

Herbicide-resistance conferred by expression of a catalytic antibody in *Arabidopsis thaliana*

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Engineering herbicide resistance in crops facilitates control of weed species, particularly those that are closely related to the crop, and may be useful in selecting lines that have undergone multiple transformation events. Here we show that herbicide-resistant plants can be engineered by designing a herbicide and expressing a catalytic antibody that destroys the herbicide *in planta*. First, we developed a carbamate herbicide that can be catalytically destroyed by the aldolase antibody 38C2. This compound has herbicidal activity on all three plant species tested. Second, the light chain and half of the heavy chain (Fab) of the catalytic antibody were targeted to the endoplasmic reticulum in two classes of *Arabidopsis thaliana* transformants. Third, the two transgenic plants were crossed to produce a herbicide-resistant F1 hybrid. The *in vitro* catalytic activity of the protein from F1 hybrids corroborates that catalytic antibodies can be constitutively expressed in transgenic plants, and that they can confer a unique trait.

Catalytic monoclonal antibodies¹, which exploit both the combinatorial diversity of the immune system and the power of programmable design, have been shown to mediate > 100 different *in vitro* reactions. They have found applications as diverse as selective organic synthesis², biosensing³, providing mechanistic insights into biocatalysis⁴ and cancer chemotherapy⁵. Yet, the prospect of modifying a plant's phenotype by expressing a gene that encodes a catalytic antibody does not appear to have been explored.

Expression of a catalytic antibody in transgenic plants to provide a beneficial trait, such as herbicide resistance, is an attractive application of this concept. Traditional methods of generating herbicide-tolerant crops are based on either breeding under selective pressure or crossing a crop with a herbicide-resistant species^{6–8}. Cell culture selection and protoplast fusion have also been used to obtain herbicide-resistant crops^{9,10}. Transgenic approaches have involved, for example, alteration or overexpression of a herbicide target, or conferring the ability to metabolize the herbicide, detoxify herbicide-derived oxygen radicals¹¹ or efflux the herbicide from the plant cell¹². Here we demonstrate that herbicide-resistant plants can be engineered by designing a herbicide that can be hydrolyzed by a catalytic antibody that is expressed *in planta*.

We have targeted the family of carbamate herbicides, which have been generally used as germination inhibitors. However, development of catalytic antibodies for carbamate hydrolysis is difficult, owing to the hydrolytic stability of this functionality. Indeed, the reported carbamate-hydrolyzing antibodies, which were elicited by immunization with organophosphorous transition state analogs, exhibit rather modest catalytic activity under neutral pH^{13–16}. We therefore pursued an alternative strategy to achieve antibody-catalyzed carbamate degradation, avoiding direct nucleophilic attack on the carbamate carbonyl. Our approach involves designing carbamates such that C–O bond cleavage produces an *N*-arylcarbamic acid intermediate, which decomposes readily in solution to give arylamine, CO₂ and an unsaturated ketone (Fig. 1a). Nine keto-carbamate derivatives (Fig. 1b and Supplementary Note 1 online), which are extended analogs of the commercial herbicides protham and chloroprotham, were designed to release an *N*-arylcarbamic acid via an antibody-catalyzed β -elimination reaction using the aldolase antibody 38C2 (refs. 5,17).

We assessed the nine herbicide candidates both as substrates of antibody 38C2 and as general herbicides. As shown by the kinetic parameters, all of the compounds were good substrates of 38C2. Acetylacetone completely inhibited the fragmentation reaction of these substrates, indicating that catalysis occurred within the antibody active site¹⁷. Unfortunately, rate enhancements with the simplest derivatives, compounds 1 and 2, were <700 owing to high rates of uncatalyzed reactions. To minimize these background reactions, we designed carbamates 3 and 4, which fragment via two 38C2-catalyzed reactions—a retroaldol reaction that produces compounds 1 and 2, respectively, followed by β -elimination reactions¹⁸. Although no background reactions could be detected using compounds 3 and 4, the catalytic rate constants were still too small for practical applications. Another disadvantage associated with carbamates 1–4 was that a decomposition product, methylvinylketone, inhibits 38C2 (ref. 19).

