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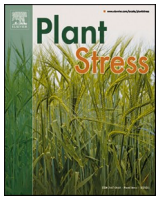

Early stress detection in forest trees using a nanobody-functionalized electrochemical biosensor for ascorbate peroxidase

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ABSTRACT

Forest environments are exposed to multiple stressful factors of both abiotic and biotic nature such as heavy metal contamination, drought, or pest infestations which may lead to their massive decline. We designed a comprehensive approach for isolating, producing and functionalizing reagents suitable for the affordable detection of forest plant stress biomarkers with the aim to provide quantitative data to assess plant stress fluctuation and, possibly, to design mitigation strategies. We first optimized a panning protocol to recover nanobodies targeting shared sequences that could cross-react with both *Pisum sativum* and *Populus nigra* ascorbate peroxidase (APX). After their production as recombinant constructs and their extensive biophysical and biochemical characterization, such reagents were exploited as the immunocapture element of an electrochemical biosensor conceived as a potential point-of-care device. Such biosensor could detect both pea and poplar APX in leaf extracts and could be used to clearly discriminate between control and heavy metal-stressed poplar plants based on their APX activity, even before the appearance of any phenotypic symptom. The combination of fast and inexpensive reagent production with the development of portable diagnostics opens the opportunity for large-scale, on-site surveys of forest trees.

1. Introduction

The resources allocated for plant research are significantly lower than those available for studying animals and this is particularly true for forest trees that have long life cycles which further complicate the investigation of their specific biology. Nevertheless, these plants are globally crucial because of their contribution in terms of CO_2 fixation and storage capacity, a factor that make them particularly worthy of attention. The past decades, characterized by increasing temperature and drought which induced tree mortality all over the world (Hartmann et al., 2022), underlined the fragility of forest ecosystems. It becomes therefore necessary to acquire as many data as possible to monitor the physiological conditions of forest trees, foresee trends and look for measures that could alleviate their decline. The present knowledge, inferred mostly by studies undertaken using herbaceous plants, is probably sufficient to identify molecular biomarkers suitable to assess stress conditions in forest trees. In contrast, what is almost totally missing are specific reagents and corresponding diagnostic technologies to set up (*in situ*) affordable analyses. Consequently, we designed an approach for the rapid isolation of immune reagents, suitable for quantifying stress biomarkers in plant extracts, starting from a phage display pre-immune library of nanobodies.

We selected ascorbate peroxidase as the model protein for this proof-of-principle. There are at least two reasons for such choice: i) the enzyme is a relevant biomarker of stress conditions; ii) the sequence is relatively conserved among plants, both herbaceous and woody and this condition might facilitate the identification of antibodies able to cross-react with the antigen isoforms present in different species. Ascorbate peroxidase (APX, EC 1.11.1.11) is a hydrogen peroxide-scavenging enzyme that belongs to class I heme-peroxidases and uses ascorbate as the specific electron donor. APX is constitutively expressed and its peroxide detoxifying activity is part of the wider ascorbate-glutathione cycle (Chew et al., 2003). The availability of such scavenging system is particularly critical in chloroplasts because such organelles must remove the large amount of H_2O_2 generated during the light phase of the photosynthetic activity, but APX activity significantly increases under oxidative stress conditions triggered by various environmental stimuli, such as drought, elevated salt content, high light radiations, high and low temperatures,

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contaminations from potentially toxic elements and pathogen attacks (Caverzan et al., 2012). Since working directly with trees is cumbersome due to their dimension and difficulty to access to reproducible samples over the year, the method to isolate specific anti-APX nanobodies has been initially set up using *Pisum sativum* (L.) and the reagents were finally validated on poplar (*Populus nigra* L.).

Biopanning is a methodology used for the recovery of binders specific for an antigen starting from large collections of constructs that differ for their sequences. This technique requires a medium, such as phages, providing a physical connection between the phenotype, namely the binder displayed on the surface, and its corresponding genotype, namely the binder DNA sequence. In phage display, each phage particle displays a unique binder and pre-immune libraries with a diversity of 10^8 – 10^{10} clones are suitable, statistically, to identify binders for any molecular target (Monegal et al., 2009; Crepin et al., 2017). In this work, a naïve phage display library of nanobodies (Monegal et al., 2009) was used because such material allows the inexpensive isolation of binders in few weeks and without the necessity to immunize animals. Nanobodies are small (15 kDa) and correspond to the variable domain of heavy chain-only IgG antibodies present in camelids. They were chosen because usually structurally stable and relatively easy to produce in bacteria even when fused to different tags (Djender et al., 2014; Muyldermans, 2021). After isolation, nanobodies were characterized for their biochemical features and finally exploited as the antigen-selective capture element of an electrochemical biosensor that successfully quantified the APX activity shift induced by stress conditions in leaves of plants grown in a soil contaminated with heavy metals.

2. Material and methods

2.1. Plant growing conditions

Pisum sativum plants were grown in non-sterile conditions in a soil with neutral pH, at 24 – 26°C, with a light/dark photoperiod of 16:8 hours. Metal stress was tested on *Populus nigra* cuttings belonging to the PN6 clone that were planted in March 2022 into 1.8 l pot using a commercial substrate (Klassman-Deilmann Gmbh, Geeste, Germany). They were kept in the greenhouse until the first week of June 2024 when they were transplanted into potential toxic element (PET)-contaminated and comparable non-contaminated (control) soil, ten plants for each treatment. Five liter pots were used for transplantation. PTE-contaminated soil originated from the abandoned farmland on the banks of the Meža river in Prevalje (14°93′73″ E, 46°54′57″ N) Slovenia, in which Pb, Zn and Cd accumulated as the result of mining and smelting activities that occurred over 300 years. Contaminated soil contained 1850, 3830 and 21 mg kg^{-1} Pb, Zn and Cd, respectively (Gluhar et al., 2021; Kaurin et al., 2021). Soil pH was 7.0 ± 0.1 and contained 3 % of C_{org} and 0.24 % of N. Control alluvial soil was obtained from the farmland on the banks of the Sava River in Zadobrova, Ljubljana (46°05′04.4"N 14°34′45.5"E). Control soil had the pH in the range 7.41-7.54 and contained 1.03-2.13 % C_{org} and 0.106-0.184 % N. Contaminated and control soil were mixed with one third of perlite to obtain the potting substrate. Transplanted poplars were kept outdoor and watered regularly to keep the soil moist all the time and used in July 2024.

