Selection, Characterization, and CDR Shuffling of Naive Llama Single-Domain Antibodies Selected against Auxin and Their Cross-Reactivity with Auxinic Herbicides from Four Chemical Families

Claudia Sheedy,[†,#] Kerrm Y. F. Yau,[†] Tomoko Hirama,[#]
C. Roger MacKenzie,[#] and J. Christopher Hall*,[†]

Department of Environmental Biology, University of Guelph, Guelph, Ontario, Canada N1G 2W1, and
Institute for Biological Sciences, National Research Council of Canada,
Ottawa, Ontario, Canada K1A 0R6

Indoleacetic acid (IAA)-binding single-domain antibodies (sdAbs) were isolated from a naive phage-display library constructed from the heavy chain antibody repertoire of a llama. The highest-affinity sdAb isolated (CSF2A) had a K_D of 5−20 μM for two IAA−protein conjugates and a K_D of 20 μM for free IAA. This sdAb also bound to a synthetic auxin analogue, 1-naphthaleneacetic acid (NAA), and to six auxinic herbicides (K_D values of 0.5−2 mM), but not to serotonin and tryptophan, which are structurally similar to IAA but have no auxinic activity. To understand how sdAb CSF2A binds IAA and to determine which complementary-determining region(s) (CDR) participate(s) most in binding IAA, CSF2A was shuffled with four other sdAb clones by staggered extension process (StEP). After panning against IAA, two shuffled sdAbs were found: sdAb CSB1A, which originated from three different parental clones, and sdAb CSE8A, derived from two parental clones. These shuffled sdAbs and CSF2A were each fused to the B subunit of the *Escherichia coli* verotoxin, resulting in the formation of the pentamerized sdAbs V2NCSB1A, V2NCSE8A, and V2NCSF2A, which were analyzed by surface plasmon resonance (SPR) along with the sdAbs previously isolated. The shuffled clones had affinity for IAA (20 μM) similar to that of the highest affinity parental clone CSF2A, but much lower affinity for the auxinic herbicides. CDR2 was instrumental in binding IAA, whereas hydrophobic CDR3 was important for binding the auxinic herbicides. A novel SPR methodology is also described for specific immobilization of pentamerized sdAbs, allowing determination of K_D values of Ab interaction with underivatized, low molecular weight haptens.

KEYWORDS: Hapten; indole-3-acetic acid; llama single-domain antibodies; pentabody; surface plasmon resonance

INTRODUCTION

Indole-3-acetic acid (IAA), also called auxin, is one of the most important hormones in the plant kingdom. Several processes such as cell elongation and division, vascular tissue differentiation, root initiation, flowering, apical dominance, and tropic responses are all influenced by auxin concentration in plant tissues (*1*). Exogenous applications of high concentrations of IAA result in growth abnormalities leading to phytotoxicity (*1*). On the basis of the chemical structure of IAA, several synthetic auxins or "auxinic" herbicides were developed (*2, 3*). The auxinic herbicides induce symptoms typical of high IAA concentrations, a phenomenon referred to as "auxin overdose".

These synthetic auxins remain among the most successful herbicides ever used in agriculture despite being discovered and developed over half a century ago (*4*).

Several proteins have been found to bind IAA, including bovine serum albumin (BSA), transport inhibitor response 1 (TIR1) proteins, and auxin-binding proteins (ABPs). TIR1 and ABPs are the two major IAA-binding proteins found in planta. TIR1 has been isolated and physiologically and biochemically characterized in detail (*5, 6*). TIR1 is part of a ubiquitin protein ligase complex, and upon binding IAA, Aux/IAA proteins are tagged with ubiquitin and thus marked for degradation. Aux/IAA proteins bind auxin response factor (ARF) proteins, a family of transcription factors that bind DNA directly and either repress or activate transcription. Thus, the auxin-mediated degradation of Aux/IAA proteins affects the reprogramming of transcription within cells and hence developmental changes for the plant. The physiological and biochemical role of the

* Corresponding author [telephone (519) 824-4120, ext. 52740; fax (519) 837-0442; e-mail jchall@uoguelph.ca].
† University of Guelph.
# National Research Council of Canada.

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interaction of ABPs with IAA is considerably less clear than that of TIR1. Most of the ABPs have been isolated by affinity chromatography on 1-naphthaleneacetic acid (1-NAA)-based or 2,4-D-based affinity columns rather than IAA-based columns. Therefore, much of the literature discussing ABPs is centered on NAA rather than IAA binding. Furthermore, there is no clear evidence yet that the auxinic herbicides unequivocally bind to ABP.

ABP-specific antibodies (Abs) (*7−15*) and anti-idiotypic Abs that bind to the ABP-specific Abs (*16*) have been used as tools to understand the mode of action of auxin. Furthermore, we have shown that expression in tobacco of a single-chain variable fragment (scFv) against the auxinic herbicide picloram can be used to immunomodulate the negative affects of this herbicide in planta (*17*). Studies have also been conducted with anti-auxin Abs, but mostly with polyclonal Abs raised for the purpose of quantitating IAA in plant tissues (*18−23*). We hypothesize that antibodies that bind to IAA may be used as surrogate ABPs to study the structure−activity relationships of IAA and the auxinic herbicides binding to their putative receptor. A similar hypothesis was previously tested and corroborated in our laboratory using a monoclonal antibody (mAb) with broad cross-reactivity against the acetyl-coenzyme A carboxylase (ACCase)-inhibiting herbicides from the cyclohexanedione and aryloxyphenoxy propionate families. This mAb was successfully used as a surrogate of the ACCase target site to identify new chemistries with ACCase activity (*24−27*).

Several polyclonal sera and monoclonal Abs against IAA have been obtained by immunizing animals. This is particularly difficult because it is believed that animals may innately develop Abs against IAA through ingestion of plant material. Furthermore, BSA is known to harbor a highly specific binding site for IAA (*28*), and so may the albumins of other animal species, thus potentially reducing the availability of the IAA-based immunogen and, concomitantly, its immunogenicity in animal. Furthermore, these albumins could contaminate polyclonal sera containing Abs against IAA, thus interfering with IAA-specific Ab binding. Therefore, naive scFv and sdAb libraries displayed on phage, yeast, or ribosome may represent a more efficient means of isolating IAA-specific Abs with high affinity, because no animal immunization is required.