Alkylated substrates, such as compounds 5–7, were designed to address both of these issues. Although these analogs exhibited reduced background reaction rates and improved rate enhancement, their decomposition rates were still low ($k_{\text{cat}} = 0.006\text{--}0.016 \text{ min}^{-1}$). A satisfactory solution to the above problems was finally found on the basis of our previous study of the 38C2-catalyzed deuterium exchange reaction at the α -position of ketones and aldehydes²⁰, suggesting that

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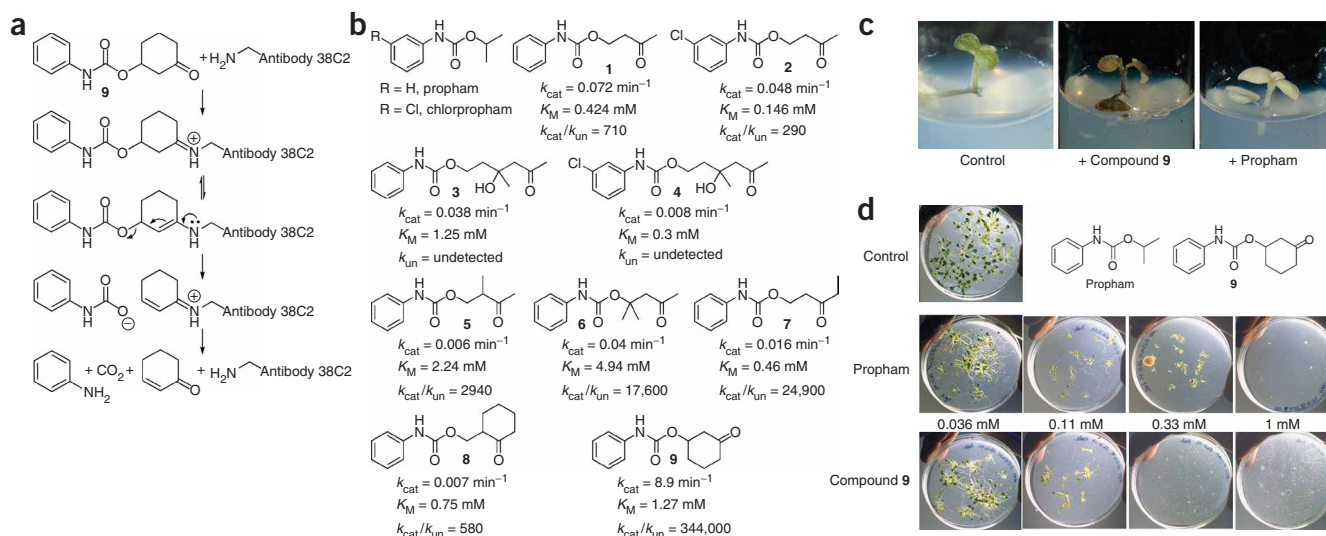


Figure 1 Herbicidal activity of keto-cartamates. (a) Antibody-catalyzed fragmentation mechanism of keto-carbamate **9** via a Schiff-base intermediate. (b) Keto-carbamate herbicide candidates, which were tested as substrates of 38C2 and general herbicides. The structure of each compound is presented together with its Michaelis-Menten kinetic parameters. (c) Herbicidal activity of compound **9** and protham with potato (cv. Desiree) shoots that were grown in the presence of 1 mM of each herbicide. (d) Herbicidal activities of compound **9** and protham with respect to germination of *A. thaliana* (ecotype Columbia) seeds. Four concentrations were examined for each compound.