2.2. Recovery of leaf extracts from control and metal-ion stressed plants

One and half g of leaves of either *Pisum sativum* or *Populus nigra* (from both control and stressed plants) were immersed in liquid nitrogen and immediately crushed in a mortar kept on ice using 2 ml of cold extraction buffer: phosphate buffer 50 mM pH 7.5, 20 µl of protease inhibitor cocktail I (Sigma Aldrich – Cat. No. 539131) and 1.5 % of Nonidet P-40 protein detergent (Sigma Aldrich – Cat. No. 492018). The resulting homogenate was filtrated through a mesh with hole diameter of 108 µm and centrifuged at 14,000x*g* for 10 min, at 4°C. The supernatant enriched in soluble proteins was used for downstream APX activity measurements and biosensor setting.

2.3. Biopanning on recombinant pea ascorbate peroxidase

Two rounds of biopanning were performed using a naïve nanobody phage library (Monegal et al., 2009). After a double depletion step against the irrelevant target SpyCatcher and the fluorescent protein mClover3 performed to eliminate binders for common epitopes, the unbound phage fraction was incubated for 120 min in the presence of 125 µl of magnetic beads coated with 100 µg of pea-APX-mClover3. Washing steps (15x) were performed with 10 ml of cold PBS to remove not specific binders and phages were eluted in two successive steps inducing partial protein denaturation: (i) Acidic elution in glycine-HCl 0.2 M, pH 2.2; (ii) Basic elution in KCl/NaOH 0.2 M, pH 12.4. Individual colonies were evaluated by ELISA using an anti-M13, HRP-conjugated detection antibody specific for phage proteins and 0.5 µg/well of pea-APX-mClover3 or BSA (control) diluted in sodium carbonate/bicarbonate 0.2 M, pH 9.5 buffer, and blocked with 100 µl/well of PBST plus milk 1 %. TMB was added as the substrate and the reaction was stopped by adding 100 µl/well of 1 N H_2SO_4. The absorbance at 450 nm was finally measured using a microplate reader (Tecan Infinite F200 - Männedorf, Switzerland).

2.4. Protein production

The sequence of the APX of *Pisum sativum* was obtained from NCBI gene bank (accession number: AAA33645), the corresponding synthetic gene was produced from Twist Bioscience (Twist Bioscience HQ - South San Francisco, USA) and the resulting DNA was cloned in a modified pET-14b vector in frame with either mClover3 or AviTag. Both constructs were expressed in *E. coli* BL21(DE3) cells. Expression was induced with 1 mM and 0.5 mM isopropyl-β-D-thiogalactoside (IPTG), respectively, when the growth medium OD_{600} was 0.6, cells were then grown for 3 h at 37°C, pelleted (30 min at 4.500x*g*) and lysed in lysis buffer (Tris-HCl 50 mM, pH 8.0, NaCl 500 mM, $MgCl_2$ 5 mM) by alternating three cycles of freezing/thawing. Lysates were then sonicated on ice (6x, amplitude 80 %, 1 min pulse, 1 min off) using a LABSONIC® M device (Sartorius, Göttingen, Germany), incubated for 30 min at room temperature with DNase I (33 U/ml) and lysozyme (100 µg/ml) and finally centrifuged at 4°C (30 min at 13,000x*g*). Proteins were purified from the resulting supernatant using a Talon Hi-Trap column (Cytiva - Marlborough, MA, USA) and an ÄKTA pure™ system (Cytiva - Marlborough, MA, USA). The purified proteins were desalted by means of a Hi-Trap Desalting column (Cytiva - Marlborough, MA, USA) and evaluated by gel filtration, Superdex™ 75 Increase 5/150 GL or Superdex™ 200 10/300 GL (Cytiva- Marlborough, MA, USA), UV scan analysis between 240 nm and 340 nm and SDS-PAGE, as previously described in details (Djender et al., 2014; de Marco et al., 2021).

Unique nanobody sequences determined by DNA sequencing were cloned in pET-14b vector using primers from Kemomed d.o.o. (Kemomed d.o.o - Ljubljana - Črnuče, Slovenia) and repliQa HiFi ToughMix® (Quantabio - Massachusetts, USA). The recombinant constructs were expressed in DiscoTune cells (Bertelsen et al., 2021) which enable the formation of disulphide bonds, purified and evaluated following the same procedures described above. Proteins were quantified by spectroscopy recording the values of UV absorption at 280 nm and calculating the concentrations by means of their molar extinction coefficient. Aliquots were stored at - 80°C.

2.5. EC_{50} evaluation

A 96-well Maxisorp plate was coated with 10 µg/ml of anti-pea-APX-AviTag resuspended in sodium carbonate/bicarbonate 0.2 M, pH 9.5 buffer. Measurements were performed in triplicate. The plate was incubated overnight, at 4°C, the day after wells were washed three times

with 200 µl of PBST and blocked with 200 µl of 2 % BSA solution in PBS for 1 h. After further three washes, nanobodies anti-pea-APX (BA5, BG12) fused to the green fluorescent protein mClover3 were added using serial dilutions in 1 % PBS/BSA, from 6 µM to 2 nM. The microplate was incubated 2 h, at 4°C under gentle mixing. After incubation, wells underwent three washing steps with 200 µl of PBS and the fluorescence signal at 485/535 nm was measured using a microplate reader (Tecan Infinite F200 - Männedorf, Switzerland). EC_{50} values were calculated with GraphPad Prism.