Llama sdAbs, also known as variable heavy domain of heavy-chain antibodies (V_HHs), have some advantageous characteristics over scFvs, the smallest stable antigen-binding fragment of a conventional IgG. scFvs consist of the heavy (V_H) and light (V_L) chain variable fragments joined by a flexible polypeptide linker. The linker stabilizes the V_H and V_L fragments in the correct configuration, but poses some limitations such as susceptibility to proteolytic cleavage, tendency to form aggregates, and difficulty in expression and purification (*29*). V_H-Hs, however, provide an alternative to circumvent these problems. V_HHs are expressed well in bacteria (*30*) and yeast (*31*), they do not tend to aggregate, and they are highly stable (*32*). Cortez-Retamozo et al. (*32*) have shown that sdAbs are highly tissue permeable in animal cells as well. V_HHs have the capacity to retain their binding specificity at temperatures as high as 90 °C (*33*) and also following 1 week of incubation at 37 °C (*30*). In addition, they can maintain their antigen-binding capacity under physiological conditions at 37 °C in plasma (*34*), demonstrating resistance to serum proteases (*35*). All of these characteristics make sdAbs useful for both assay development and expression in hosts such as animals and plants for the purpose of immunomodulation, tissue penetration, etc. Furthermore, sdAbs that bind haptens can be isolated from both naive

and immune libraries: For example, a sdAb binding to the azo-dye RR6 (mol mass 728 Da) was isolated from an Ab library constructed from an immunized camel (*31, 36*). Furthermore, sdAbs binding to the auxinic herbicide picloram (mol mass 230 Da) were isolated in our laboratories from a naive ribosome-display library (*37*).

Through gene mutagenesis, IAA-specific sdAbs with different cross-reactivity properties, higher expression levels, and higher affinity may be obtained, thereby allowing for a more complete characterization of IAA-binding requirements. Ab mutagenesis has been performed to determine hapten-contacting residues (*38, 39*) and to probe the importance of second-sphere residues in binding haptens (*40*). Mutagenesis of hapten-specific sdAb clones can be accomplished by several means: random (*41, 42*) or site-directed (*43−45*) mutagenesis, scanning saturation mutagenesis (*46*), and codon-based mutagenesis (*47*) as well as bacterial surface display (*48*) and chain (*49*) and DNA shuffling (*50*).

Traditionally, mutagenesis was performed by error-prone Polymerase Chain Reaction (PCR), where several rounds of PCR with a low-fidelity Taq polymerase introduce mutations over the whole length of the gene (*51, 52*). Such random mutagenesis, however, will result in the majority of clones being either nonfunctional or having lower affinity than the parental clone, therefore requiring an extensive screening process to isolate the improved binders. Another strategy involves DNA shuffling, where the genetic material is fragmented by DNase I and randomly reassembled by PCR (*50, 53*). Although DNase I digestion requires several rounds of PCR for the reassembly of full-length genes to occur, the percentage of functional molecules compared with random mutagenesis is large (*54*). DNA shuffling of three llama sdAb genes specific for the hapten azo-dye RR6 was performed successfully to improve the production, affinity, and stability of sdAbs (*54*).

DNA shuffling by staggered extension process (StEP) was developed by Zhao et al. in 1998. StEP consists of the priming of template sequences followed by several cycles of denaturation and shortened annealing/polymerase-catalyzed extension (*55*). During each cycle, the DNA fragments can anneal to different templates on the basis of sequence complementarity and extend further to create recombinant genes. Because of the template switching, the growing polynucleotide chains can contain information from one or more parental clones (*55*). The whole process can be performed with a single PCR reaction, and the majority of the mutants are functional.

The objectives of this research were to (i) isolate and characterize sdAbs against IAA and (ii) determine whether these Abs can cross-react with several auxinic herbicides. These objectives are a first step in the process of creating reagents for immunomodulation of IAA activity in planta and to create potential surrogates of ABP(s). With this in mind, sdAbs binding IAA were isolated from a phage-displayed, naive llama sdAb library by biopanning against IAA conjugated to a carrier protein, BSA. sdAb affinities for two different IAA conjugates were determined by SPR. We also describe a novel SPR methodology that allows for the specific immobilization of pentamerized sdAbs and the binding of free hapten to the immobilized sdAbs. This methodology was used to determine dissociation constants for the interaction of two pentamerized sdAbs with free IAA, as well as with an analogue of auxin, 1-naphthaleneacetic acid (1-NAA), six synthetic auxins (the herbicides clopyralid, dicamba, 2,4-dichlorophenoxyacetic acid, MCPA, mecoprop, and quinclorac), and two structurally related molecules that have no auxin activity, namely, serotonin (a
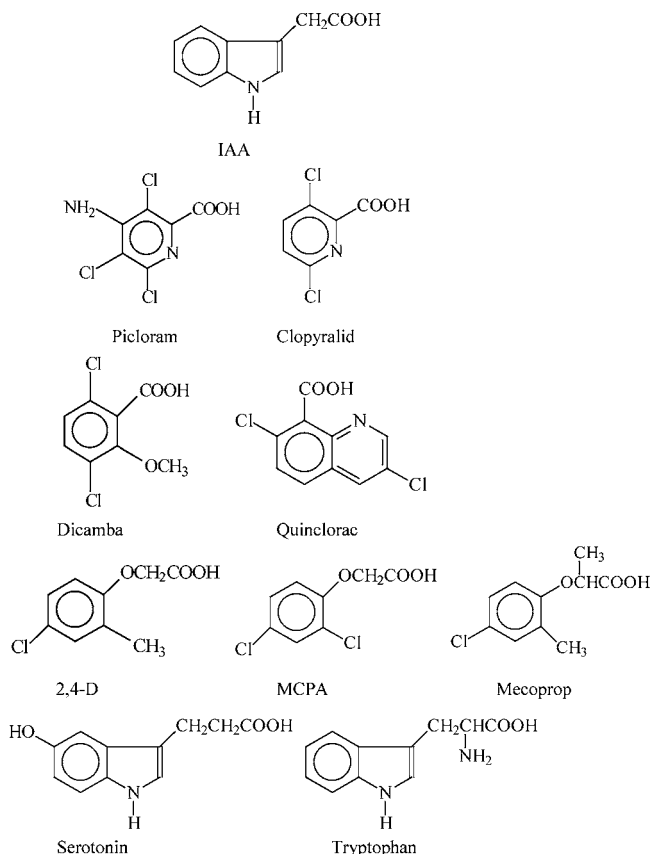
Figure 1. Chemical structures of the plant hormone indole-3-acetic acid (IAA), the auxinic herbicides clopyralid and picloram (pyridine family), dicamba (benzoic acid family), quinclorac (quinolinecarboxylic acid family), and 2,4-dichlorophenoxyacetic acid, MCPA, and mecoprop (phenoxyacetic acid family), the neurotransmitter serotonin, and the amino acid L-tryptophan.

neurotransmitter) and L-tryptophan, an aromatic amino acid believed to be a precursor of auxin in plants (**Figure 1**) (*56, 57*). The SPR analysis of hapten binding by sdAbs and pentamerized versions thereof is compared. Finally, to understand how the highest affinity sdAb, CSF2A, binds IAA and to determine which complementary-determining region(s) (CDR) participate(s) most in binding IAA, the CDRs of CSF2A were shuffled with those of four other IAA-binding sdAb clones using StEP. A comparison of the affinities of parental clones versus that of shuffled and selected mutants is presented.