cyclohexanone derivatives, such as compounds **8** and **9**, could exhibit high catalytic rates. Although the keto-carbamate compound **8** decomposed quite slowly ($k_{cat} = 0.007 \text{ min}^{-1}$) as was the case with compound **5**, probably as a result of steric hindrance at the α -position, compound **9** decomposed in neutral saline with excellent kinetic parameters ($k_{cat} = 8.9 \text{ min}^{-1}$, $k_{cat}/k_{un} = 344,000$) to produce aniline and cyclohexenone. Although the two enantiomeric forms of compound **9** could, in principle, exhibit different kinetic parameters, we did not observe any evidence that they had different reaction velocities. Thus, the above-mentioned parameters refer to catalytic hydrolysis of the racemic mixture of compound **9**. Notably, no inhibition of 38C2 by cyclohexenone could be observed under the reaction conditions used¹⁷.

The relative herbicidal activities of compounds **1–9** were evaluated by examining seed germination of *A. thaliana* seeds, as well as development of potato plantlets, and propagation of *Lemna* fronds (Fig. 1c,d, Supplementary Figs. 1–3 and Supplementary Note 2 online). Studies involving all three species indicated that compound **9**, which was the best substrate of 38C2, was also the most effective herbicide with an activity comparable to that of protham (Fig. 1c,d see Supplementary Fig. 1 online). Compounds **1**, **2**, and **6** also exhibited similar herbicidal activity as protham, whereas compounds **3**, **4**, **5** and **8** had minor or no herbicidal activity.

To verify the *in vitro* activity of the catalytic antibody 38C2, we cloned genes that encode the light chain and half of the heavy chain

(Fab) of this antibody into pET29 vectors and expressed each gene separately in *Escherichia coli*. Although the Fab of 38C2 was previously expressed in *E. coli*^{21–23}, those reports expressed both chains from the same plasmid. For our purpose, however, as we planned to express each chain in separate *Arabidopsis* lines, it was important to verify that the two separately expressed chains could assemble in solution to form a functional catalyst. Accordingly, we expressed each chain in a separate cell line. After dialysis of protein extracts from the inclusion bodies of each strain, we used compound **10** (methodol)²⁴, which undergoes catalytic fragmentation to generate a fluorescent product (compound **11**; Fig. 2), to examine whether association of the two peptides can form a functional Fab with retroaldolase activity. High activity was obtained when the two extracts were mixed and dialyzed together. Interestingly, extracts of the heavy chain alone exhibited measurable catalytic activity. This observation is not too surprising because this chain contains the low-pKa Lys-H93 residue, which catalyzes the retro-aldol reaction through a Schiff base intermediate (Fig. 1a).

The successful expression of a functional 38C2 in *E. coli* from separately encoded chains set the stage for *in planta* expression. Previously, noncatalytic antibodies were expressed in plants and their stoichiometric binding properties were used to modify existing phenotypes. These included alteration of metabolic routes to study plant growth and development²⁵, medicinal applications^{26,27},

Figure 2 *In vitro* retroaldolase activity of 38C2 (Fab) extracted from *E. coli*. Methodol (compound **10**, 50 μM) was added to three dialyzed solutions, 25 μl each, containing: protein extract of light chain (LC)-producing bacteria; protein extract of heavy chain (HC)-producing bacteria; and a dialyzed mixture of both chains (HC&LC). The mixtures were incubated overnight at 30 °C and then tested for fluorescence (irradiation at 320 nm, emission at 452 nm). The figure shows data from three independent experiments of each mixture placed as a droplet on a glass plate. C, control; LC, light chain-producing bacteria; HC, heavy chain-producing bacteria; HC&LC, dialyzed mixture of both chains.