2.6. Immunoprecipitation

Twenty-five µl of M-450 Epoxy Dynabeads™ (Invitrogen – Cat. No. 14011) were washed twice with 500 µl of PBS, pH 7.4. Magnetic beads were coated with 10 µg of the antigen (pea-APX-mClover3) diluted in PBS and incubated overnight with gentle rotation at 4°C. The day after, coated beads were washed twice with 500 µl of PBS and 15 µg of anti-pea-APX-AviTag (BA5, BG12) nanobodies were added. The samples were incubated 1 h at RT under rotation, washed twice as previously described and samples were finally resuspended in 30 µl of PBS, pH 7.4, before being mixed with loading buffer for SDS-PAGE evaluation.

2.7. Nano differential scanning fluorimetry (NanoDSF)

One µl, corresponding to 1 µg, of each sample (Nb-BA5-AviTag, Nb-BG12-AviTag, pea-APX-AviTag) was mixed with 9 µl of different buffers (Bis Tris 20 mM pH 6.5; Bis Tris 20 mM pH 7; Hepes 20 mM, NaCl 300 mM pH 7; 20 mM Hepes 20 mM, NaCl 300 mM pH 7.5; Tris 50 mM, NaCl 250 mM pH 7.6; Tris 50 mM, NaCl 50 mM pH 7.5; Tris 20 mM, NaCl 500 mM pH 7.5; Tris 20 mM, NaCl 300 mM, 10 % glycerol pH 7.5; PBS pH 7.4) to obtain final concentrations of 0.1 mg/ml. Samples were subjected to temperature gradient from 20°C to 95°C inside a thermostated block and the intrinsic fluorescence was measured at 330 and 350 nm. First derivative of the fluorescence ratio 330/350 nm was plotted versus the gradient temperature and the melting temperature (Tm) of each sample was calculated.

2.8. Dynamic light scattering (DLS)

Nb-antigen complexes (1:1) were prepared by mixing 10 µM of pea-APX-AviTag with 10 µM of BA5-AviTag or BG12-AviTag and analyzed by DLS (de Marco et al., 2021). Prior to DLS measurements, the proteins were incubated 30 min at 20°C under gentle rotation to allow the formation of the complex. The quartz cell was filled with 20 µl of each sample solution and the scattered light was detected at 20°C using a Zetasizer Nano ZS90 (Malvern Panalytical Ltd., UK).

2.9. Mass photometry

Protein complexes were prepared as described for DLS analysis. Samples were diluted to 100 nM in filtered PBS pH 7.4 buffer. First, fresh buffer was flowed into the chamber to find the focus, then 20 µl of sample were introduced into the flow chamber for each acquisition using the Mass Photometer TwoMP (Refeyn - Massachusetts, USA).

2.10. Biosensor preparation, use and data analysis

Amperometric biosensor was used to assess the interaction between Nb-BA5 and pea-APX. Then, the same protocol was applied to detect the presence of ascorbate peroxidase in both *P. sativum* and *P. nigra* protein extracts. First, the surface of the screen-printed carbon electrode (SPCE) was washed with 500 µl of ethanol and dried with air. Then, the electrochemical deposition of gold nanoparticles (AuNPs) was carried out by drop-casting 40 µl of a solution of tetrachloroauric acid ($HAuCl_4$) 1 mM in H_2SO_4 0.5 M and applying 0.18 V for 50 sec. Then, the SPCE surface was washed twice with PBS pH 7.4 and dried with air. Ten µl of 1 µM of Nb-BA5-AviTag were immobilized on the AuNPs-modified carbon working electrode by adsorption for 30 min, at RT. Then, after two washing steps with PBS pH 7.4, the surface was blocked with 10 µl of BSA 0.5 % solution for 30 min. The electrode surface was washed twice as above and finally covered with 10 µl of the sample provided at different concentrations: (i) increasing amounts (0, 0.5, 1, 1.5 µM) of pea-APX; (ii) different dilutions (0.01, 0.05, 0.5, 1) of *P. sativum* protein extract; (iii) different dilutions (0.5, 0.125, 0.06, 0.03, 0.01) of *P. nigra* protein of both healthy and stressed plants. The interaction was detected by adding 60 µl of ascorbic acid (500 µM) for 1 min and applying -0.5 V for 60 sec. Since the sensor can measure variations of current intensity, the formation of the nanobody-antigen complex must correspond to a change in the electric signal. We exploited the peroxidase activity of APX that can catalyze reactions using molecular oxygen, although its primary function is scavenging hydrogen peroxide or organic peroxides. Hence, it was possible to correlate the decreased oxygen−reduction response upon increasing the quantity of bound APX. Specifically, the activity of APX was assessed through amperometric cathodic detection of oxygen depletion during the enzymatic reaction. This particular design is also guarantee of signal specificity because only a peroxidase can trigger the reaction.

