Research Article

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Development of enzymes for robust aryloxyphenoxypropionate and synthetic auxin herbicide tolerance traits in maize and soybean crops

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Abstract

BACKGROUND: Effective management of weedy species in agricultural fields is essential for maintaining favorable growing conditions and crop yields. The introduction of genetically modified crops containing herbicide tolerance traits has been a successful additional tool available to farmers to better control weeds. However, weed resistance challenges present a need for additional herbicide tolerance trait options.

RESULTS: To help meet this challenge, a new trait that provides tolerance to an aryloxyphenoxypropionate (FOP) herbicide and members of the synthetic auxin herbicide family, such as 2,4-dichlorophenoxyacetic acid (2,4-D), was developed. Development of this herbicide tolerance trait employed an enzyme engineered with robust and specific enzymatic activity for these two herbicide families. This engineering effort utilized a microbial-sourced dioxygenase scaffold to generate variants with improved enzymatic parameters. Additional optimization to enhance in-plant stability of the enzyme enabled an efficacious trait that can withstand the higher temperature conditions often found in field environments.

CONCLUSION: Optimized herbicide tolerance enzyme variants with enhanced enzymatic and temperature stability parameters enabled robust herbicide tolerance for two herbicide families in transgenic maize and soybeans. This herbicide tolerance trait for FOP and synthetic auxin herbicides such as 2,4-D could be useful in weed management systems, providing additional tools for farmers to control weeds.

Supporting information may be found in the online version of this article.

Keywords: agriculture biotechnology; herbicide tolerance trait; FOP herbicide tolerance; 2,4-D herbicide tolerance; applied enzyme engineering

1 INTRODUCTION

In agricultural systems, the management of weedy species in crop fields is one of many essential components to ensure favorable crop yields. Many weedy species are well adapted to compete with crops for sunlight, water, soil nutrients, and physical space. Farmers control weeds by utilizing a variety of weed management strategies, including herbicides and genetically modified herbicide-tolerant crops. However, weeds have evolved resistance to herbicides and this continues to present a challenge in agriculture today.[1] One of many strategies to manage resistant weeds is to employ multiple herbicide modes of action.

Two herbicide families of interest are the aryloxyphenoxypropionate (FOP) herbicides and members of the synthetic auxin herbicide family. FOP herbicides inhibit an enzyme variant of acetyl CoA carboxylase (ACCase) found in grasses.[2] Due to this selective nature, FOP herbicides are commonly used to control

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grass weeds in dicot crops. The addition of a FOP herbicide tolerance trait in maize would enable the use of FOP herbicides to control grass weeds in maize fields. The synthetic auxin family of herbicides includes 2,4-dichlorophenoxyacetic acid (2,4-D), which is often used to control dicot weeds.[3] Synthetic auxin herbicides disrupt plant hormone regulatory pathways, resulting in plant death.

Two approaches have been useful in building herbicide-tolerant crops.[4] The first is to utilize a transgene expressing an insensitive variant of an enzyme that is the herbicide's target in a sensitive crop. The second approach is to utilize a transgene expressing an enzyme that deactivates the herbicide chemistry. The oxygenase enzyme α-ketoglutarate-dependent (*R*)-dichlorprop dioxygenase (RdpA), which was isolated from the common soil microbe *Sphingobium herbicidovorans*, could be utilized for the second approach.[5,6] RdpA is part of a large superfamily of dioxygenases found in microbes, plants, and animals.[7,8] RdpA catalyzes the oxidative metabolism of chemistries in the FOP (selective for the *R* enantiomers, the active form of these herbicide chemistries) and synthetic auxin herbicide groups, and has been shown to provide tolerance to these herbicides when expressed as a transgene in sensitive plants.[5,6,9]

A FOP and 2,4-D herbicide tolerance trait, when paired with other herbicide tolerance traits and selective herbicides, can aid in managing and preventing herbicide-resistant dicot and grass weeds, and provides an additional tool for effective weed management. Crops display differences in sensitivity to herbicides in the FOP and 2,4-D families. For example, maize is sensitive to FOP herbicides but displays incomplete tolerance to 2,4-D. Dicots, such as soybeans, are fully tolerant to FOP herbicides, but sensitive to 2,4-D. Therefore, we were interested in engineering an enzyme that could robustly inactivate both FOP and 2,4-D herbicides.