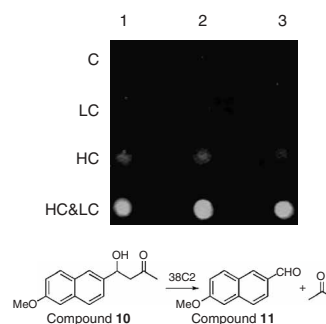
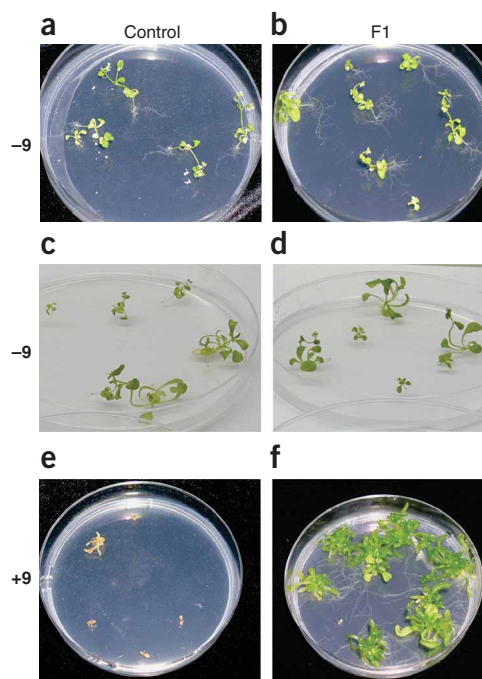


Figure 3 Influence of herbicide **9** on the rooting and development of seedlings of F1 hybrids and control *A. thaliana* plants. (a–f) The control plants are shown in a, c and e, whereas the hybrid plant lines (F1) expressing both light and heavy chains are shown in b, d and f. Plantlets grown on MS medium without herbicide **9** are shown in a–d, whereas those grown in the presence of herbicide **9** (0.05 mM) are shown in e and f.



pathogen control²⁸, pesticide activity, as well as herbicide resistance²⁹. However, the use of catalytic antibodies to create new phenotypes has not yet been reported³⁰.

To accomplish this goal, we cloned the genes encoding the chains required for functional antibody 38C2 (Fab) in separate binary vectors. Both chains were fused at the N-terminus to an endoplasmic reticulum (ER)-targeting sequence and at the C-terminus to an ER retention signal. *A. thaliana* plants were transformed using *Agrobacterium tumefaciens*-mediated transformation. Over 20 independent transgenic lines containing the light chain were isolated and characterized. In contrast, only two transgenic lines containing the heavy chain were produced using the same experimental protocols. Notably, the latter two lines exhibited poor germination, short internodes and slow growth rates. Such defects do not appear to have previously been associated with the expression of antibodies in plants. DNA and mRNA analyses verified transformation and transcription of the transgenes. Homozygous plants, each containing either the light or heavy chain, were crossed to yield F1 hybrids. RT-PCR analyses verified that the F1 hybrids expressed both subunits in the same plant tissue.

We examined the retroaldolase catalytic activity and herbicide resistance of the hybrid plants expressing both chains. *In vitro* plantlet development was determined on medium with or without herbicide **9**. Germination and growth of the F1 hybrid in the absence of the herbicide was similar to that of the control *A. thaliana* plants (Fig. 3a–d). It appears that association of the heavy and light chains alleviates the adverse effect of heavy chain expression on plant growth and development. However, the presence of herbicide **9** (0.05 mM) in the medium caused substantial inhibition of shoot development and effective prevention of root growth in control plants (Fig. 3e–f). In contrast, the F1 hybrid exhibited striking tolerance of the herbicide, both in terms of plant development and root growth (Fig. 3f).

Quantitative analyses of root development indicated an ~74% reduction in the number of rooting plants in the control lines, whereas only 20% of the F1 plants failed to develop roots.

To verify that the observed herbicide resistance is associated with the catalytic activity of 38C2 produced *in planta*, we used the methodol assay to compare protein extracts from the F1 plants with those from the control lines. Whereas a bright fluorescent signal was detected with the protein extract from the F1 hybrids, no fluorescence was observed with protein extracts from the control, nontransgenic plants (Fig. 4).

