H. brasiliensis* is localized to latex lutoids and Frey-Wyssling complexes (Coupé et al., 1972) and is 5- to 34-fold higher in lutoids than in Frey-Wyssling complexes (Wititsuwannakul et al., 2002). It has also been proposed that PPOs play a role in the coagulation of *H. brasiliensis* latex, since the exogenous application of substances interfering with PPO activity, such as reducing agents or a nitrogen atmosphere, prolongs the latex flow (Hanower et al., 1976; Hanower and Brzozowska, 1977; Brzozowska-Hanower et al., 1978). In contrast to the situation in dandelion, we showed that PPO is a minor component of the *H. brasiliensis* latex we studied, although we cannot exclude the possibility that PPO activities differ among *H. brasiliensis* accessions. Nevertheless, the rate of latex coagulation in *H. brasiliensis* (with low PPO activity) and *T. kok-saghyz* (with high PPO activity) is very similar, indicating that other factors contribute to the coagulation of *H. brasiliensis* latex. Some of these factors have recently been identified (Gidrol et al., 1994; Silpi et al., 2006).

The latex from certain plants is a good source of natural rubber, but rubber extraction is made more complex by the tendency of latex to coagulate in contact with air (a property that allows it to seal open wounds, forming a barrier to microorganisms). The rubber tree *H. brasiliensis* is the predominant commercial source of natural rubber, but the Russian dandelion *T. kok-saghyz* also contains large amounts of rubber and has been investigated as a potential alternative source (van Beilen and Poirier, 2007). One challenge that must be overcome before *T. kok-saghyz* can be considered as a commercial rubber source is the rapid coagulation and browning of extruded latex. These limitations could be circumvented through the use of *T. kok-saghyz* plants with reduced PPO activity,
since the latex could be harvested by low-speed centrifugation from mechanically macerated material, as is currently the case for guayule (Parthenium argentatum) latex (Cornish, 1996, 1998; Cornish and Brichta, 2002), and the rubber could be extracted with the process currently used for H. brasiliensis. To demonstrate this principle, we generated transgenic T. officinale and T. kok-saghyz plants in which the pre-PPO1 mRNA was targeted by RNAi. Among 50 transformed lines, 25 for each species, two T. officinale and two T. kok-saghyz transgenic lines were selected based on their strong knockdown activity. PPO activity was significantly depleted in these lines, which correlated well with increased coagulation times when latex was extracted and tested. In contrast, latex fluidity was not affected in transgenic T. officinale or T. kok-saghyz lines showing no or very weak PPO silencing, where coagulation times were very similar to wild-type values (data not shown). This indicates that PPO enzymatic activity must be strongly depleted in order to increase latex fluidity and suggests that there might be a threshold above which the normal coagulation phenotype is displayed. The introduction of T. kok-saghyz varieties with PPO silencing could provide an opening for the development of alternative rubber sources, since four to five times more latex could be harvested compared with wild-type accessions. However, all currently available accessions are wild-growing isolates (i.e. major breeding programs are needed to produce elite T. kok-saghyz varieties for use as a cash crop).