Here, we characterize novel optimized dioxygenase enzyme variants in maize and soybean. These new variants, named FT enzymes (FOP and 2,4-D tolerance enzymes), were engineered to improve enzymatic parameters, including enhanced enzymatic activity on selected herbicides and improved temperature stability of the enzymes. These enhanced variants could be useful in building a new generation of transgenic maize and soybeans with improved herbicide tolerance and agronomic performance when FOP and 2,4-D herbicides are used in a weed control system.

2 MATERIALS AND METHODS

2.1 Purification, enzyme assays and characterization

FT variants were synthesized and cloned into a modified pET28a vector which included a TVMV cleavable N-terminal His tag or a C-terminal His tag (Novagen, Burlington, MA). These variants were transformed into Rosetta 2 cells (Novagen) or BL21 DE3 cells (New England Biolabs, Ipswich, MA) for protein expression overnight at 20 °C. Cell pellets were lysed and FT proteins extracted using B-PER™ and Y-PER™ (ThermoFisher, Waltham, MA) plus lysozyme and benzonase (Sigma Aldrich, St. Louis, MO). Clarified extracts were Immobilized metal affinity chromatography (IMAC) purified using nickel resin (Sigma Aldrich) and eluted using 30 mM Tris pH 8, 200 mM imidazole buffer. Further purification, if needed, included dialysis or size exclusion using 30 mM Tris pH 8 and 200 mM NaCl buffer (ThermoFisher).

Enzyme assays were completed with FT proteins using a modified phenolic-based colorimetric detection assay.[10] Purified proteins (or in the case of the high-throughput screening assays, clarified *E. coli* extracts) were tested with selected substrates. Chemicals were obtained from Sigma Aldrich. For quizalofop (QFOP) and synthetic auxins, *in vitro* assays used the acid form (the active QFOP molecule in plant cells) in the racemic form (if applicable). The reaction buffer contained 20 mM MOPS pH 6.75, 50 µM $(NH_4)_2$ $Fe(SO_4)_2$, 50 µM ascorbic acid (Na salt), and 1 mM α-ketoglutarate (α-KG). Reactions were performed in 150 µL final reaction volumes in 96-well assay plates. All reactions for kinetic characterization were completed with four replications while high-throughput screening reactions for enzyme optimization utilized fewer replications. Reactions were stopped with 15 µL of stop solution (50 mM boric acid, 50 mM KCl, pH 10.0 (KOH) with 2% 4-aminoantipyrine w/v (in water) freshly diluted 1:10 into stop buffer). Color development agent was added (15 µL of an 8% potassium ferricyanide w/v (in water), freshly diluted 1:10). Following color development, assays were recorded with a SpectraMax spectrometer at absorbance of 510 nm. Back calculations were completed with product standards (Sigma Aldrich or custom synthesis) in simulated reactions. Control reactions without α-KG were inactive, and reactions with purified enzyme without $(NH_4)_2$ $Fe(SO_4)_2$ and ascorbic acid were significantly inhibited. Unless indicated, all reactions were completed at 22–23 °C. For the temperature stability assays shown in Fig. 2(b), the enzymes were equilibrated at the indicated temperature for 5 min before substrate (also equilibrated at the same temperature) was added and the reaction allowed to proceed at the same temperature. During the high-throughput screens focused on enhanced temperature stability, screens were run at 25 °C and 40 °C to select variants with strong activity at both temperatures. Kinetic parameters were calculated with nonlinear regression analysis using GraphPad software with default settings.

Protein melting temperatures were determined by fluorescence tracking of the hydrophobic protein binding dye SYPRO Orange (Invitrogen, Waltham, MA) with progressive increases in temperature. The assays were run in a 20 mM Tris, pH 7.75 and 150 mM NaCl buffer. Assays were also run with the addition of $(NH_4)_2$ $Fe(SO_4)_2$ and α-KG since some substrate stabilization was observed. The FT protein and a 1:100 dilution of SYPRO Orange was combined in buffer in a Bio-Rad CFX96 PCR thermocycler with a fluorescence detector. The temperature was progressively increased from room temperature to full protein denaturation. The temperature at which the protein was 50% denatured was calculated from the first derivative of the change in fluorescence in Bio-Rad CFX Manager 2.1 software.

