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# Selective inhibition of plant serine hydrolases by agrochemicals revealed by competitive ABPP

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ABSTRACT

Organophosphate and -phosphonates and their thio derivatives are often used in agroindustry as herbicides and insecticides, but their potential off-targets in the plant are poorly investigated. Here, we use competitive activity-based protein profiling (ABPP) of serine hydrolases (SHs) to detect targets of these agrochemicals and other compounds in *Arabidopsis thaliana*. Using broad-range and specific probes, and by overexpression of various SHs *in planta*, we are able to confirm eight SH-compound interactions, including selective inhibition of carboxylesterase CXE12, prolyloligopeptidase, methylesterase MES2 and tripeptidyl peptidase TPP2. These observations can be used for the design of novel probes and selective inhibitors and may help to assess physiological effects of agrochemicals on crop plants.

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## 1. Introduction

Serine hydrolases (SHs) compose a wide range of enzymes in plants and animals that carry an activated serine residue in the catalytic site. SHs are mostly hydrolytic enzymes (e.g., esterases and proteases), but not exclusively, for example, acyltransferases. SHs are involved in a wide range of physiological processes, including metabolism, development, and immunity. In plants, for example, SHs were found to regulate stomatal density (e.g., SDD1), immune responses (e.g., saspase), and are involved in detoxification processes (e.g., carboxylesterase CXE12) and secondary metabolism (e.g., acyltransferase SNG1).[1]

The activity of SHs in complex proteomes can be analyzed by activity-based protein profiling (ABPP). ABPP uses chemical probes that covalently react with active site residues.[2] ABPP exploits the availability and reactivity of enzymes, which is a hallmark for enzyme activity.[3] Profiling enzyme activities rather than abundance has led to important discoveries in biomedical research,[4] and plant biology.[5] In plant immunity, for example, ABPP identified defence-induced activities of Cys proteases,[6a] and the proteasome,[6b] and demonstrated a suppression of these enzymes by pathogen-derived inhibitors.[6a,c–h]

Probes for ABPP on SHs are typically fluorophosphonates (FPs), which react with the conserved serine active site nucleophile.[7] FP-based probes have been instrumental for the discovery of SHs that serve as markers and targets for cancers and infectious diseases,[8] the annotation of novel SHs,[9] and the identification of selective inhibitors.[10] In plants, FP profiling revealed activities of over 50 SHs in leaf extracts of the model plant *Arabidopsis thaliana*, and displayed several differential SH activities upon fungal infection.[11]

SHs are important targets of agrochemicals that are massively used in agriculture as herbicides, insecticides and fungicides. The targeted SHs of these compounds in crops and their consumers are, however, only poorly investigated. Most SH targeting agrochemicals are organophosphate or -phosphonate compounds, and their thio-derivatives. Studies with these chemicals on mammalian lipases and SHs from mouse brain revealed that each serine hydrolase has different sensitivities for these agrochemicals.[10g,12] Here, we investigate the selective inhibition of plant SHs by agrochemicals and other SH inhibitors using competitive ABPP. Competitive ABPP evaluates inhibitors based on their ability to prevent labeling by the probe in a competitive assay, and has been routinely used to identify and optimize selective SH inhibitors.[10]

## 2. Results and discussion

A collection of 12 putative SH inhibitors was assembled (Fig. 1A), containing nine organophosphate and -phosphonate

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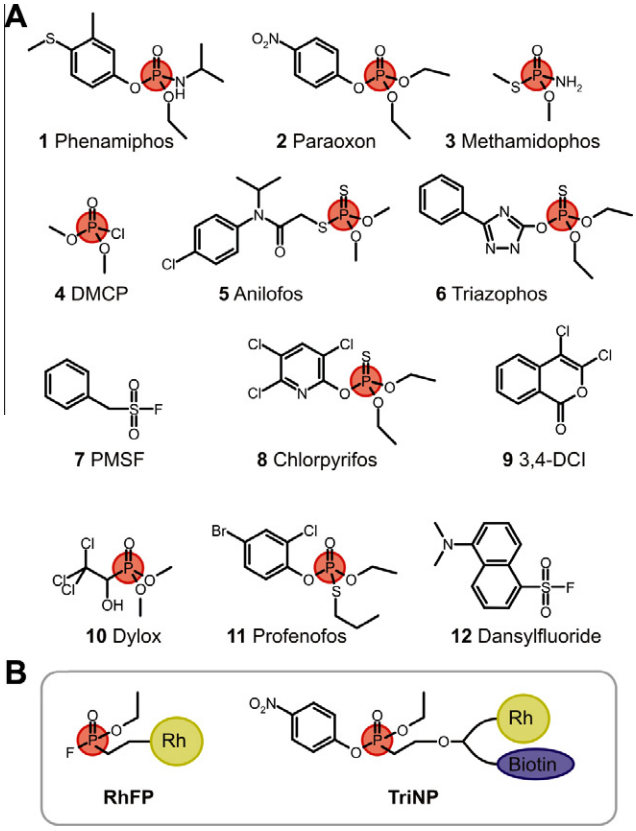