MATERIALS AND METHODS

Auxin Conjugate Synthesis. IAA (Sigma, St. Louis, MO) was conjugated to BSA by Mannich condensation (IAA-N–BSA), in which active hydrogen is condensed with formaldehyde and amine groups on a carrier protein (*58*). Briefly, 200 μL of a 10 mg of BSA/mL 0.1 M morpholinoethanesulfonic acid, 0.15 M NaCl, pH 4.7, solution was added to 200 μL of 10 mg of IAA/mL of absolute ethanol. Fifty microliters of 37% formaldehyde was added, and the reaction mixture was incubated at 37 °C for 12 h.

IAA was also conjugated to BSA according to a protocol by Fleeker (*59*), whereby IAA is conjugated through its COOH group to amine groups on BSA (IAA-C–BSA). Briefly, 0.19 mmol of *N*-hydrosuccinimide and *N,N'*-dicyclohexylcarbodiimide were dissolved in dioxane (separately). IAA (33 mg) was added to the *N*-hydrosuccinimide solution, after which 0.5 mL of the dicyclohexylcarbodiimide solution was added. The solution was mixed and allowed to stand for 12 h at 25 °C. The conjugate was filtered through glass wool, and solvent was removed by evaporation in a water bath at 35 °C under a stream of nitrogen gas for 1 h. BSA (500 mg) in 3 mL of 0.1 M boric acid, pH

9.0, was added dropwise to the residue with constant stirring. The mixture was incubated for 2 h at 25 °C with constant stirring.

Similar reactions were conducted using a mixture of IAA and 3-[5(n)-^{3}H]indolylacetic acid (specific activity = 999 GBq/mmol; Sigma-Aldrich, Oakville, ON) for both conjugates. The auxin conjugates were dialyzed against phosphate-buffered saline (10 mM H$_3$PO$_4$, 150 mM NaCl, pH 7.4; PBS) after filtration through glass wool. The amount of IAA conjugated to BSA was measured using liquid scintillation spectroscopy and a protein assay. The protein conjugates 2,4-D–OVA and picloram–OVA (PIC–OVA) were obtained from Dr. J. Christopher Hall's laboratory. 2,4-D–OVA was prepared according to Fleeker's method, as described by Clegg et al. (*60*), and picloram–OVA was prepared according to the mixed anhydride method, as described by Yau et al. (*37*).

Isolation of sdAbs. sdAbs binding to IAA conjugated to BSA were isolated from a sdAb library constructed from the heavy-chain Ab repertoire of a nonimmunized llama as described by Tanha et al. (*61*). Briefly, microtiter-plate (Nunc, Naperville, IL) wells were coated with 150 μL of 100 μg of IAA-N–BSA or BSA (for subtractive panning, in which the phage library is preincubated with the carrier protein prior to being added to the carrier protein–hapten conjugate) per milliliter of PBS and allowed to bind overnight at 4 °C. Wells were each washed three times with PBS prior to blocking with 350 μL of 2% milk–PBS (MPBS) for 2 h at 37 °C. Wells containing the carrier protein were rinsed three times with PBS containing 0.02% Tween 20 (PBST) and three times with PBS. Phages (10^{12} transducing units) were added in a total volume of 150 μL of 2% MPBS per well. Phages were incubated for 30 min at 25 °C. IAA–BSA-containing wells were rinsed three times each with PBST and PBS. Phage supernatant from each BSA-coated well (150 μL) was transferred to a corresponding IAA–BSA-coated well, and phages were allowed to bind for 1.5 h at 25 °C. Supernatants were discarded and wells rinsed 10 times with 350 μL of PBST, followed by 10 times with 350 μL of PBS. Bound phages were eluted by adding 200 μL of 100 mM triethylamine (TEA) (35 μL of 7.18 M TEA in 2.5 mL of water) followed by 10 min of incubation at 25 °C. Eluted phages were neutralized by adding 100 μL of 1 M Tris-HCl, pH 7.4. For further rounds of panning (two rounds, same conditions as above), 10 mL of *Escherichia coli* TG1 in log phase was infected with 150 μL of eluted phages for 30 min at 37 °C. Phages were purified as described by Tanha et al. (*61*).

Phage ELISA. Microtiter wells were each coated with 150 μL of 5 μg of IAA-N–BSA/mL PBS overnight at 4 °C. Wells were washed five times each with PBST and PBS and blocked for 2 h with 300 μL of 2% MPBS. Phage-infected *E. coli* TG1 grown in 2× YT was centrifuged for 10 min at 1000g and the supernatant transferred to BSA- or IAA–BSA-coated wells and incubated for 2 h at 37 °C. Wells were washed five times each with PBST and PBS, and 150 μL of mouse anti-M13 Ab (Amersham Biosciences, Piscataway, NJ) diluted 5000 times in PBS was added and incubated for 1 h at 25 °C. Wells were washed five times each with PBST and PBS, and 150 μL of goat anti-mouse Ab conjugated to horseradish peroxidase diluted 5000 times in PBS was added; the mixture was incubated for 30 min at 25 °C. Wells were washed five times each with PBST and PBS, and 150 μL of HRP substrate (KPL, Gaithersburg, MD) consisting of 1:1 TMB peroxidase/H$_2$O$_2$ was added per well. The reaction was stopped by adding 150 μL of 1 M H$_3$PO$_4$ per well, and the absorbance (405 nm) was measured.