2.2 In-plant controlled environment herbicide tolerance and temperature stability assays in maize

Several different expression vectors containing FT variants were used to transform maize immature embryos using *Agrobacterium*-mediated transformation.[11] Plants containing a single copy of the transgenic insert were identified through molecular and segregation analysis and advanced to seed production for further experimentation.[12] The FT enzymes were expressed either without a targeting peptide or targeted to the chloroplast using the malate dehydrogenase (MDH) chloroplast targeting peptide (CTP).[13] A growth chamber experiment at two different temperatures was conducted to evaluate the tolerance of F1 hybrid transgenic maize expressing MDH/FT_5, MDH/FT_T, MDH/FT_R, FT_T, or FT_R and control plants to postemergence applications of 2,4-D. For QFOP (Assure II®, formulated as an ester in the *R* enantiomer) postemergence tolerance, plants expressing MDH/FT_5, MDH/FT_C, MDH/FT_T, MDH/FT_R, FT_T, or FT_R, and control plants were assayed. The first growth chamber was set to 20 °C

day and 20 °C night and the second growth chamber was set to 38 °C day and 30 °C night, both with 50% relative humidity and 16-h photoperiod. For each variant/targeting peptide combination tested, three different transformation events were evaluated with eight replications for each temperature regime. The herbicide treatment consisted of 2,4-D amine at 4500 g ha^{-1} (4X) applied to V3/V4 maize and QFOP at 360 g ha^{-1} (4X) plus 0.25% v/v Chemsurf™ nonionic surfactant (NIS) containing a 90% mixture of alkylarylpolyoxykane ether, isopropanol and free fatty acids applied to V2 maize in a track sprayer. Plants were visually rated 11 days after treatment (2,4-D) and 9 days after treatment (QFOP) on a scale of 0% to 100% with 0% being no injury and 100% being plant death. This scoring system is based on classical herbicide injury rating systems and includes injury to plant tissue such as chlorosis, necrosis, malformation, stunting and plant death. Injury data were averaged with standard error bars calculated in Excel.

2.3 Field-based herbicide tolerance assays in maize

Field experiments were conducted at two independent locations in Illinois, USA, to evaluate herbicide tolerance of maize expressing MDH/FT_5, MDH/FT_C, MDH/FT_T, MDH/FT_R, FT_T, or FT_R, and control maize to postemergence applications of QFOP and 2,4-D. Experimental design was a randomized complete block design with two replications. For QFOP, herbicide treatments consisted of (i) QFOP at 360 g ha^{-1} plus 0.25% v/v NIS applied to maize multiple times within the same plot, VE-V2 followed by (fb) V4 fb V8, (ii) QFOP at 720 g ha^{-1} plus 0.25% v/v NIS applied to VE-V2 fb V4 fb V8 maize, and (iii) QFOP at 1440 g ha^{-1} plus 0.25% v/v NIS applied to VE-V2 fb V4 fb V8 maize. For 2,4-D, herbicide treatments consisted of (i) 2,4-D amine at 2250 g ha^{-1} plus 0.25% v/v NIS applied to VE-V2 followed by (fb) V4 fb V8 maize, (ii) 2,4-D amine at 4500 g ha^{-1} plus 0.25% v/v NIS applied to VE-V2 fb V4 fb V8 maize, (iii) 2,4-D amine at 9000 g ha^{-1} plus 0.25% v/v NIS applied to VE-V2 fb V4 fb V8 maize, and (iv) 2,4-D amine at 18 000 g ha^{-1} plus 0.25% v/v NIS applied to VE-V2 fb V4 fb V8 maize. Herbicide treatments were applied with a CO_2 backpack or tractor-mounted sprayer calibrated to deliver 140 L ha^{-1} using air-inducted Teejet® TTI nozzles with water as the herbicide carrier. Plots were visually rated 10–14 days after V4 and V8 applications for crop injury. All data were subjected to analysis of variance and means separated at LSD or P value < 0.05.

2.4 In-plant herbicide tolerance in soybeans

Several different vectors containing FT variants were transformed into soybean using *Agrobacterium*-mediated transformation. Individuals (a transformation event) containing a single copy of the transgenes were identified and plants were advanced to seed production for further experimentation. Homozygous FT expressing plants were grown to the V3 growth stage and treated with 1680 g ha^{-1} of 2,4-D amine in controlled environment studies. Plants were assessed for visual injury approximately 7 days after treatment. Non-traited control is an average injury of four plants and the experimental treatments are the average injury of 19–39 plants. In field treatments, homozygous FT expressing plants were treated with 2240 g ha^{-1} of 2,4-D at V3 and R1 growth stages.