3. Results and discussion

During preliminary activities we experienced some shortcomings associated with the experimental work performed using deciduous forest plants, the most annoying of which was the extremely short period in which reproducible biological material is available, even using cuttings grown under controlled conditions. Of course, herbaceous plants can be useful models for adapting technologies initially conceived for experiments with animals or microorganisms to plant research, but we wished to avoid the risk of failing in the successive step, namely the transfer of the methodology from model herbaceous plant to target forest tree. To minimize such risk, we first searched for biomarkers that could be not only physiologically meaningful, but also have sequences conserved among model and target species, with the aim of isolating nanobodies able to cross-react with the different isoforms. This condition should allow the exploitation of the same reagents and platforms to analyze the biomarker isoforms in different species. Specifically, we noticed that APX sequences were highly conserved in pea and poplar (Fig. 1a), a characteristic that is favourable for the isolation of binders targeting conserved epitopes. Since poplar APX possesses a C-term sequence extension missing in pea APX, nanobody panning was performed with the shorter pea APX clone to avoid the potential isolation of binders specific for the poplar-exclusive C-term region. The clone was produced recombinantly as fusion proteins with either AviTag or mClover3, yielding 2-4 mg/l and the quality of such constructs was analyzed in terms of purity and monodispersity. The quality control performed by SDS-PAGE and gel filtration indicated that the recombinant APX-AviTag was highly homogeneous, with no apparent degradation or aggregation but a tendency to dimerize (Fig. 2a and 2b). Similarly, APX-mClover3 was pure and present as both, monomer and dimer forms (Suppl. Fig. 1). AviTag is a short (15 aa) sequence that enables 1:1 biotinylation at a specific residue, namely the production of a reagent suitable for being used in combination with streptavidin/avidin reagents, whereas mClover3 is a green fluorescent protein that simplifies the construct identification in any downstream application. APX-mClover3 was used for biopanning in combination with a large and well-validated nanobody naïve phage display library (Monegal et al., 2009). Although the clones present in pre-immune libraries like this did not undergo somatic maturation, they usually provide binders with affinities in the nanomolar range that are suitable for most of the research uses and, if necessary, their features can be further optimized *in silico* (Soler et al., 2018; Li et al., 2023). Nanobody libraries obtained from immunized animals can offer stronger binders, but creating a new library for each new antigen is time consuming (several months instead of few weeks),

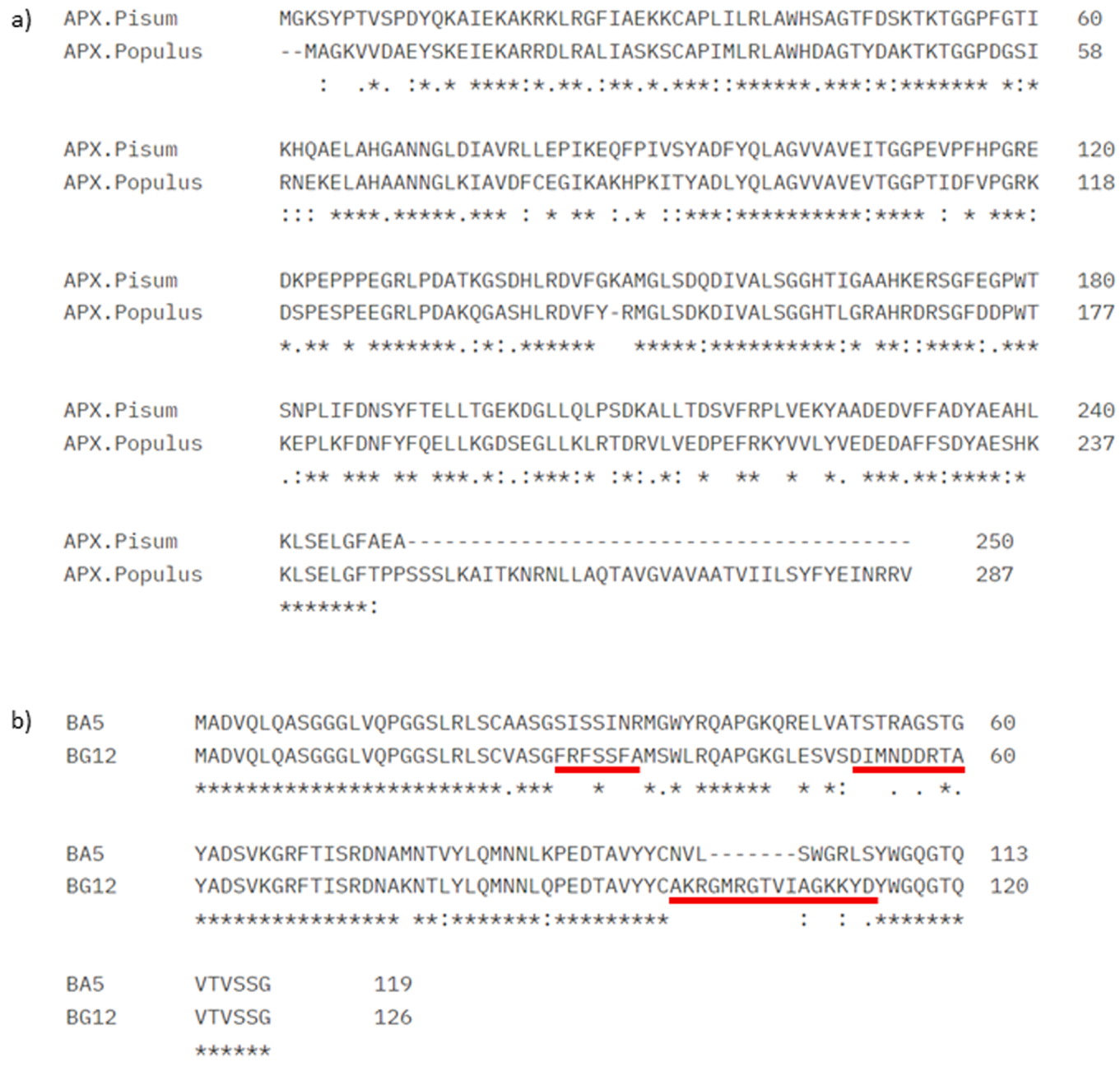

Fig. 1. Sequence comparisons
a) Alignment of APX sequences from *Pisum sativum* and *Populus nigra*; b) Alignment of the sequences corresponding to the nanobodies BA5 and BG12. Red lines correspond to nanobody hypervariable CDRs.