Expression, Extraction, and Purification of sdAbs. IAA-binding clones isolated from the naive library were transferred to the expression vector pSJF2, which added C-terminal c-myc and His$_5$ tags (*62*). Following transformation into *E. coli* strain TG1, clones expressing sdAbs were grown in 1 L of Luria broth (LB) containing ampicillin for 24 h at 37 °C, prior to induction with 1 mM isopropyl-β-D-thiogalactopyranoside followed by a 12 h incubation at 37 °C. Expression was confirmed by SDS-PAGE and Western blotting. sdAbs were extracted from bacterial periplasm according to the protocol of Anand et al. (*62*) and purified by immobilized metal affinity chromatography (IMAC) as described elsewhere (*63*). Briefly, sdAbs were purified by IMAC using a Hi-Trap column (Amersham Biosciences), and the purified fraction was dialyzed against SPR analysis buffer (10 mM Hepes, 150 mM NaCl, 3 mM EDTA; HBS-E) prior to concentration using a Centricon 10 ultrafiltration device (Millipore, Bedford,

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MA) and size exclusion chromatography on Superdex 75 (Amersham Biosciences) to remove aggregates and impurities.

Pentamerization of sdAbs. sdAb DNA was cloned into the expression vector pVT2 as described elsewhere (*35*) using standard cloning techniques (*64*). Briefly, sdAb DNA was amplified (for ligation into pentamerization/expression vector pVT2) using primers VT-ApaR (5′ATTATTATGGGCCCTGAGGAGACGGTGACCTGGGTC) and VT-BbsF (5′TAATAAGAAGACACCAGGCCGATGTGCAGCTG-CAGGCGTCTG) prior to digestion by restriction enzymes *Apa*I and *Bbs*I. Gel-purified digestion product was ligated in the pVT2 vector and thereby fused to the verotoxin B subunit. The ligation product was used to transform *E. coli* TG1.

Expression, Extraction, and Purification of Pentamerized sdAbs. Clones expressing pentabodies were grown according to the protocol described above for sdAbs. Pentabodies were extracted by whole cell lysis. Briefly, cells were pelleted by centrifugation (20 min, 6000*g*, 4 °C) and resuspended in 25 mL of ice-cold lysis buffer (10 mM Hepes, 500 mM NaCl, 20 mM imidazole, pH 7.4). Cells were lysed, and cell lysate was centrifuged for 1 h at 6000*g*. Supernatants were filtered using a 0.22 μM filter (Millipore). Protein extracts were centrifuged for 60 min at 6000*g*. Pentabodies were purified by IMAC as described above, and aggregated pentabodies were removed by size exclusion chromatography on Superdex 200 (Amersham Biosciences).

StEP. DNA preparations (20 ng) of the sdAbs with affinity for IAA were mixed together and incorporated in the following PCR reaction (100 μL total volume) as described in Zhao et al. (*55*): 30 pmol of forward primer VH *Bam*H1 (5′TATGGATCCTGAGGAGACGGT-GACCTG) and reverse primer VH *Bbs*1 (5′TATGAAGACACCAG-GCCGATGTGCAGC), 1× Taq buffer, 0.25 mM dNTPs (Roche), 1.5 mM MgCl$_2$, water, and 1.0 unit of Taq polymerase (Sigma). The following program was used: 5 min of 95 °C, followed by 80 cycles of 30 s, 94 °C, and 5 s, 55 °C. StEP PCR was performed with a Perkin-Elmer thermocycler model GeneAmp 9700. A prominent band at ∼450 bp was obtained and gel-purified using a QIAquick purification kit (QIAgen, Mississauga, ON).

StEP Phage-Display Library Construction. StEP product was further amplified by PCR using the primers llamaApal1 and llamaNot1, which add the corresponding restrictions sites to the DNA, thereby allowing cloning into phage vector fd-tetGIIID (*65*). Following gel purification, StEP product was digested with restriction enzymes *Not*1 and *Apa*1 and purified. StEP library was ligated into phage vector fd-tetGIIID using standard cloning techniques (*64*) and transformed in *E. coli* TG1.

sdAbs Isolation from StEP Library. SdAbs binding to IAA conjugated to a carrier protein (either BSA or OVA) were isolated from the shuffled library as described above for the naive library but with a few modifications. Specifically, panning was performed in parallel on IAA conjugated to carrier protein with two different chemistries, IAA-N−BSA and IAA-C−OVA, with preincubation of the library with carrier protein in each instance. Bound phages were eluted by adding 200 μL of 100 mM glycine, pH 2.2. Phage was purified as described previously (*61*). Three rounds of panning were performed on both IAA conjugates, with increasing stringency: the second and third rounds were performed with 10 and 1 μg of IAA conjugate/mL of PBS, using 20 and 30 washes, respectively.

SPR Analysis of sdAbs. A Biacore 3000 instrument (Biacore Inc., Piscataway, NJ) was used for all SPR analysis, and collected data were analyzed using BIAevaluation 3.0 software (Biacore Inc). Approximately 7500 resonance units (RUs) of IAA-N−BSA or IAA-C−BSA conjugates and 10000 RUs of control protein (BSA) were immobilized on CM4 sensor chips (Biacore Inc.) by amine coupling, according to the manufacturer's instructions. SdAbs were passed over the sensor chip surface in HBS-E buffer containing 0.005% P-20 (HBS-EP). All analyses were performed at 25°C in HBS-EP buffer at a flow rate of 40 μL/min. IAA was injected at concentrations ranging from 0.8 to 8 μM. Cross-reactivity experiments were performed with sdAb CSF2A, where CSF2A was injected over 2,4-D−ovalbumin (2,4-D-OVA) or PIC−OVA conjugates to determine whether IAA-isolated sdAb would bind to other auxinic herbicides conjugated to OVA. Cross-reactivity experiments were performed as described above. Approximately 7000

RUs of 2,4−D-OVA or PIC−OVA conjugate and 8000 RUs of control protein (BSA) were immobilized on sensor chips.

Inhibition experiments were performed by injecting sdAb CSF2A at K_D concentration (5 μM) mixed with 100−2000 μM of free inhibitor. Inhibition experiments were carried out on the IAA-C−BSA conjugate, and the following haptens were injected: IAA, 1-NAA, tryptophan, clopyralid, 2,4-D, mecoprop, MCPA, or dicamba. IC$_{50}$ (50% inhibition) was defined as the concentration of hapten required to decrease binding of sdAb CSF2A to IAA-C−BSA by half (115 RUs) of that without hapten present (230 RUs).