This work demonstrates that a herbicide designed to be targeted by an antibody can be used together with the latter to produce transgenic herbicide-tolerant plants. The *in vitro* catalytic activity of the protein extract from F1 hybrids corroborated that catalytic antibodies can be constitutively expressed in transgenic plants, and that they can thereby confer a unique trait that alters plant physiology. Together with reports of the expression of the chorismate mutase antibody, 1F7, in yeast^{31,32}, this study suggests that *in vivo* expression of catalytic antibodies could become a general strategy for phenotype modification not only in plants but also in other organisms.

METHODS

Synthesis of keto-carbamates, 1–9. Compounds **1** and **2** were prepared by reacting 2-(methoxymethoxy)butan-1-ol with phosgene and either aniline or 3-chloroaniline, respectively, followed by deprotection of the secondary alcohol and oxidation to ketone. Compounds **3** and **4** were prepared from **1** and **2** by condensation with acetone in the presence of lithiumdiisopropyl amide (LDA). Compounds **5–8** were prepared from the appropriate ketoalcohol and phenyl isocyanate in the presence of triethylamine. Compound **9** was prepared from 3-hydroxycyclohexanone³³ and phenyl isocyanate without base. All compounds were purified either by crystallization or by column chromatography and characterized by ¹H and ¹³C NMR and by mass spectrometry.

Determination of kinetic parameters. All antibody-catalyzed reactions were carried out in either PBS (50 mM phosphate, 100 mM NaCl, pH 7.4) or saline

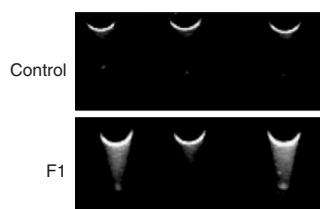


Figure 4 Retroaldolase activity 38C2 (Fab) extracted from transgenic plants. Six tubes containing methodol and protein extracts from either the nontransgenic plants (control, top three tubes) or from the F1 hybrid plants (bottom three tubes) were irradiated with UV light as described in Figure 2, showing that a fluorescent product was formed only with the F1 extracts. Fresh leaves (200 mg) were collected and ground in 600 μ l protein extraction buffer (50 mM Tris-Cl pH 7.4, 1 mM EDTA, 1% NP-40). The extract was centrifuged at 20,000g for 20 min at 4 $^{\circ}$ C. The soluble fraction was collected and centrifuged again, after which 250 μ l were filtered through cellulose Millipore YM-100 to give mixtures of proteins smaller than 100 kDa. These mixtures were filtered through Millipore YM-10 to give mixtures of proteins larger than 10 kDa. The catalytic activity assay was performed with the latter mixtures in a total volume of 100 μ l (50 μ l protein extract + 35 μ l ddH₂O + 15 μ l 750 μ M methodol solution).

(100 mM, NaCl) containing 10% organic cosolvent. Antibody active site concentrations ranged between 2.7 and 26.7 μM and substrate concentrations ranged between 50 and 2,500 μM . The progress of the reactions was monitored by high-performance liquid chromatography (HPLC) using an RP Merck LC18 column and kinetic parameters were determined by Lineweaver-Burk analysis of the HPLC data.

Determination of herbicidal activity of compounds 1–9. Compounds 1–4 were assayed using potato (cv. Desiree) plantlets. Plant material was grown in MS medium containing 3% sucrose and 0.8% agar, pH 5.8 in the presence of either protham or a keto-carbamate (0.012–1.00 mM). Plant assays included root development and leaf chlorosis. Experiments with seeds of *A. thaliana* (ecotype Columbia) involved comparison of the effects of compounds 5, 6, 8 and 9 with those of protham on both germination and postgermination development. For the latter assay, plantlets were transferred to MS-agar plates containing the herbicide (0.012–1.00 mM) and analyzed for growth inhibition, chlorosis and root development.