MATERIALS AND METHODS

Plant Material and Cultivation Conditions

Seed and plant materials were obtained from the Botanical Gardens in Karlsruhe, Germany (Taraxacum kok-saghyz), and the Botanical Gardens of the Westphalian Wilhelms-University of Münster, Germany (Hevea brasiliensis, Ficus elastica, Sonchus asper, and Taraxacum officinale). For all subsequent experiments, plants were cultivated in greenhouses. S. asper, T. officinale, and T. kok-saghyz were grown in the institute greenhouse at 16°C with a 16-h photoperiod, and H. brasiliensis and F. elastica in the tropical greenhouse of the Botanical Gardens of the Westphalian Wilhelms-University of Münster at 30°C with relative air humidity of 80% under natural light conditions. For molecular characterization of the T. officinale and T. kok-saghyz major latex proteins, latex was harvested from 9-month-old plants by abscising petioles (or roots) with a razor blade. For the PPO activity/coagulation experiments, latex was harvested and processed as described above and the aqueous phase was fractionated by 10% SDS-PAGE (Laemmli, 1970), so that proteins could be visualized by standard Coomassie Brilliant Blue staining. N-terminal and internal peptide sequences were obtained by blotting proteins onto a polyvinylidene difluoride membrane, followed by staining with sulforodamin B (Sigma) and analysis using an ABI Proci 491 Protein Sequencer (Applied Biosystems). Homology searching was carried out using the advanced BLAST program (Altschul et al., 1997). For the detection of PPO activity, unheated latex proteins were separated by 10% SDS-PAGE and the gel was washed three times for 20 min each in 100 mM sodium phosphate buffer (pH 6) containing 1% Triton X-100 and three times for 20 min each in 100 mM sodium phosphate buffer (pH 6). The gel was then incubated in 100 mM sodium phosphate buffer (pH 6) containing 20 mM L-DOPA (Sigma). When a brown color developed, the reaction was stopped by replacing the L-DOPA solution with 100 mM sodium phosphate buffer (pH 6). For dot-blot analysis, 2 μg of latex protein was spotted onto a nitrocellulose membrane (Schleicher & Schuell) and placed onto an agarose gel containing 100 mM sodium phosphate buffer (pH 6) and 20 mM L-DOPA. For in situ PPO assays, semithin sections of T. officinale or T. kok-saghyz plants were selected based on the conditions described above by scoring the bark or abscising petioles (or roots) with a razor blade.

Latex Collection and Purification of the Aqueous Latex Phase

Freshly tapped latex was collected in ice-cold Eppendorf tubes containing 50 mM sodium phosphate buffer (pH 6). The latex was centrifuged (15,700 g, 10 min, 4°C) to separate the aqueous latex phase from the pellet and (in some species) an upper viscous layer. In some species, the centrifugation step was repeated to remove impurities from the aqueous phase.

RNA Isolation and RT-PCR

Total RNA was isolated from T. officinale and T. kok-saghyz tissue and latex using the RNAmagic kit (Biobudget) following the manufacturer's instructions, and 500 ng of total RNA was used as the template for SuperScript II reverse transcriptase (Invitrogen) using an oligo(dT) primer. PCR amplification was performed with Thermoprime DNA Polymerase (Thermo) and gene-specific primers Tk-PPO-I fwd (5'-AAATCTCGAGGATCTCCGCTCCGCAAC-3'), Tk-PPO-I rev (5'-GACTTACCATGGTGTGATGTGAC-3'), GAPDH-I fwd (5'-CTACGTGACATCCATCCATAC-3'), and GAPDHRev (5'-CACACGGTGCTGAGGATGAC-3').

Analysis of PPO Activity and Localization, DsRed and GUS Assays, and Western Blots