SPR Analysis of Pentamerized sdAbs. Anti-c-myc IgG (50 μg/mL 10 mM acetate, pH 4.5) was immobilized on the surface of a research grade Biacore CM5 chip by amine coupling at a surface density of 12000 RUs, according to the manufacturer's instructions. IAA-binding pentabodies V2NCSF2A and V2NCSE6E and a reference pentabody IV12 (a peptide binder) were each captured on a different flow cell, at densities of 3500, 4000, and 3290 RUs, respectively, by injecting each at 50 μg of pentabody/mL of HBS-EP buffer. The surface baseline was allowed to stabilize prior to the injection of IAA or each auxin analogue over the reference and IAA-binding pentabody surfaces. All analyses were performed at 25 °C in HBS-EP buffer at a flow rate of 10 μL/min. IAA was injected at concentrations ranging from 2 to 100 μM, and auxinic herbicides were injected at concentrations ranging from 25 to 1000 μM, with a minimum of 10 concentrations for each analogue. BIAevaluation 3.0 software (Biacore Inc) was used to analyze collected data.
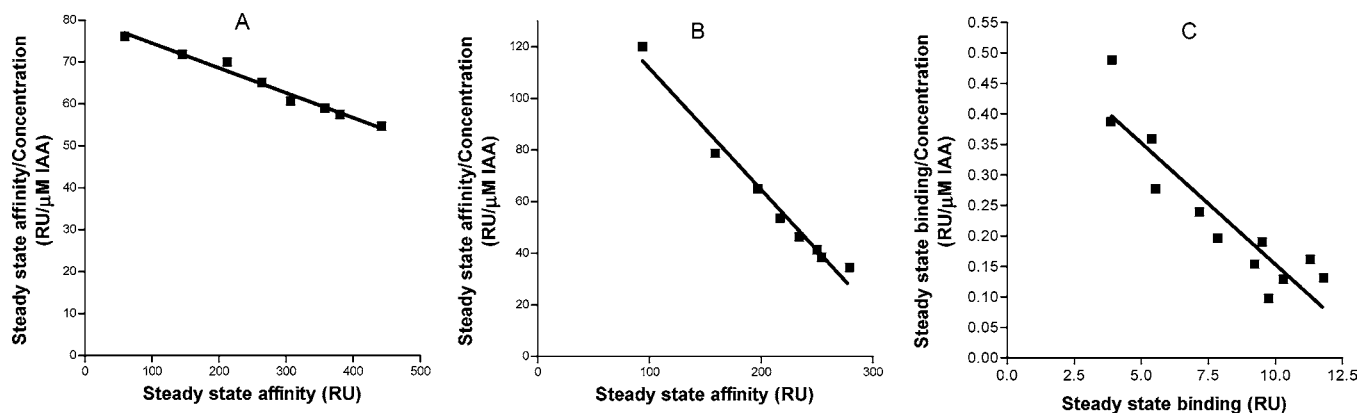
RESULTS

Screening of Naive Llama Library. *Auxin Conjugate Synthesis.* The final IAA-N−BSA concentration after dialysis was 5.5 mg of IAA-BSA/mL of PBS. Approximately five molecules of IAA were bound per molecule of BSA. IAA was also conjugated to BSA by Fleeker's reaction, whereby the IAA is conjugated through its carboxylic acid group to free amine groups on BSA (IAA-C−BSA). The final IAA-C−BSA concentration was 11 mg of IAA-C−BSA/mL of PBS, and approximately 15 molecules of IAA were bound per molecule of BSA.

Isolation of sdAbs. IAA-N−BSA was used throughout panning because the current literature indicates that the carboxylic acid group of IAA and the auxinic herbicides are required for auxinic activity. After three rounds of panning against IAA-N−BSA, phage culture supernatants were tested for binding to IAA-N−BSA and BSA (negative control) by phage ELISA. Most phage clones showed positive binding to IAA-N−BSA and little if any cross-reactivity with BSA. Absorbance values (450 nm) ranged from 0.2 to 0.8, and only clones with absorbance on IAA-N−BSA-coated wells at least 3 times greater than that of corresponding BSA-coated wells were characterized further. Sequencing of all positive clones revealed that five different sdAbs were isolated from the llama naive library (**Table 1**). These sdAbs possess relatively long CDR3s (12−19 residues), and sdAb CSF2A, the highest affinity IAA binder isolated, also possesses a high number (7) of aromatic residues, mainly tyrosine, in its CDR3. Following transformation in *E. coli* TG1, several transformants were obtained for each sdAb or pentamer clone. All transformants positive for the sdAb insert expressed the 17 kDa protein, whereas clones expressing the pentamerized sdAbs produced a 25 kDa fusion protein (sdAb-B subunit monomer). Expression levels were ∼1 mg of sdAb or pentabody/L.

SPR Analyses: SdAbs and IAA−Protein Conjugates. Analyses were performed for each sdAb isolated from the library, but K_D values could be obtained for only three of the five sdAbs. The K_D values for sdAbs CSB9D, CSE6E, and CSF2A were determined to be 20, 4o, and 5 μM, respectively, on IAA-C−

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Table 1. Sequence Alignments of Five sdAbs Isolated from the Naive Phage-Display Library after Panning against IAA-N–BSA (Hereafter Called Parental Clones)[a]

sdAb Name	FR1	CDR1	FR2	CDR2	FR3	CDR3	FR4
	•1 •10	•25	•36	•65	•75	•94	•105
CSF2A	DVQLQASGGGLVQAGGSLKLSCAAS	GRTFSSVGMG	WFRQAPGKEREFVA	AISRRGASTIYADSVKG	RFTISRDNAKNTVYLQMNSLKPGDTAVYYCAG	TSATYYSSSYYYRSNNYNY	WGQGTQVTVSS
CSB10F	....	......R...	....	V···A-SV·····	·A···············...S····	--AYTFQNVPPMNSRQYAY	....
CSD7C	............R............	...G·T···	·········G·	··R··SGNAY·····	········S···K····	----KETAFLTRNYAY	....
CSE6E	....	......R···	....	V····A-SV·····	·VL·····K·RVD···F·G·	--RDGYPGIYKRENDYRR	....
CSB9D	........T·D··S...........	·····DYA··	···EV······	···WTGSHTR·····	·······G····V···G	--RPNYTPPYNRAQSYNY	....

[a] Numbers refer to Kabat numbering system (73). Dashes represent the absence of a residue. Dots represent a residue identical to that of sdAb CSF2A. All CDRs are color-coded, whereas framework regions are shown in black.

Table 2. SPR-Measured Dissociation Constants (K_D) of sdAbs Isolated from the Llama Naive Library

sdAb clone	conjugate immobilized on chip	K_D (μM) for the respective conjugate
	IAA Conjugates	
CSB9D	C[a] or N[b]	20, nd[c]
CSB10F	C or N	>1000, >1000
CSD7C	C or N	>1000, >1000
CSE6E	C or N	40, nd
CSF2A	C or N	5, 20
	Other Conjugates	
CSF2A	2,4-D-C–OVA	15
CSF2A	PIC-C–OVA	5

[a] C = IAA-C–BSA conjugate. [b] N = IAA-N–BSA conjugate. [c] Not determined.