**Figure 1.** Structures of the used chemicals. (A) Twelve compounds used as competitor. Organophosphates and -phosphonates and their thio-derivatives are indicated with red circles and are used in agroindustry as herbicides and insecticides. (B) Two probes used in this study: fluorophosphonate-rhodamine (RhFP,[13]) and the paraoxon-derived probe TriNP.[14] Reporter groups are encircled green (rhodamine) and blue (biotin).

compounds and their corresponding thio-derivatives that are used in agroindustry as insecticides and herbicides. Three other compounds were added because they are known SH inhibitors: phenylmethanesulfonylfluoride (**7**, PMSF), 3,4-dichloroisocoumarin (**9**, 3,4-DCI), and 5-dimethylaminonapthalene-1-sulfonyl fluoride (**12**, dansylfluoride). Two probes were used to detect the selective inhibition of SH activities (Fig. 1B). RhFP is a rhodamine-tagged FP probe (Ref. 13); and TriNP is a trifunctional nitrophenol phosphonate probe (Ref. 14), with structural similarities to paraoxon, tagged with both biotin and rhodamine.

Labeling of Arabidopsis leaf extracts with RhFP, followed by detection of fluorescent proteins on protein gels by fluorescence scanning robustly displays ~16 signals (s1–s16 in Fig. 2A, first lane). Preincubation of leaf extracts with 100 μM compound **1–12**, followed by RhFP labeling and detection, reveals reproducible and selective suppression of the labeling of 24 signal-compound combinations (Fig. 1A and SI Fig. S1). Signals s1, s2, s5 and s16 are specifically suppressed by **7**; s3 by **2**, **4**, and **6**; s4 by **11**; s8 by **2**, **4**, **5** and **10**; s11 by **2**, **6–8** and **10–12**; s12 by **2** and **11**; and s15 by **2**, **10** and **11**.

To annotate the different signals, we used the more selective paraoxon-based probe (TriNP, Fig. 1B), which labels only a few proteins in Arabidopsis leaf extracts.[14] We previously identified these proteins as tri-peptidyl-peptidase-2 (TPP2, At4g20850), prolyl-oligo-peptidase (POPL, At1g76140), serine carboxy-peptidase-like 48 (SCPL48, At3g45010) and carboxylesterases-7 and -12 (CXE7, At2g03550 and CXE12, At3g48690).[14] Preincubation with compounds **1–12**, followed by TriNP labeling revealed selective inhibition of TPP2 by **7** and POPL by **11** (Fig. 2B). TriNP-labeling