work intensive and expensive. Furthermore, antibody development *in vivo* is strongly dependent on the antigen immunogenicity, whereas *in vitro* selection of existing unbiased collections provides more flexibility and enables the identification of binders for epitopes "neglected" by somatic maturation (D'Agostino et al., 2022). In our case, we tested 92 colonies for each elution condition and initially selected fifteen clones obtained by acidic elution and five recovered by basic elution because their ELISA signal was at least five times above the background value. This arbitrary threshold is commonly used during screening of recombinant binders. Eleven clones from the acidic elution shared the same nucleotide sequence, therefore this protocol provided five unique sequences. Two unique clones were identified among those eluted at basic conditions. Among these 7 unique clones, three were chosen after another ELISA assay performed with a higher number of replicates (GenBank: PQ159473, PQ159474, PQ159475) because their absorbance values were particularly high, namely at least 14 times above the background signal. These were: i) the most repeated clone from the acidic elution (A11) and ii) the two clones recovered from the basic elution (BA5 and BG12). After subcloning, they were produced as fusion proteins with either AviTag or mClover3. Due to the low productivity of the clone A11, only BA5 and BG12, that yielded more than 1 mg/l of culture, were considered for further characterization (Fig. 1b and Suppl. Fig. 2). SDS-PAGE revealed only one band corresponding to the expected mass of the nanobodies BG12 and BA5 (15 kDa). The absence of contaminants and aggregates in the sample was confirmed by UV scan and gel filtration analyses that showed single, well-defined peaks for both nanobodies (Fig. 2c and 2d, Suppl. Fig. 2). NanoDSF was performed to determine the protein melting temperature (Tm) in different buffers because this parameter positively correlates with protein stability. The collected data (Suppl. Fig. 3) evidenced that nanobodies clearly

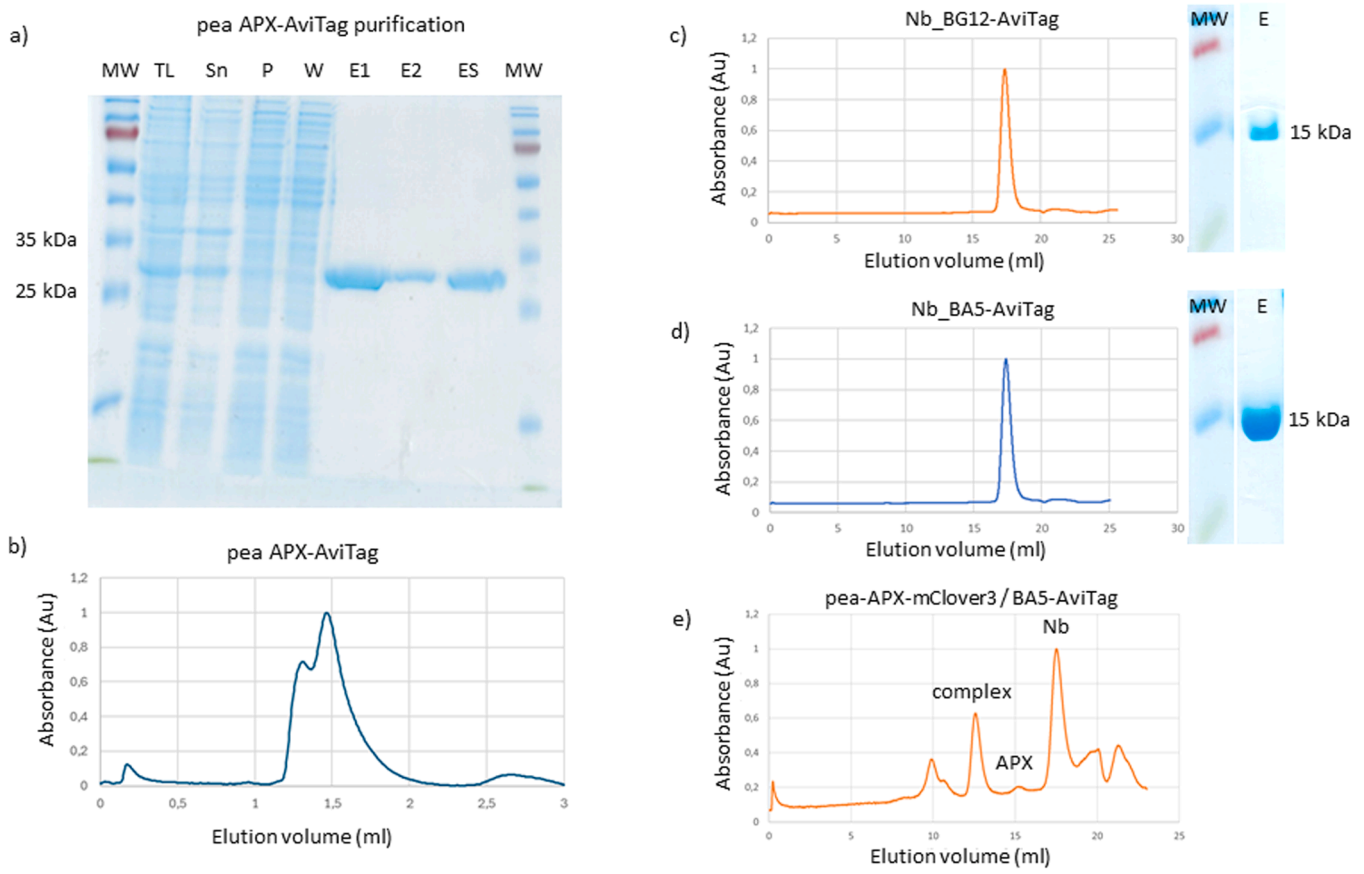

Fig. 2. Characterization of recombinant pea APX and corresponding nanobodies BG12 and BA5
a) Pea APX was expressed fused to AviTag-6xHis tag and purified by IMAC. The homogeneity of the resulting sample was analyzed by SDS-PAGE. The different fractions corresponded to: TL (total lysate), Sn (supernatant), P (pellet), W (washing), E (eluate), MW (molecular weight); b) The purified sample was then evaluated by gel filtration; c) Purified anti-pea APX nanobody BG12 fused to AviTag (gel filtration profile and SDS-PAGE lane corresponding to the elution fraction); d) Purified anti-pea APX nanobody BA5 fused to AviTag (gel filtration profile and SDS-PAGE lane corresponding to the elution fraction); e) gel filtration profile of the sample containing APX-mClover3, BA5-AviTag and their complex.