Table 3. Inhibition of sdAb CSF2A Binding to IAA-C–BSA Conjugate by IAA, Synthetic Auxins, Serotonin, and Tryptophan

inhibitor	inhibitor concn to reach 50% inhibition (μM)
auxin	
indole-3-acetic acid	800
synthetic auxins	
clopyralid	>>>[a]
2,4-D	500
mecoprop	200
MCPA	500
1-naphthaleneacetic acid	650
dicamba	1800
others	
tryptophan and serotonin	NIO[b]

[a] Fifty percent inhibition could not be reached. [b] No inhibition observed.

BSA. The K_D for IAA-N–BSA was 20 μM for CSF2A, whereas values for CSB9D and CSE6E could not be determined (**Table 2**). Scatchard plots are shown in **Figure 2**, panels **A** (IAA-N–BSA) and **B** (IAA-C–BSA). In cross-reactivity experiments, sdAb CSF2A bound both 2,4-D and picloram, two auxinic herbicides, with K_D values of 15 μM (2,4-D-OVA) and 5 μM (PIC–OVA; **Table 2**).

Inhibition Experiments with Free Haptens and sdAbs. Inhibition experiments were performed solely with IAA conjugate IAA-C–BSA and sdAb CSF2A, because this combination of reagents yielded the highest binding affinity. Inhibition experiments consisted of injecting sdAbs (at K_D concentrations) premixed with increasing concentrations of free hapten. IC_{50} values were obtained for IAA and all auxinic herbicides except clopyralid. Although clopyralid did inhibit binding of CSF2A to IAA-C–BSA, the IC_{50} could not be reached even at a concentration of 2 mM. No inhibition was observed for tryptophan and serotonin, even at the highest concentrations injected. Inhibition results are summarized in **Table 3**.

Pentabodies and Free Haptens. The Scatchard plot for V2NCSF2A binding to IAA is shown in **Figure 2C**. In terms of IAA binding, the K_D of the pentabody V2NCSF2A was determined to be 20 μM and that of pentabody V2NCSE6E to be 140 μM. 1-NAA, an auxin analogue, and six auxinic herbicides were each injected at concentrations ranging from 25 to 1000 μM. 1-NAA and all of the auxinic herbicides were bound by V2NCSF2A. Results are shown in **Table 4**. Identical experiments were performed with serotonin and tryptophan, but no binding was observed.

CDR Shuffling of sdAbs. *StEP PCR and Library Construction.* The StEP library was sampled to evaluate its diversity prior to panning. Thirty phage sdAbs were sampled, and their

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Figure 2. Scatchard plots of data for equilibrium binding of sdAb CSF2A to IAA–N–BSA (**A**), CSF2A to IAA–C–BSA (**B**), and IAA to pentabody V2NCSF2A (**C**).

Table 4. SPR-Determined K_D Values of Pentabody V2NCSF2A Interaction with Soluble IAA, 1-NAA, Six Auxinic Herbicides, Serotonin, and Tryptophan[a]

hapten	mol mass (Da)	K_D (μM)
auxin		
indole-3-acetic acid	175	20
synthetic auxins		
clopyralid	192	500
2,4-D	221	1×10^3
mecoprop	214	1×10^3
quinclorac	242	1×10^3
MCPA	200	2×10^3
1-naphthaleneacetic acid	186	1×10^3
dicamba	221	2×10^3
others		
serotonin	212	no binding[b]
tryptophan	204	no binding[b]

[a] The c-myc-tagged pentabody was bound to the chip via immobilized anti-c-myc IgG. [b] No binding detected at hapten concentrations ranging from 100 to 2000 μM.

sequences revealed that the StEP PCR resulted in a diverse library where all parental clones are represented at various CDR positions. CDRs from clones CSF2A and CSD7C, however, were present at lower percentages than those from the three other clones. The shuffled, unpanned, sdAb library had 46, 35, and 10% of the clones derived from CDRs from one, two, and three parents, respectively, whereas 10% of the clones had point mutations in at least one of the CDRs (**Figure 3**).

SdAb Isolation. After three rounds of panning the StEP library against IAA conjugated to either BSA or OVA, each phage eluate was sampled to monitor differential selection on both auxin conjugates. Sequence diversity is shown in **Figure 3**. Sequencing revealed that panning on IAA-N−BSA favored the isolation of sdAbs shuffled from two parental clones (58%), with a fair percentage of original parents (18%), triple shuffled (21%), and a few clones with point mutations that occurred during the StEP PCR (5%). Panning on IAA-C−OVA also yielded two parental clones (75%) and triple mutants (25%), but did not select for parental clones or point mutations.

Phage culture supernatants (47 from each panning) were tested for binding to the corresponding IAA conjugates and carrier protein alone (negative control) by phage ELISA. Most phages showed positive binding to IAA conjugates and little if any cross-reactivity to BSA. Sequencing of the clones showing the highest absorbance revealed that several different sdAbs were isolated from the StEP library, in both pannings. Two sdAb clones (**Table 5**) showed high absorbance values: CSB1A (from IAA-C−BSA panning) and CSE8A (from panning on IAA-N−
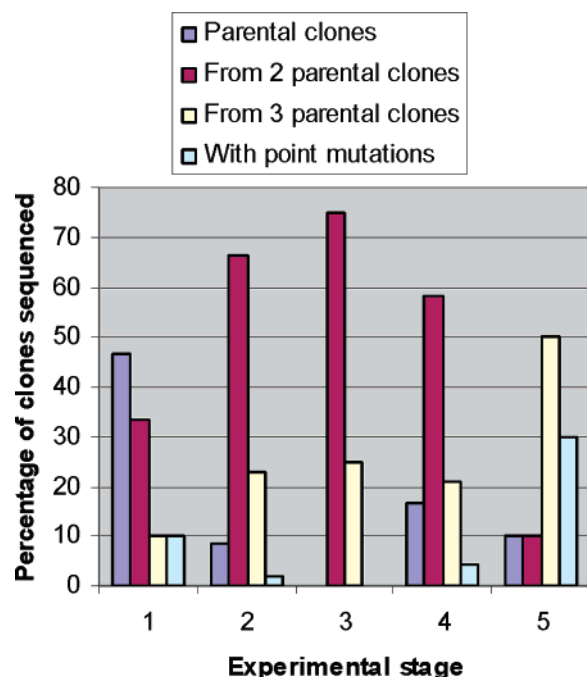
Figure 3. Clonal lineage throughout post-StEP selection process showing the percentage of clones at every experimental stage that either have the same sequence as one of the parental clones shown in **Table 1**, have CDRs from two or three different parental clones, or contain point mutation(s). Experimental stages (numbers in parentheses refer to the total number of clones sequenced at each experimental stage): 1, shuffled library before panning (30); 2, shuffled library after panning with IAA-C−OVA plus IAA-N–BSA (48); 3, shuffled library after panning with only IAA-C–OVA (24); 4, shuffled library after panning with only IAA-N–BSA (24); 5, shuffled library after phage ELISA with IAA-C–OVA plus IAA-N–BSA (20).