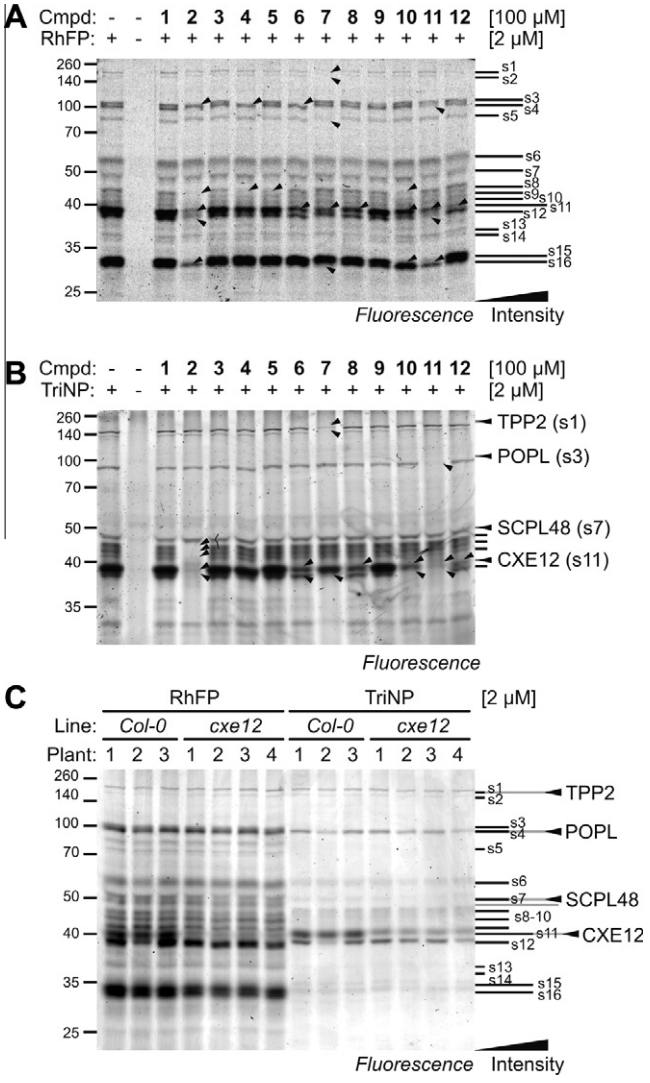


**Figure 2.** Selective inhibition of plant SHs. (A) Selective inhibition of RhFP labeling. Arabidopsis leaf extract was preincubated with 100 μM compound for 30 min and then labeled with 2 μM RhFP for 1 h. Fluorescently labeled proteins were detected from protein gels by fluorescent scanning. The 16 signals are numbered on the right with lines of which the length indicates the signal intensity in the no-inhibitor-control (lane 1). (B) Selective inhibition of TriNP labeling. The experiment was done as described in (A) but with TriNP instead of RhFP. (C) Comparison of labeling profiles with different probes on different proteomes. Leaves from individual wild-type (*Col-0*) or CXE12-mutant (*cxe12*) plants were labeled with RhFP or TriNP and labeling was detected by fluorescence scanning. The reduced signal intensity of the TriNP-labeled proteome is caused by the reduced reactivity and increased selectivity caused by TriNP when compared to RhFP.

of SCPL48 is not inhibited by any of the tested compounds, whilst CXE7/12 labeling is inhibited by **2** and **11**, and suppressed by **6**, **8**, **10**, and **12** (Fig. 2B). Three additional signals at 45 kDa are inhibited by **2** only, but have not been annotated. Interestingly, these data show that TPP2, POPL and SCPL48, which are labeled by TriNP that features high similarities to paraoxon, are not sensitive to inhibition by paraoxon (compound **2**).

To compare if these inhibition profiles are consistent with those observed with RhFP labeling, we performed a comparative analysis by labeling leaf extracts from three independent Arabidopsis plants (Col-0 ecotype) with RhFP and TriNP. We included leaves from four plants of the *cxe12* mutant line[1a] to determine the contribution of CXE12 to the RhFP and TriNP profiles. Comparison of the labeling profiles indicates that some RhFP targets are also labeled by TriNP (Fig. 2C). TPP2, POPL, and SCPL48 for example, correspond to s1, s4,