2.5 Data availability

Sequence information is available in GenBank:

FT_5: MH043108; FT_C: MH043109; FT_R: MH043110 and MH043111; FT_T: MH043112 and MH043113; FT_Tv3: MH043114; FT_Tv7: MH043115. Records for RdpA (AAM90965) and MDH (BT000621) have been previously submitted by other groups.[5,13]

The crystallography data referred to in this study are available in the Protein Data Bank (PDB, https://www.rcsb.org/):

6D0O, RdpA dioxygenase holoenzyme; 6D1O, FT_5 dioxygenase apoenzyme; 6D3H, FT_T dioxygenase with bound dichlorprop; 6D3I, FT_Tv7 dioxygenase with 2,4-D bound; 6D3J, FT_T dioxygenase holoenzyme; and 6D3M, FT_T dioxygenase with bound QFOP.

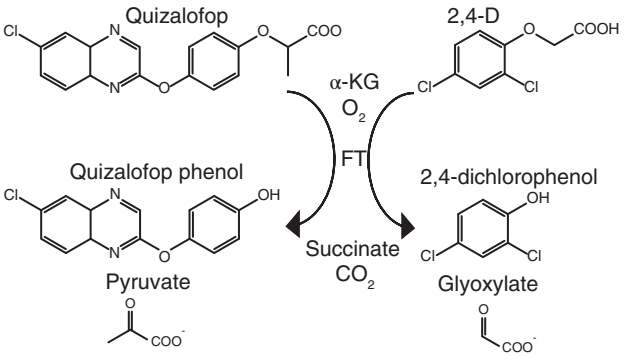

Figure 1. FT enzymes catalyze herbicide deactivation. The dioxygenase, FT, catalyzes the initial metabolism of chemistries in two herbicide chemistry families: FOP, represented by quizalofop (QFOP), and members of the synthetic auxins, represented by 2,4-D.

3 RESULTS

3.1 Introduction

The dioxygenase RdpA has been characterized as an α-ketoglutarate-dependent non-heme iron-dependent dioxygenase.[6] This enzyme utilizes α-KG, molecular oxygen and a variety of herbicide compounds to catalyze a classical dioxygenase reaction (Fig. 1). This reaction results in the release of CO_2, succinate and two breakdown products from the herbicide chemistry. However, the natural enzymatic activity of RdpA could be improved through enzyme engineering to provide tolerance to both herbicide chemistries in both monocot and dicot crops in typical field conditions. An enzyme engineering effort was thus undertaken to design optimized enzymes with enhanced activity on selected herbicide chemistries. RdpA was used as a base dioxygenase scaffold for engineering design and the optimized FT enzyme variants were screened for robust enzymatic activity on the herbicides QFOP, a FOP herbicide, and 2,4-D, a synthetic auxin. In-plant analysis of maize and soybean plants expressing selected FT variants in both controlled environment and field-based testing was essential for informing additional optimization parameters. This included a need to stabilize the enzyme for field temperature extremes and optimizing the activity of the enzyme towards the substrate 2,4-D.

3.2 Engineered FT variants demonstrated FOP tolerance in maize, but low enzyme temperature stability

At the outset of this engineering effort a series of now well-established engineering approaches were applied to RdpA to optimize activity for QFOP and 2,4-D separately and in combination (see the supporting inofrmation). Engineering approaches included homology modeling and homologous amino acid changes, scanning mutagenesis, computational protein design, rational protein design and data-driven design. At the onset of the engineering, no crystal structure of RdpA or a close

homolog was available and therefore a homology model was generated with ICM-pro using a distant dioxygenase homolog, TauD (30% sequence identity), as a template.[14] This homology model was used for substrate docking and identification of putative active site residues for rational design to modulate protein–substrate interactions. As discussed below, we later used structural characterization of enzyme variants as crystal structures were determined to help inform additional engineering. This coupled with the enzymatic characterization of early variants described below helped further drive the computational, rational and data-driven design efforts of additional optimized variants.