In order to test the involvement of PPO in latex browning, the substrate L-DOPA (Sigma-Alrich) and the potent PPO inhibitor tropolone (Fuerst et al., 2006) were used in a photometric assay. L-DOPA was dissolved in 100 mM citrate buffer (pH 5) and applied at a final concentration of 20 mM. Its conversion to dopaquinone was followed at 478 nm (ε = 3313 M⁻¹ cm⁻¹) using an Uvikon943 spectrophotometer (Kontron Instruments). All values were obtained by subtraction of the blank value. Tropolone was used at a final concentration of 0.1 mM to arrest PPO activity. Tropolone was preincubated with the aqueous latex phase for 30 min at room temperature prior to photometric measurements and was included at the stated concentrations in substrate solutions. All photometric measurements were carried out at 30°C with prewarmed substrate solutions. For MLP characterization, latex from T. officinale and T. kok-saghyz plants was harvested and processed as described above and the aqueous phase was fractionated by 10% SDS-PAGE and the gel was washed three times for 20 min each in 100 mM sodium phosphate buffer (pH 6) containing 1% Triton X-100 and three times for 20 min each in 100 mM sodium phosphate buffer (pH 6). The gel was then incubated in 100 mM sodium phosphate buffer (pH 6) containing 20 mM L-DOPA (Sigma). When a brown color developed, the reaction was stopped by replacing the L-DOPA solution with 100 mM sodium phosphate buffer (pH 6). For dot-blot analysis, 2 μg of latex protein was spotted onto a nitrocellulose membrane (Schleicher & Schuell) and placed onto an agarose gel containing 100 mM sodium phosphate buffer (pH 6) and 20 mM L-DOPA. For in situ PPO assays, semithin sections of T. officinale or T. kok-saghyz leaves were cut with a razor blade and incubated in 20 mM L-DOPA solution. For inhibition of the PPO enzyme in latex bladders, thin sections were preincubated for 20 min with 0.1 mM tropolone prior to the L-DOPA stain. Histochemical localization of GUS activity in transgenic dandelion plants and detection of GUS activity in the aqueous latex phase was performed using the substrate 5-bromo-4-chloro-3-indolyl-O-glucuronide without an oxidative catalyst, according to established protocols (Jefferson et al., 1987). The stained thin sections were visualized with an Axioskop 40 microscope (Zeiss). Detection of DsRed fluorescence (excitation, 556 nm; emission, 586 nm) in the chloroplasts or cytoplasm of transfected protoplasts was carried out using a TCS SP confocal microscope (Leica Microsystems). DsRed fluorescence was displayed in red, and chloroplast autofluorescence was rendered in green. For the immunological detection of GUS, proteins of the aqueous latex phase were separated by 10% SDS-PAGE and electrobotted onto nitrocellulose membranes. Membranes were incubated with a 1:600 dilution of a polyclonal rabbit anti-GUS serum (Invitrogen), washed three times in 1× phosphate-buffered saline, and incubated in a 1:10,000 dilution of a polyclonal goat anti-rabbit GUS serum (Invitrogen), followed by detection using nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (Roche).

Identification and Cloning of ToPPO-1 and TkPPO-1, and Assembly of pToPPO-1, pToPPO-prom1/GUS, 35STP/DsRed, and 35S/RNAi Constructs

Genomic DNA was extracted from young T. officinale leaves using the cetyltrimethyl-ammonium bromide method (Doyel and Doyle, 1990). For inverse PCR, 1 μg of T. officinale genomic DNA was digested with different restriction enzymes at 37°C for 2 h. Following phenol/chloroform extraction and ethanol precipitation, the digested DNA was self-ligated at a concentration of 0.3 to 0.5 μg ml⁻¹ in the presence of 3 units ml⁻¹ T4 DNA ligase (Promega) overnight.


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at 15°C. The ligation mixture was extracted with phenol:chloroform, precipitated with ethanol, and resuspended in water to a concentration of 20 ng µL\(^{-1}\). In order to amplify ToPOPO-1 fragments, we designed two degenerate primers based on the peptide amino acid sequences. These were named LZ50 (5'-CAAGCGTTCACTCTCGG-3') and LZ45 (5'-TGGTGIAAGGRC-GICAACAATC-3'). The inverse PCRs were prepared using 200 ng of circularized genomic DNA in a final volume of 50 µL containing 50 mU MgCl\(_2\), 0.25 mU of each deoxynucleoside triphosphate, 10 pmol of each primer, and 1 unit of Taq DNA polymerase. Undigested genomic DNA was used as the control. PCR was initiated by a denaturation step at 94°C for 15 min, followed by 36 cycles of denaturation (30 s, 94°C), primer annealing (30 s, 50°C–60°C), and extension (3 min, 72°C), with a final extension step at 72°C for 10 min. Inverse PCR fragments were cloned in the pGEM-T vector (Promega) and sequenced.