Construction of the plant vectors. *E. coli* strain XL1blue was used for routine cloning. A plant-derived ER-targeting sequence was excised by *EcoRI* restriction enzyme from the pRTL-mG5-ER plasmid (kindly provided by K. Oparka, Scottish Crop Research Institute) and was ligated to the plasmids pBS KS and pART7 at the same sites, resulting in the vectors pBS-SP and pART-SP, respectively. The genes encoding for half of the heavy chain (HC) and the light chain (LC) were amplified through PCR from the plasmids pBS-D1-HC and pBS-D2-LC, respectively, using the following primers: 5'-end primer (5'HC2) AAACCCGGGCATATGGCCCTCGAGGTG-3' and 3'-end primer (3'HC2) TTTGGATCCTTAAAGCTCATCCTTGATGCATGCTCGAGC-3' for the heavy chain, and 5'-end primer (5'LC2) AAACCCGGGCATATGCCCAGAGCT-3' and 3'-end primer (3'LC2) TTTGGATCCTTAAAGCTCATCCTTACACTCTCCCTGT-3' for the light chain. Primers 5'HC2 and 5'LC2 contained a *SmaI* site at their 5' end and a *BamHI* site at their 3' end (underlined). In addition, a KDEL retention signal (bold) was added to the primers, in-frame to the 3' end of both chains. The amplified fragments (HC: 747 bp, LC: 684 bp) were purified from an agarose gel, digested with the appropriate restriction enzyme, and ligated simultaneously into *SmaI/BamHI*-digested pART-SP and pBS-SP to generate the vectors pBS-SP-HC and pART-SP-LC, respectively. Sequencing reactions were used to verify that the nucleotide sequences of the SP were in-frame with those of the HC and LC. The SP-HC cassette was excised from pBS-SP-HC to add the control of the CaMV-35S promoter and the Nos terminator. Digestion of the pBS-SP-HC at the *BamHI* site was also performed by Klenow treatment to form a blunt end. The 5' end of the SP-HC cassette was restricted at the *SalI* site. The 1,000-bp fragment (SP-HC) was inserted into the *SalI* (5' end) and *SmaI* (3' end) sites in pJD330 under the control of the CaMV-35S constitutive promoter and Nos terminator.

The complete cassettes containing CaMV35S-SP-LC/HC were ligated to the binary vector pMLBart, which is suitable for transformation in plants, and contains the selective marker gene, *Bar* that confers resistance to the herbicide Basta. The CaMV35S-SP-HC-Nos cassette was digested from the pJD-SP-HC by *XbaI* (1,700 bp) and was inserted into the pMLBart binary vector processed at the same restriction site. *NotI* digestion was used to excise the cassette CaMV35S-SP-LC-Ocs (3,080 bp) from the pART-SP-LC, which was ligated into the pMLBart plasmid at the same sites, resulting in pMLBart-SP-HC and pMLBart-SP-LC plasmids, respectively. The binary vectors were introduced into *A. tumefaciens* strain GV3101 for *A. thaliana* plant transformation³⁴.

Total RNA was extracted from leaves using the Tri-reagent (Sigma) according to the manufacturer's protocol. One microgram of total RNA was used for cDNA synthesis with 46-mer oligo(dT) primer and ImpromII reverse transcriptase (Promega). Two specific primers within the conserved region of the LC, 5'-AAACCCGGGCATATGGCCGAGCT-3' and 5'-TTGGATCCTTAAAGCTCATCCTTACACTCTCCCCTGT-3' forward and reverse, respectively, and two specific primers within the conserved region of the HC, 5'-GCTT GAGTGGGTGCTGAA-3' and 5'-TGGGCTCAACTTCTTGTCC-3' forward and reverse, respectively, were used for the PCR amplification. The resulted fragments were of 684 bp and 525 bp for the LC and HC genes, respectively.

Homozygous lines were characterized based on resistance of all progenies collected from specific T1 generation to glufosinate herbicide (0.5% Basta).

PCR analyses on 20 plants were further used to verify that there was no segregation in the selected T2 homozygous plant lines.

Accession numbers. The GenBank Accession numbers of the heavy and light chains are AF242215 and AF242214, respectively.

Note: Supplementary information is available on the Nature Biotechnology website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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