The enzyme variants were assayed in a bacterial expression system and tested in a high-throughput, plate-based enzymatic assay using clarified bacterial lysates. This assay enabled screening of approximately 4000 enzyme variants in a semi-quantitative manner. Those variants identified with improved activity relative to bacterial lysate expressing RdpA were further combined in multiple rounds of optimization. The highest performing variants in the bacterial lysate screen (depending on the optimization objectives described below) were confirmed with purified protein assays. A small subset (<1% in total) of these confirmed variants were selected for *in planta* efficacy analysis.

The enzyme variant FT_5 was the first variant selected for in-plant analysis based on first round *in vitro* kinetic characterization. The FT_5 variant showed good activity with QFOP (0.44 μmol mg^{-1} min^{-1} at V_{max}) and weaker activity with 2,4-D (0.03 μmol mg^{-1} min^{-1} at V_{max}) in enzyme assays. Although this activity was lower than that observed for RdpA, FT_5 was selected early in the optimization process to benchmark in-plant tolerance assays and to direct the optimization efforts. A transformation vector with the FT_5 transgene linked to a plant CTP at the N-terminal end of the enzyme was used to transform maize. A CTP was included because FOP herbicides inhibit the enzyme ACCase. In grasses such as maize, the herbicide sensitive ACCase enzyme isoform is localized to the chloroplast.[15] Therefore, we hypothesized that localizing the FT_5 enzyme to the chloroplast would provide improved FOP tolerance.

Maize immature embryos from the germplasm LH244 were transformed with FT_5 transformation vectors using *Agrobacterium tumefaciens*. The regenerated transformed plants were spray challenged with a commercial formulation of QFOP. Following spray applications, the transformed plants were rated for tolerance using a percentage injury rating. FT_5 plants showed tolerance to the herbicide application while the control plants showed extensive tissue damage and eventual plant death (Fig. 2(a)). Treated plants with low injury ratings were grown to set seed and advanced for additional testing in the following generations.

Greenhouse testing for FT_5 was encouraging; however, a robust herbicide tolerance trait requires good performance in field-relevant environmental conditions. Therefore, the FT_5 maize plants were screened for herbicide tolerance in Midwestern USA field research plots that mimicked typical maize cropping conditions. In these trials, FT_5 expressing plants were tolerant to both QFOP and 2,4-D, showing that the FT proteins can provide tolerance to these herbicides in field conditions (Fig. 3(a) and (c)). However, tolerance in field conditions using the FT_5 variant was not as robust as desired, with injury rates exceeding 20% in some treatments. Therefore, additional characterization of the FT_5 enzyme variant and FT_5 maize plants was undertaken.

Further FT_5 enzyme characterization showed that FT_5 was unstable at elevated temperatures *in vitro* and in plants,