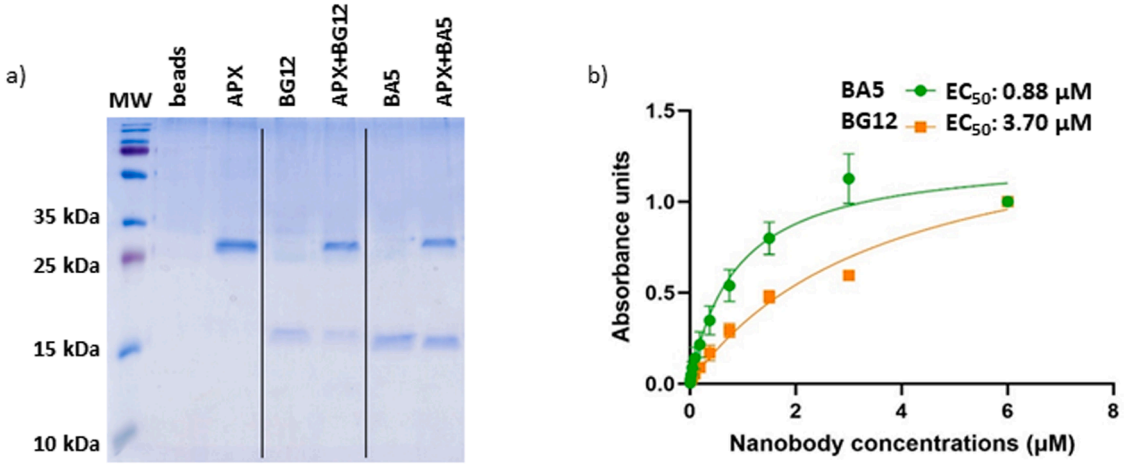

Fig. 3. Characterization of the complex between APX and the corresponding nanobodies - I
a) SDS-PAGE with the samples used for immunoprecipitation. Lanes were loaded with epoxy beads alone, beads plus APX, and the same APX-functionalized beads plus BG123 or BA5. Nanobodies alone were loaded as controls; b) ELISA test performed using immobilized APX and decreasing nanobody concentrations to calculate their EC_{50}.

preferred Tris buffers, high salt concentrations and glycerol, whereas pea APX was better stabilized by HEPES buffer and high salt content. Based on these preliminary results, both AviTag- and mClover3-fusion constructs were used in combination with the pea APX to study the interaction between binders and antigen.

The formation of a complex between the nanobodies and the antigen was initially confirmed by an immunoprecipitation experiment that demonstrated the capacity of beads functionalized with APX to capture

BG12 and BA5 resuspended in solution (Fig. 3a). Next, an ELISA test performed using immobilized APX and variable nanobody concentrations as analytes enabled to evaluate their EC_{50} binding values (Fig. 3b). BA5 showed higher affinity (EC_{50} = 0.88 μM) with respect to BG12 (EC_{50} = 3.70 μM) and the strength of the binding with APX was sufficient to maintain the complex stability in gel filtration (Fig. 2e). These data were further confirmed by the dynamic light scattering experiments that showed the formation of a larger structure when nanobodies and APX were simultaneously present in solution (Fig. 4a). Interestingly, both APX and nanobodies are too small to be detected by mass photometry, but their 1:1 complex provided a well-defined peak (Fig. 4b). Altogether, this set of independent results represents a strong evidence for the formation of a stable nanobody-pea APX complex. We wish to underline that the nanobodies used in these experiments were monovalent and that they can be expressed as well fused to domains that promote their assembling into multimeric constructs with significantly higher avidity (Djender et al., 2014). APX activity has been often used to evaluate plant stress, also induced by heavy metals (Sydam-Aydin et al., 2015; Niu et al., 2018; Kumar, 2022), and the impact of remediation strategies against stress factors (Yang et al., 2015; He et al., 2022). It has also been exploited biotechnologically to assess ascorbate content or, coupled to antibody fragments, to detect the presence of pathogens (Sherwood & Hayhurst, 2022; Shi et al., 2023). Nevertheless, in all these cases APX activity is either measured by conventional biochemical assays or inferred by applying quantitative real-time PCR, namely methods that require lab equipment and are not suitable for field measurements. Effective large-scale forest surveys would, in contrast, benefit from portable devices that require minimal training for being operated. Consequently, we conceived an electrochemical biosensor with the aim of obtaining a proof-of-concept for a point-of-care (POC) diagnostic device able to quantify the APX content in vegetal samples and, potentially, convert the signal in values that can be directly displayed on a phone display (Boonkaew et al., 2024).