To clone the entire coding region, we used the ToPOPO-1 primers LZ62 (5'-AAGATTGTTCGCGCCA-GATCT-3'), LZ59 (5'-CATATCGAATGTCGATAAGT-3'), and LZ56 (5'-CCACCTCTGCTGAATGTCGCA-3'). Total RNA was isolated from T. officinale latex using Trizol Reagent (Invitrogen) and processed using the OneStep RT-PCR system (QIAGEN) and the primers LZ74 (5'-AAGCTCTGA-GATATCCATCTCCAGAC-3') and LZ73 (5'-CAACCCATATCCTCATGTAAT-3') following the manufacturer's instructions. The missing 5' and 3' ends of the ToPOPO-1 mRNA were identified using the BD SMART RACE cDNA amplification system (Clontech) and primers 5'-CCGCTTACTCCCATGACCAC-3' (5' RACE') and 5'-AAGATTTGTTT-TCCCGGGACTGTTGA-3' (5' RACE) according to the manufacturer's recommendations. The clone TkPOPO-1 was obtained by RT-PCR on total RNA from T. kok-saghyz using the primers 5'-AAGCTCTGAATGTCGATAGAATG-3' and 5'-AAAACTCGAGATATGGCA--3' and ligated into the pTRAK vector. The pTR/DsRed plasmid was obtained after PCR amplification of the leader peptide with the primers LZ74 and LZ75 (5'-AAACCATGCGAATGTCGATAGAATG-3') and LZ73 (5'-CACTCCCTATCTTGGGGC-3') and transcriptional fusion to the DsRed cloning vector where the DsRed gene is under the control of a double CaMV 35S promoter (C. Schulze Gronover, unpublished data). For the RNAi construct, a 1,525-bp fragment was isolated using a gene-specific primer (5'-CCGCTTACTCCCATGACCAC-3') and the Universal GenomeWalker Kit (Clontech). This fragment was named pToPOPO-1 (Supplemental Fig. S1B) and represents the sequence immediately upstream of the ToPOPO-1 translational start codon. For the construction of pToPPO-prom-1/GUS, the uidA (gusA) gene was placed under the control of pToPOPO-1 and ligated into the pTRAK vector. The pTP/DsRed plasmid was obtained after PCR amplification of the leader peptide with the primers LZ74 and LZ75 (5'-AAACCATGCGAATGTCGATAGAATG-3') and LZ73 (5'-CACTCCCTATCTTGGGGC-3') and transcriptional fusion to the DsRed cloning vector where the DsRed gene is under the control of a double CaMV 35S promoter (C. Schulze Gronover, unpublished data). For the RNAi construct, a 1,414-bp PCR fragment was amplified using the RNAi-dicer optimized primers RNAi_for (5'-TTTGTTACACATCCTGGCCT-TGAGTC-3') and RNAi_rev (5'-TTTG-GCGCGCCTGTTGGAATCGAGTGAATGCGAC-3') and ligated into the pTRAK vector. The PCR fragment was inserted into the KpnI and NotI sites of the Gateway vector pENTR4 (Invitrogen) and transferred to the destination plasmid pFGC9491 (www.chromDB.org) containing the chalcone synthase intron.

The integrity of all constructs was verified by sequencing. Database analysis was carried out with the Wisconsin Package (Genetics Computer Group). Chloroplast transit and thylakoid lumen targeting signals were analyzed using the ChloroP algorithms (Emanuelsson et al., 1999).