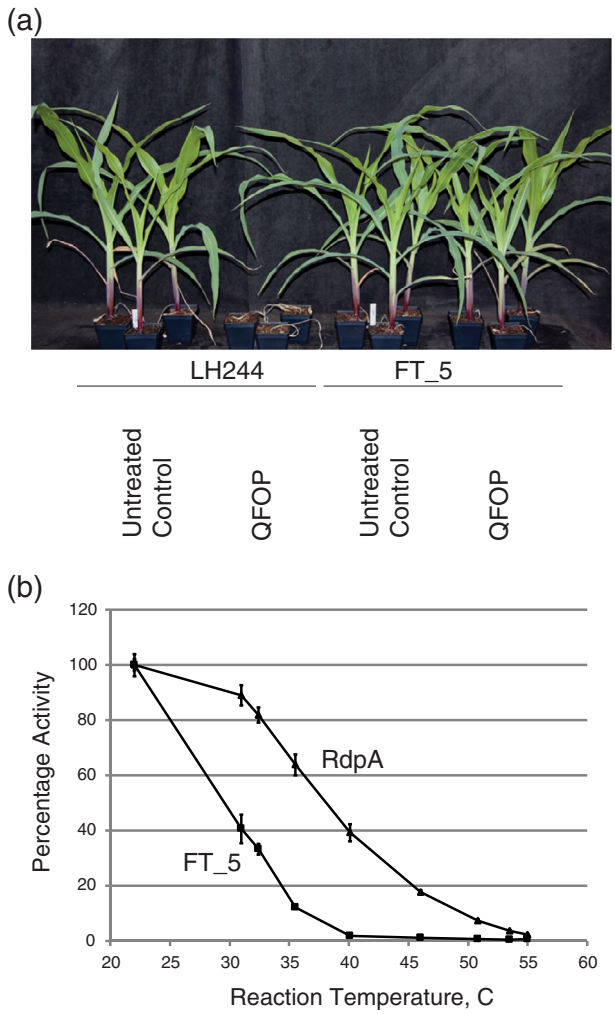

Figure 2. Characterization of FT_5. (a) Plants expressing FT_5 showed tolerance to QFOP applications in controlled environment experiments. Plants were treated with QFOP at 90 g ha^{-1}. The transformation background, LH244, served as a sensitive control while LH244 plants expressing the FT_5 enzyme (FT_5) were the test plants. Image taken 18 days after treatment. (b) Enzyme activity of FT_5 relative to RdpA with QFOP. Both FT_5 and RdpA show a progressive loss of activity with increasing reaction temperature (relative to room temperature control, which was set at 100% for both enzymes). Error bars indicate the standard error (SEM) of three replications.

suggesting that temperature stability is essential for improved in-crop performance. In the field environment, plants are exposed to large temperature swings from night to day and throughout the season. In addition, daytime temperatures in Midwestern fields commonly exceed 30 °C and can exceed 37.5 °C, the leaf surface temperature at which net photosynthesis begins to decline in well-watered maize plants.[16] A robust herbicide tolerance trait is dependent on a tolerance enzyme that can function throughout a real-world temperature range. FT_5 enzyme characterization revealed a progressive loss of enzyme function at temperatures above 30 °C with nearly complete enzyme inactivity at 40 °C with *in vitro* testing (Fig. 2(b)). While the original RdpA enzyme variant displayed somewhat more temperature stability *in vitro* than FT_5, RdpA was also temperature sensitive, with nearly 40% loss of activity at 35 °C (Fig. 2(b)). We also pre-exposed the FT_5 enzyme to an elevated temperature of 35.5 °C (a temperature which results in nearly 90% loss of activity) for various time lengths of 0 to 35 min before returning the enzyme to 22 °C for