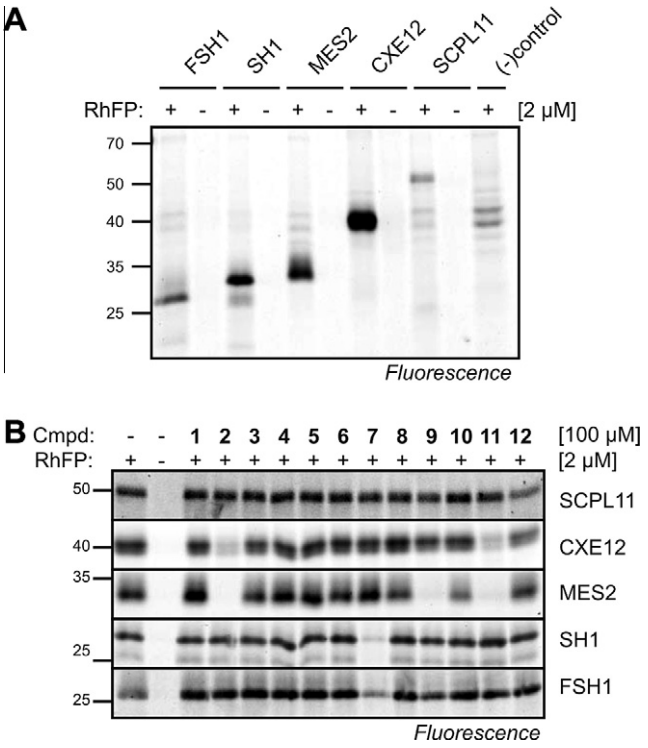


**Figure 3.** Heterologously expressed SHs confirm selective inhibition. (A) Five Arabidopsis SHs were transiently overexpressed in *Nicotiana benthamiana* leaves by agroinfiltration. Extracts of agroinfiltrated leaves were labeled with and without 2 μM RhFP for 1 h. Fluorescently labeled proteins were detected in protein gels by fluorescent scanning. (B) Selective inhibition of various Arabidopsis SHs. Extracts from agroinfiltrated leaves were preincubated with 100 μM compound for 30 min and then labeled with 2 μM RhFP for 1 h. Fluorescently labeled proteins were detected form protein gel by fluorescent scanning.

s7, respectively, in the RhFP profile. CXE12/7 was labeled by TriNP and corresponds to s11 in the RhFP profile. This signal is reduced but not absent in the *cxe12* mutant line, indicating that CXE12 contributes to the s11 signal, together with another SH (possibly CXE7), which is labeled by both RhFP and TriNP. The SCPL48 and CXE12/7 signals run slightly higher upon TriNP labeling, presumably since this probe is larger when compared to RhFP. This MW shift is probably also visible for signals s12, s15 and s16, but not for proteins with higher MW, consistent with the fact that the relative contribution of a heavy probe on the total mass is less on large proteins.

The comparative annotation of the profiles is consistent with the compound sensitivity: TPP2 and s1 are both sensitive only to **7**, whereas POPL and s4 are both sensitive to **11** (Fig. 1A and B). SCPL48 and s7 are both insensitive to all compounds. The situation for CXE7/12/s11 is more complicated since the *cxe12* mutant analysis demonstrates that these signals are composed of multiple SHs. Compounds **2** and **11** are surely effective inhibitors of both CXE7/12 and s11 because signals are absent. In contrast, compounds **6**, **8**, **10** and **12** lead to incomplete labeling of the proteins in this region, making it uncertain if CXE12 or another SH is inhibited.

To confirm selective inhibition of CXE12 and other SHs, we cloned and overexpressed five SHs in plants and used these for competitive ABPP assays. We chose CXE12 (At3g48690), methylesterase-2 (MES2, At2g23600), SCPL11 (At2g22970), and two more SHs: FSH1 (At5g65400) and SH1 (At5g20060). These five SHs represent different SH families and were previously identified as FP-labeled proteins in an Arabidopsis leaf proteome.[11] The SHs were transiently overexpressed by infiltration of *Agrobacterium tumefaciens* strains carrying the SH-encoding genes on binary plasmids into leaves of *Nicotiana benthamiana*, in the presence of the p19 silencing inhibitor to boost over-expression.[15] Labeling of leaf extracts expressing the SHs with RhFP revealed specific signals that are absent in the control (Fig. 3A). The size of these signals is consistent with the expected molecular weight of each of these SHs: 28, 27, 30, 36, and 48 kDa for FSH1, SH1, MES2, CXE12 and SCPL11, respectively. Signals in the negative control (leaves overexpressing p19), are from endogenous SHs and partially overlap with the CXE12 signal.

These five SH-containing extracts were preincubated with compounds **1–12** and then labeled with RhFP to detect selective inhibition. None of the compounds prevents labeling of SCPL11 (Fig. 3B). This insensitivity is similar to that observed for SCPL48, which is also insensitive to these compounds. Labeling of CXE12 can be inhibited by **2** and **11** (Fig. 3B), consistent with the absence of signals in this region with RhFP and TriNP labeling (Fig. 2A and B). MES2 labeling can be inhibited by **2**, **9** and **11**. MES2 was previously identified from gels in the region corresponding to signals s15 and s16.[11] The s15 signal is, however, sensitive to **2**, **10**, and **11**, whereas s16 is sensitive to only **7** (Fig. 2A), indicating that the s15 and s16 signals do not represent MES2. Labeling of FSH1 and SH1 can be blocked only by **7**, similar to signals s1, s2, s5, and s16, but the s1 and s2 signals are too high in the protein gel to be caused by FSH1 or SH1. It is therefore unknown if FSH1 and SH1 contribute signals to the RhFP profile.