BSA). CSB1A is a triple mutant derived from clones CSE6E (CDR1), CSB9D (CDR2), and CSB10F (CDR3). CSE8A is a double mutant arising from clones CSF2A (CDRs 1 and 2) and CSB10F (CDR3). All transformants positive for the pentabody insert expressed the 25 kDa fusion protein (sdAb-B subunit monomer). Expression levels were ∼1 mg pentabody/L or less.

SPR. The K_D values of pentabodies V2NCSB1A and V2NCSE8A for free IAA are both 20 μM. 1-NAA and all of the auxinic herbicides were bound by V2NCSB1A and V2NCSE8A, but K_D values were large (low to high millimolar range). Identical experiments were performed with the neurotransmitter serotonin and the amino acid tryptophan, but no binding was observed. Steady-state affinity and Scatchard plots

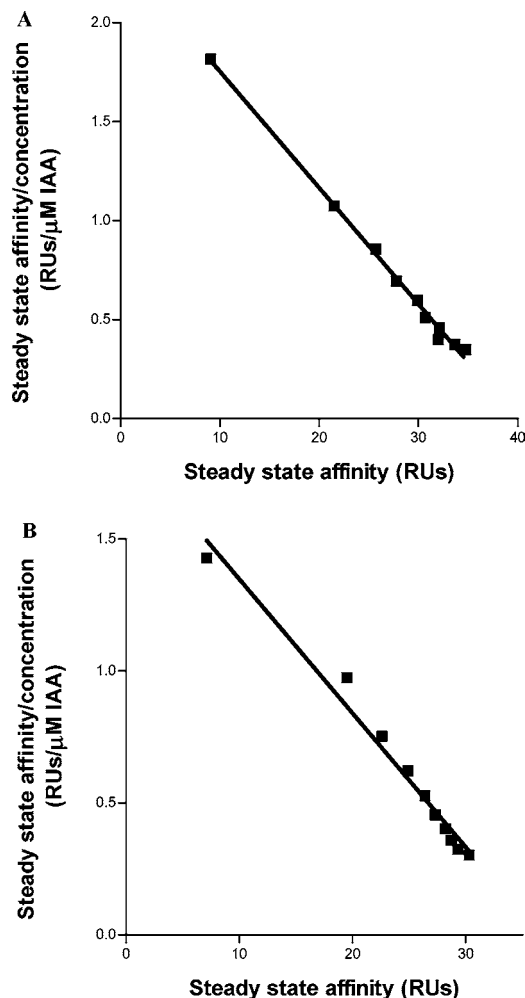**Table 5.** Sequence Alignment of sdAbs CSB1A and CSE8A Isolated from the StEP Library[a]

SdAb	←Framework Region (FR) 1→ ↓ CDR1 → ↓ FR2 ↓ ↑ → ↑ ↓ CDR2 → ↓ FR3 → ↓ ↑ ↓ CDR3 → ↓ FR4 →
	•1 •10 •25 •36 •65 •75 •94 •105
CSB1A	EVQLQASGSGGGLVQAGGSLKLSCAAS GRTFSSRAMG WFRQAPGKEREFVA AISWTGSHTRYADSVKG RFTVSRDNAKNTVYLQMNSLKPEDTAVYSCAA --AYTFQNVPPMNSRQYAY WGQGTQVTVSS
CSE8A	....VG. ....RR.AS.I...... .... --AYTFQNVPPMNSRQYAY ....

Origin of CDRs:

- ▪ sdAb CSB10F
- ▪ sdAb CSE6E
- ▪ sdAb CSF2A
- ▪ sdAb CSB9D

[a] Numbers refer to the Kabat numbering system (73). Dashes represent the absence of a residue. Dots represent a residue identical to that of sdAb CSB1A. All CDRs are color-coded, whereas framework regions are shown in black. Block arrow color indicates origin of CDR.

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Figure 4. Scatchard plots of data for equilibrium binding of shuffled pentabodies V2NCSB1A (**A**) and V2NCSE8A (**B**) to IAA.

based on overlaid sensorgrams were used to determine K_D values. The Scatchard plots of V2NCSB1A and V2NCSE8A binding to IAA are shown in **Figure 4**.

DISCUSSION

In the present study, a naive llama sdAb library was panned against IAA-N−BSA, in which IAA is conjugated to BSA through the indole nitrogen, to obtain sdAbs binding to the acetic acid moiety of IAA. For SPR analyses, IAA-N−BSA and IAA-C−BSA were immobilized on sensor chips, to confirm that the sdAbs were specific for IAA rather than the linkage region. This seems to be the case for sdAb CSF2A, which bound to both conjugates. Moreover, SPR experiments on auxinic herbicide conjugates 2,4-D−OVA and PIC−OVA confirmed that CSF2A binds specifically to IAA or auxinic herbicide conjugated to the protein, rather than to the protein (**Table 2**). However, the quality of the two IAA conjugate surfaces degraded rapidly. The surface density was low and the baseline unstable because the IAA conjugate gradually leached from the surface. The sdAbs also interacted nonspecifically with the dextran surface. As a result of these problems, competitive inhibition experiments with free IAA were conducted by linking the sdAbs to the chip to ensure that the sdAbs were specifically binding the free hapten.