The biosensor design is depicted in Fig. 5a and its operational functioning described in Fig. 5b. The specific recognition of APX is provided by the presence of the anti-APX nanobodies as the immunocapture element. In details, BA5 nanobodies are coated on the electrode surface and unspecific binding is prevented by a treatment with BSA that saturates the electrode. Once a sample is loaded above the biosensor, only the APX in solution will be selectively bound by the nanobodies. Since the sensor can measure variations of current intensity, the formation of the nanobody-antigen complex should be able to induce a change in the electric signal. We exploited the peroxidase activity of APX to catalyze the oxidation of ascorbic acid to dehydroascorbic acid. Hence, it was possible to correlate the decreased oxygen−reduction response to increasing quantities of bound APX. This particular design is also a guarantee of signal specificity because only the peroxidase specifically captured to the electrode can trigger the reaction. In other

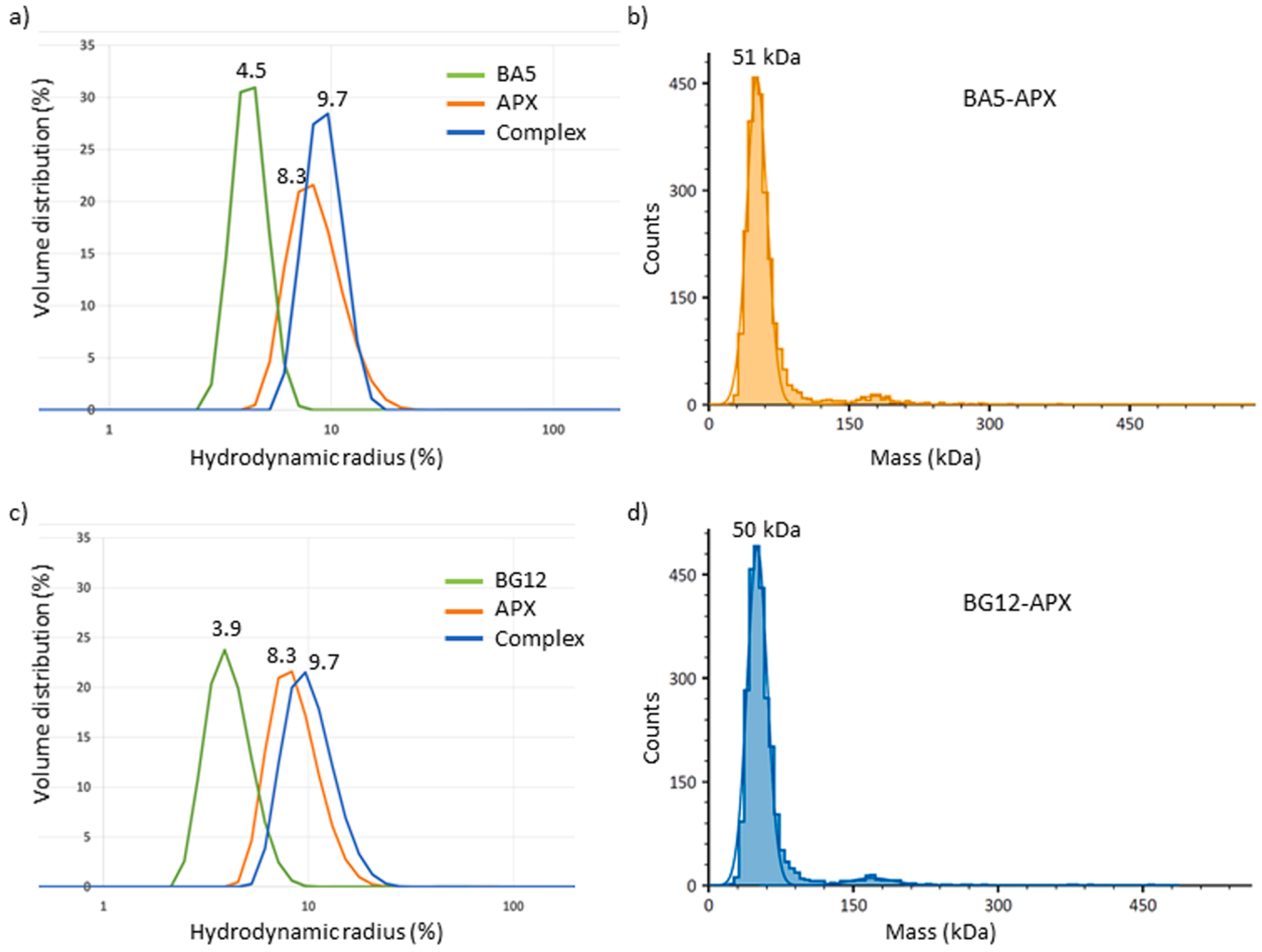

Fig. 4. Characterization of the complex between APX and the corresponding nanobodies - II
a) Dynamic light scattering (DLS) results relative to BA5, APX and their complex; b) Mass photometry profile of the BA5-APX complex; c) DLS results relative to BG12, APX and their complex; d) Mass photometry profile of the BG12-APX complex.