## 3. Conclusion

Taken together, we have detected and confirmed selective inhibition of CXE12 by paraoxon (**2**) and profenofos (**11**), and showed selective inhibition of TPP2, FSH1 and SH1 by PMSF (**7**); POPL by profenofos (**11**); and MES2 by paraoxon (**2**), 3,4-DCI (**9**) and profenofos (**11**). This study demonstrates that each SH has different sensitivities for inhibitors, and is consistent with studies on animal SHs using enzymatic assays,[12] and competitive ABPP.[10g] It is not surprising that paraoxon (**2**), PMSF (**7**) and profenofos (**11**) were found to be efficient inhibitors. The phosphorous in **2** and **11** as well as the sulfur in **7** are very electrophilic because they are directly linked to good leaving groups. This facilitates the attack of the hydroxyl group of the active site serine. The other tested inhibitors are less reactive ('disarmed') because they lack a good leaving group, or contain a less-reactive phosphorothionate ester. For example, the aromatic ring of phenamiphos (**1**) has reduced reactivity, and activation of this compound requires in vivo oxidation of the thiomethyl group into a sulfone or sulfoxide.[17,18] In compounds derived from phosphorothionate esters (**5**, **6** and **8**), the polarity of the P═S bond is weaker when compared to a P═O bond. These compounds require in vivo conversion into their corresponding organophosphorous esters to become more reactive.[17,18] These properties have to be considered when using these inhibitors for chemical knockout studies, but also for the design of selective probes. A different decoration of the leaving group of paraoxon-derived probes, for example, could lead to a POPL-selective probe since POPL is sensitive to profenofos (**11**) but not paraoxon (**2**). Apart from these practical implications, this study also indicates that insecticides paraoxon (**2**) and profenofos (**11**) may alter the physiology of crop plants by inhibiting CXE12, POPL and MES2 (-like) proteins.

## 4. Experimental

### 4.1. Chemicals and labeling

Agrochemicals and inhibitors were purchased from Sigma (Steinheim, Germany). RhFP[13] and TriNP[14] were synthesized as described elsewhere. *A. thaliana* leaf extracts were generated by grinding 2 g of frozen leaves of 4-week-old Arabidopsis thaliana ecotype Col-0 in a mortar at room temperature (22–24 °C) to a homogenous green paste. The paste was mixed with 5–6 mL of distilled water and cleared by centrifugation (5 min at 16,000×*g*). The

protein concentration was determined by using the RC/DC Protein Assay (Bio-Rad) following the manufacturer's instructions.

### 4.2. Competitive ABPP and detection

Extracts were diluted to 1 mg/mL protein concentration in 1× phosphate buffered saline (1× PBS, 1.1 mM $KH_2PO_4$, 155 mM NaCl, 3 mM $Na_2HPO_4$, pH 7.4) and supplemented with 3 mM dithiothreitol. Aliquots (50 μL) were then preincubated with 100 μM compounds s1 to s12 or DMSO for 30 min. This was followed by addition of either RhFP (final concentration 2 μM) or TriNP (final concentration 4 μM) and further incubation at room temperature in the dark for 1 h. The reaction was stopped by adding 15 μL of 4× SDS electrophoresis gel loading buffer (280 mM SDS, 400 mM Tris, 40% glycerol, 1.4 M β-mercaptoethanol, 0.6 mM Bromophenol Blue, pH 6.8). Proteins were separated on 12% SDS polyacrylamide gels. Gels were washed three times 15 min with pure water and then scanned on a Typhoon 8600 scanner (GE Healthcare, Munich, Germany). Excitation wavelength was set to 532 nm, emission was measured using a TAMRA filter (580 nm BP30).

### 4.3. Cloning and expression of Arabidopsis SHs

Full-length cDNAs for FSH1, SH1, MES2, CXE12 and SCPL11 were amplified from an *A. thaliana* cDNA library (kindly provided by Dr. Hans Sommer, Max Planck Institute for Plant Breeding Research) using primers as indicated in Supplemental Table I. The PCR fragments were cloned into the cloning vector pFK26 and then shuttled into the binary vector pTP5 as summarized in Supplemental Table II.[6a] The binary vectors were transformed into *A. tumefaciens* strain GV3101 pMP90.[16] Transient overexpression of SHs was achieved by co-infiltrating cultures of Agrobacterium strains carrying the binary vectors together with cultures carrying silencing inhibitor p19 into fully expanded leaves of 4-week-old *N. benthamiana*.[15] Leaves were harvested after 3 days, and the proteins were extracted and labeled as described above.

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### Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2011.06.040.

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