Inhibition experiments with free haptens confirmed that CSF2A specifically bound IAA and the synthetic auxinic analogues. Inhibition experiments required large quantities of

sdAbs because the K_D of sdAb CSF2A for IAA and the synthetic auxins was high, that is, at least 5 μM, which limited the number of experiments that could be performed with a given sdAb. Pentamerization of the sdAbs, however, allowed for a much more convenient SPR analysis method, whereby the direct binding of the hapten to the immobilized sdAb pentabody could be measured. This is the first report of using SPR when an Ab is immobilized on a chip in this manner. Furthermore, this study also provides the first evidence of the direct and selective interaction of auxin and several auxinic herbicides to a single, IAA-selected, antibody-based binding site. Specifically, CSF2A possesses broad cross-reactivity to 1-NAA and all four chemical families of auxinic herbicides, that is, benzoic acids, pyridines, phenoxyalkanoic acids, and quinolinecarboxylic acids. Furthermore, our results suggest that the isolation of a sdAb against IAA rather than against its analogues 1-NAA and auxinic herbicides may be important to obtain an Ab possessing cross-reactivity to all synthetic auxins.

The affinity of V2NCSF2A for free IAA is 20 μM, which is identical to the K_D obtained for CSF2A binding to IAA-N−BSA. This low affinity is typical for sdAbs isolated from a naive llama library (*35*). Similar K_D values (low micromolar range) were obtained for sdAbs against the auxinic herbicide picloram (230 Da) that were previously isolated from the same naive library (*37*). CSF2A also bound 1-NAA and all of the auxinic herbicides, although with much lower affinity (~1000-fold less), compared to that for IAA (**Table 4**). Neither serotonin nor tryptophan was bound by CSF2A, despite their structural similarity to IAA. Another sdAb isolated from the llama naive library, CSE6E, has a similar pattern of selectivity for IAA, 1-NAA, and the auxinic herbicides, although the K_D for IAA and the herbicides was much higher (lower affinity) than that of CSF2A (results not shown).

The herbicide quinclorac (**Figure 1**) is a member of a newer auxinic herbicide family, the substituted quinolinecarboxylic acids (*1*). Symptoms of auxin overdose have been observed with this herbicide, but compared to the three other families of auxinic herbicides, the phenoxyalkanoic acids (2,4-D, mecoprop, MCPA), the benzoic acids (dicamba), and the pyridines (picloram and clopyralid), which target broadleaf weeds, quinclorac also targets some graminaceous weeds, for example, selective control of barnyard grass in rice. Furthermore, the structure of the quinolinecarboxylic acids differs significantly from that of IAA. Nonetheless, both CSF2A and CSE6E (albeit with low affinity) bind quinclorac. Such broad cross-reactivity of a single Ab toward IAA, 1-NAA, and all of the major chemical families of auxinic herbicides has not been observed with polyclonal antiserum, mAbs, or scFvs against auxinic herbicides previously studied in our laboratory. For example, Abs and fragments thereof against the auxinic herbicides picloram and dicamba have not shown any significant cross-reactivity to either IAA (Yau et al., unpublished results) or other auxinic herbicides (*60, 66, 67*). IAA-specific polyclonal Abs developed for ELISAs have also been tested for their cross-reactivity to other auxins, namely, 1-NAA and 2,4-D, as well as to tryptophan. Polyclonal antisera raised against IAA conjugates in both rabbits and mice had significant cross-reactivity to 1-NAA (*18, 23, 68*), but little or no cross-reactivity to 2,4-D, other synthetic auxins, and tryptophan (*21, 69−71*).

Although StEP performed well in shuffling the five parental sdAbs CDRs, biopanning of the resulting library yielded no binders with improved affinities or higher expression levels. However, the sequences and SPR-measured affinity of the two sdAbs isolated from the StEP library, CSB1A and CSE8A,

indicate that for sdAb CSF2A CDR2 is most likely involved in binding IAA, whereas the CDR3 provides broad cross-reactivity to the auxinic herbicides. To our knowledge, this is the first time StEP has been performed with sdAbs for sequence–activity relationship purposes.

The pentamerized c-myc tag allowed for very tight, stable, and oriented capture of pentabodies by an anti-c-myc IgG immobilized onto the dextran surface of the Biacore sensor chip. Only nanogram quantities of pentabody were required for capture, and relatively high surface densities (3500–4000 RUs) could be obtained. A single injection of pentabody provided a highly stable surface with which all of the hapten experiments could be performed. Also, because a one-to-one interaction (one sdAb binding one molecule of IAA) was observed, the data collected reflect the true affinity of the sdAb for the free hapten; that is, no IAA conjugate is required and no inhibition experiments are necessary to show sdAb binding to the free hapten. To our knowledge, this is the first time pentamerization of a sdAb has been used to perform SPR-based characterization of low molecular weight haptens. We believe this new method will prove to be very useful in the analysis of sdAb–hapten interactions by SPR in the future.

sdAbs CSF2A and CSE6E can discriminate between active and nonactive auxin analogues, a feature that makes them potentially interesting for screening and identifying novel agrochemicals and peptides with auxinic activity. Such screening is generally performed using bioassays that are often difficult to perform and/or time-consuming, thus limiting throughput of new chemistry (*72*). sdAb CSF2A could provide a platform for high-throughput screening, as demonstrated by Webb and Hall (*24*–*26*) and Webb et al. (*27*), who showed that an Ab-based surrogate of the enzyme ACCase could be used to screen and select diverse chemistries with ACCase inhibitor activity. Furthermore, these sdAbs may be useful for in planta immunomodulation of IAA activity, thus furthering our understanding of its mode and mechanism of action.

ABBREVIATIONS USED

1-NAA, 1-naphthaleneacetic acid; 2,4-D, 2,4-dichlorophenoxyacetic acid; Ab, antibody; ABP, auxin-binding protein; ACCase, acetyl coenzyme A carboxylase; ARF, auxin response factor; BSA, bovine serum albumin; CDR, complementarity determining region; HBS, Hepes-buffered saline; HBS-E, Hepesbuffered saline–EDTA; IAA, indole-3-acetic acid; IMAC, immobilized metal affinity chromatography; K_D, dissociation constant; LB, Luria broth; MPBS, milk-phosphate-buffered saline; OVA, ovalbumin; PBS, phosphate-buffered saline; PBST, phosphate-buffered saline–Tween; PCR, Polymerase Chain Reaction; RR6, reactive red 6; RU, resonance unit; scFv, singlechain variable fragment; sdAb, single-domain antibody; SPR, surface plasmon resonance; StEP, staggered extension process; TIR1, transport inhibitor response 1; V_HH, variable heavy domain of heavy-chain antibody.

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Supporting Information Available: Tables 6 (sequence alignment of randomly sampled sdAbs from the StEP library prior to panning), 7 (sequence alignment of sdAbs sampled from the StEP library after panning), and 8 (parental origin of predominant CDRs found in sdAbs from the StEP library). This material is available free of charge via the Internet at http://pubs.acs.org.

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