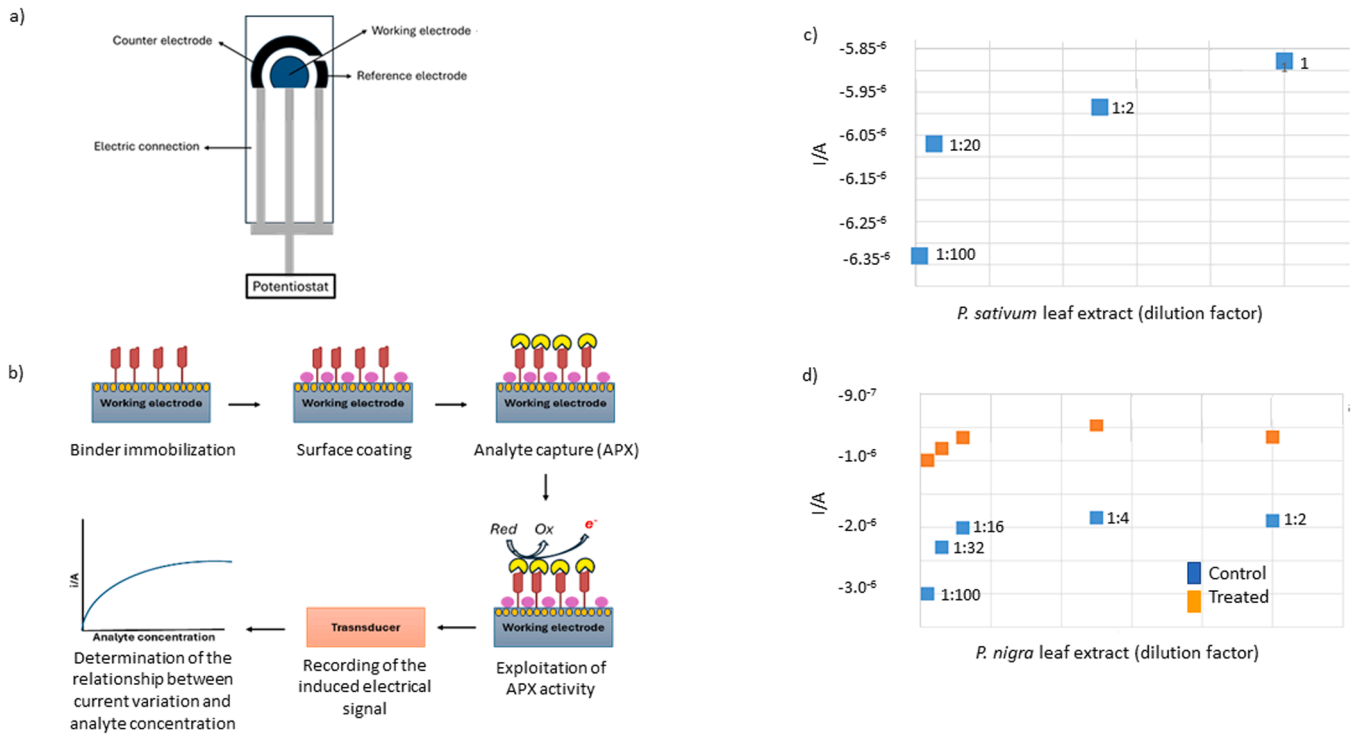

Fig. 5. Evaluation of APX activity using an electrochemical biosensor
a)Schematic organization of the screen-printed carbon electrode; b) Functionalization of a gold nanoparticle-decorated electrode with capture nanobodies that can intercept and block APX from the samples. The peroxidase activity of the enzyme is exploited to modify the current and such variations are recorded and translated into numeric values useful for quantification and comparison; c) Application of the biosensor to measure APX activity in pea leaf extracts at different dilutions; d) Application of the biosensor to measure APX activity in leaf extracts of control and heavy-metal stressed poplar trees. The signal intensity was dependent on the extract dilutions.

words, if the biosensor would interact not specifically with several antigens, the intensity of the electrochemical signal of an APX containing sample would be probably close to the background noise. In a preliminary test performed to assess the biosensor function, different concentrations of recombinant APX were used to verify the capacity of the nanobody-based biosensor to bind to the antigen and to calculate its detection limit (1.37 μM), confirming the reliability of the biosensor for APX quantification. Nanobodies are very stable and preserve their functionality for several weeks, both in solution and when conjugated to different materials (Ambrosetti et al., 2017; Ambrosetti et al., 2021), a condition that makes them ideal capture elements inside biosensors. Next, we moved to the analysis of real plant samples. The biosensor detected APX in pea leaf extract and the signal intensity varied according to the sample dilution (Fig. 5c). This result confirmed as well the capacity of BA5 to recognize the native pea APX and not only its recombinant form. Next, the same experiment was performed using poplar leaf extract and also in this case the binding capacity was confirmed, indicating that BA5 recognizes an APX epitope conserved in both pea and poplar (Fig. 5d). Furthermore, it was possible to show that the biosensor clearly discriminated between the APX content in control poplar plants and APX content in leaves of plants that underwent metal ion stress (Fig. 5d). The signal intensity variation is remarkable and this despite the phenotypes of the two plant groups did not show apparent difference at the moment of sampling (Suppl. Fig. 5).

4. Conclusion

Forest trees, because of their long-life cycle, are among the species that will suffer most for climate change. Many stress factors can occur, even simultaneously, and it would be useful to quantify their effect on the plant physiology by extensive monitoring of biomarker activities. This possibility is restricted at the present by the poor information relative to suitable biomarkers and the shortage of both available reagents and suitable diagnostic tools for on-site analyses. The results reported in this work, despite being only a proof-of-principle, show that is feasible to obtain reliable diagnostic reagents, effectively and in a short time. We demonstrated that it is possible to exploit pre-immune libraries to quickly isolate nanobodies that cross-react with APX from different species (in our case, pea and poplar) and use them to prepare a biosensor that allows inexpensive POC analysis using directly leaf extracts. The recovery of new binders for further antigens using the same panning approach is conceivable and the production of recombinant nanobodies in bacteria keeps low the price of reagent production. Of course, the prototype needs further tests to evaluate its technical reproducibility at different conditions, to optimize the on-site leaf sample preparation as well as to identify biologically relevant activity thresholds and correlate them to specific metabolic conditions. Also alternative electrochemical detection methods, based for instance on impedance, should be considered and tested using the now available reagents, since they resulted very effective to monitor other environmental factors (Oloketuyi et al., 2020). Nevertheless, the feasibility of the approach has been demonstrated and including other relevant biomarkers would allow obtaining even more complete information. We expect that the opportunity to collect more data will facilitate the evaluation of the forest tree physiology and will allow envisaging more effective mitigation policies aimed at plant protection against biotic and abiotic stress conditions.

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CRediT authorship contribution statement

Claudia D'Ercole: Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation. **Rossella Svigelj:** Writing – review & editing, Validation, Resources, Methodology, Investigation, Data curation. **Tanja Mrak:** Writing – review & editing, Resources, Methodology, Investigation. **Ario de Marco:** Writing – review & editing, Writing – original draft, Supervision, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare to have no conflict of interests

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.stress.2025.100844.

Data availability

Data will be made available on request.

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