**Agrobacterium-Mediated Transformation of Taraxacum Species and Dandelion Protoplast Preparation and Transfection**

_Agrobacterium tumefaciens_ strain LBA4404, carrying the binary constructs, was cultured in 100 mL of induction broth (5 g L\(^{-1}\) Suc, 5 g L\(^{-1}\) peptone, 5 g L\(^{-1}\) casein hydrolysate, 1 g L\(^{-1}\) yeast extract, 10 mM MES, and 2 mM MgSO\(_4\), pH 7) containing the appropriate antibiotics (50 mg L\(^{-1}\) kanamycin, 100 mg L\(^{-1}\) rifampicin, and 300 mg L\(^{-1}\) streptomycin). The bacteria were cultured at 28°C to the end of the log phase and then centrifuged, and the pellet was resuspended in coculture medium (4.4 g L\(^{-1}\) Murashige and Skoog salt solution including vitamins, 20 g L\(^{-1}\) sucrose, and 18 g L\(^{-1}\) Glc, pH 5.6), supplemented with 200 µM acetosyringone. Leaf discs (approximately 1 cm\(^2\)) were punched from the leaves of 6- to 10-week-old _T. officinale_ and _T. kok-saghyz_ plants grown under sterile conditions on solid medium (2.2 g L\(^{-1}\) Murashige and Skoog salt solution, 10 g L\(^{-1}\) Glc, and 8 g L\(^{-1}\) agar, pH 5.8) and inoculated in the coculture medium with _A. tumefaciens_ for 30 min. The leaf discs were then placed on wet filter paper for 14 to 20 h at 26°C. To induce regeneration, the leaf discs were placed on regeneration medium (4.4 g L\(^{-1}\) Murashige and Skoog salt solution including vitamins, 20 g L\(^{-1}\) sucrose, 9 g L\(^{-1}\) agar, and 250 mg L\(^{-1}\) Clorox, pH 5.8) supplemented with 1 mg L\(^{-1}\) 6-benzyladenine and 0.2 mg L\(^{-1}\) naphthaleneacetic acid for callus and shoot induction. The elongation of shoots was maintained by the addition of 2 mg L\(^{-1}\) zeatin, 20 µg L\(^{-1}\) naphthaleneacetic acid, and 20 µg L\(^{-1}\) gibberellic acid. Rooting was induced on regeneration medium without hormones, whereas all regeneration media contained 3 mg L\(^{-1}\) phosphinotrichin for selection. All chemicals and reagents were purchased from Duchefa. Transgenic dandelion plants were transferred to soil and cultivated at 16°C with a 16-h photoperiod. Protoplasts were isolated from _T. officinale_ and _T. kok-saghyz_ according to established protocols (Negrutiu et al., 1981). Ca(NO\(_3\))\(_2\)/polyethylene glycol-mediated DNA transfer was performed as described (Negrutiu et al., 1987) using 3.3 × 10\(^3\) protoplasts and 10 µg of pTP/DsRed or pDsRed (basic cloning vector) plasmid DNA per transfection experiment.

**Measurement of Latex Coagulation**

For the measurement of latex coagulation, roots of _T. officinale_ and _T. kok-saghyz_ wild-type and RNAi plants as well as bark from _H. brasiliensis_, roots from _S. asper_, and petioles from _F. elastica_ were dissected and the latex allowed to exude from the cut. After 10 s, the latex was collected in a glass capillary (ringcaps, 1.25 x 5 µL; Hirschmann Laborgeräte). The coagulation rate of the latex was determined by measuring the csd.

**Measurement of Latex Quantity, Rubber Content, and Molecular Weight**

The quantity of harvested latex was determined by collecting the latex extruded from abscessed pedicels and measuring its weight using a fine balance. For the measurement of rubber content, 100 µL of latex was dissolved in 100 µL of sodium phosphate buffer (pH 6) and centrifuged at 13,500 rpm, 4°C. The upper rubber fraction was coagulated by adding 50 µL of glacial acetic acid. The coagulated rubber was dried and weighed. The molecular size distribution of rubber was determined by size exclusion chromatography (SEC) using an Agilent Series 1100 with a column system comprising three PSS SDV 5µ columns (100–100,000 Å, Polymer Standard Service). Samples were prepared by centrifuging 100 µL of latex in sodium phosphate buffer (pH 6) at 13,500 rpm, 20 min, 4°C to separate the rubber from soluble material. The upper rubber fraction was transferred to a fresh vial and dissolved in tetrahydrofuran, which was also used as the solvent and eluent in subsequent SEC steps with a flow rate of 1 mL min\(^{-1}\). The SEC system was calibrated with standard polysisoprenes in the range 1,090 to 1,040,000 D (Polymer Standard Service).

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession number EU154993 (ToPOPO-1).

**Supplemental Data**

The following materials are available in the online version of this article.

**Supplemental Figure S1.** Amino acid sequence of ToPOPO-1 and TkPPO-1 and promoter sequence of ToPOPO-1.

**Supplemental Figure S2.** Semiquantitative RT-PCR analysis of RNAi-knockdown _T. officinale_ and _T. kok-saghyz_ plants.

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**LITERATURE CITED**