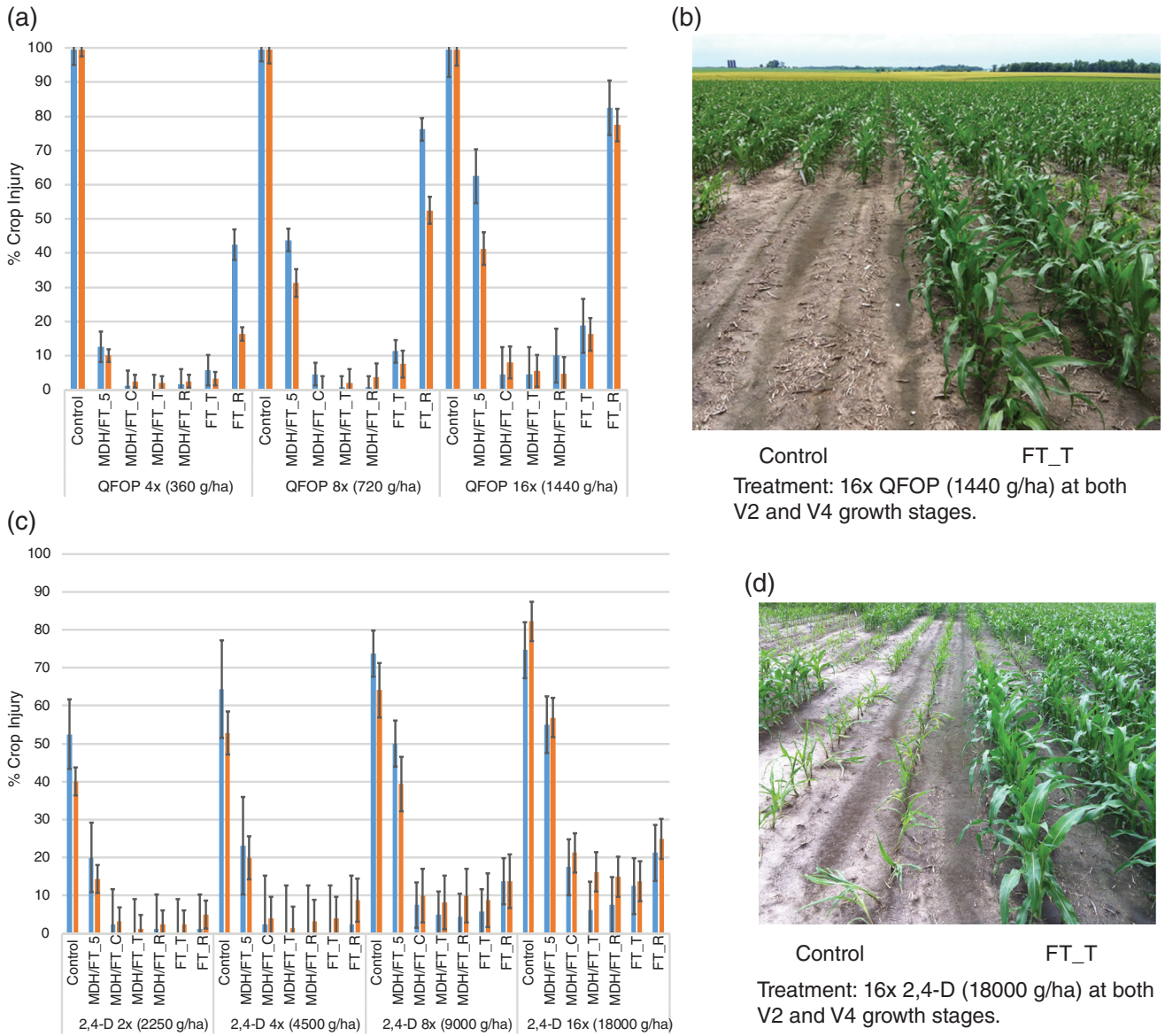

Figure 3. Field testing of FT variants. (a) Field testing confirms robust QFOP tolerance in maize. F1 plants expressing the indicated FT variants were treated at V2 and V4 (early vegetative) and V8 (late vegetative) with the indicated QFOP treatments. Injury scores were taken approximately 1 week post-treatment. The temperature-stable variants performed well when paired with a CTP. Injury ratings increased when the enzymes were expressed without a CTP. The blue bars indicate crop injury following the V4 treatments while the orange bars are following the V8 treatment, error bars indicate LSD at $P < 0.05$ with four replications. (b) Field view of MDH/FT_T (chloroplast targeted FT_T) expressing plants following the V4 application of QFOP. (c) Field testing confirmed robust 2,4-D tolerance. F1 plants expressing the indicated FT variants were treated at V2, V4 and V8 with the indicated 2,4-D treatments. Injury scores were taken approximately 1 week post-treatment. The temperature stabilized variants performed well in this assay either with or without a CTP. The blue bars indicate crop injury following the V4 treatments while the orange bars are following the V8 treatment, error bars indicate LSD at $P < 0.05$ with four replications. (d) Field view of MDH/FT_T expressing plants following V4 2,4-D application.

a traditional activity assay with QFOP. In this assay, the 10-min heat pre-treatment reduced enzyme activity by 50%, suggesting that the loss of activity at elevated temperatures is at least partly irreversible (data not shown).

To confirm that the temperature sensitivity observed in the FT_5 *in vitro* assays correlated with incomplete tolerance in plants, a series of growth chamber treatments was undertaken. Maize seedlings were moved to two temperature regimes before application of QFOP or 2,4-D herbicides. The first was a 20 °C day and night regime (non-stressed) and the second was a 38 °C day and 30 °C night regime (heat stress). FT_5 transgenic plants displayed tolerance to both herbicide applications at the non-stressed temperature regime, but in the heat stress regime these plants demonstrated increased plant injury for both herbicides (Fig. 4). Together, the *in vitro* and in-plant results confirmed that temperature sensitivity of the FT_5 enzyme contributed to incomplete FOP and 2,4-D herbicide tolerance that was observed in maize field experiments. Therefore, enzyme optimization was conducted with a focus on improving temperature stability.

3.3 Crystal structure analysis informed subsequent designs

To enhance our ability to engineer additional FT enzyme variants, crystal structures of several FT enzymes (discussed below) were analyzed. The engineered FT enzyme variants and RdpA were

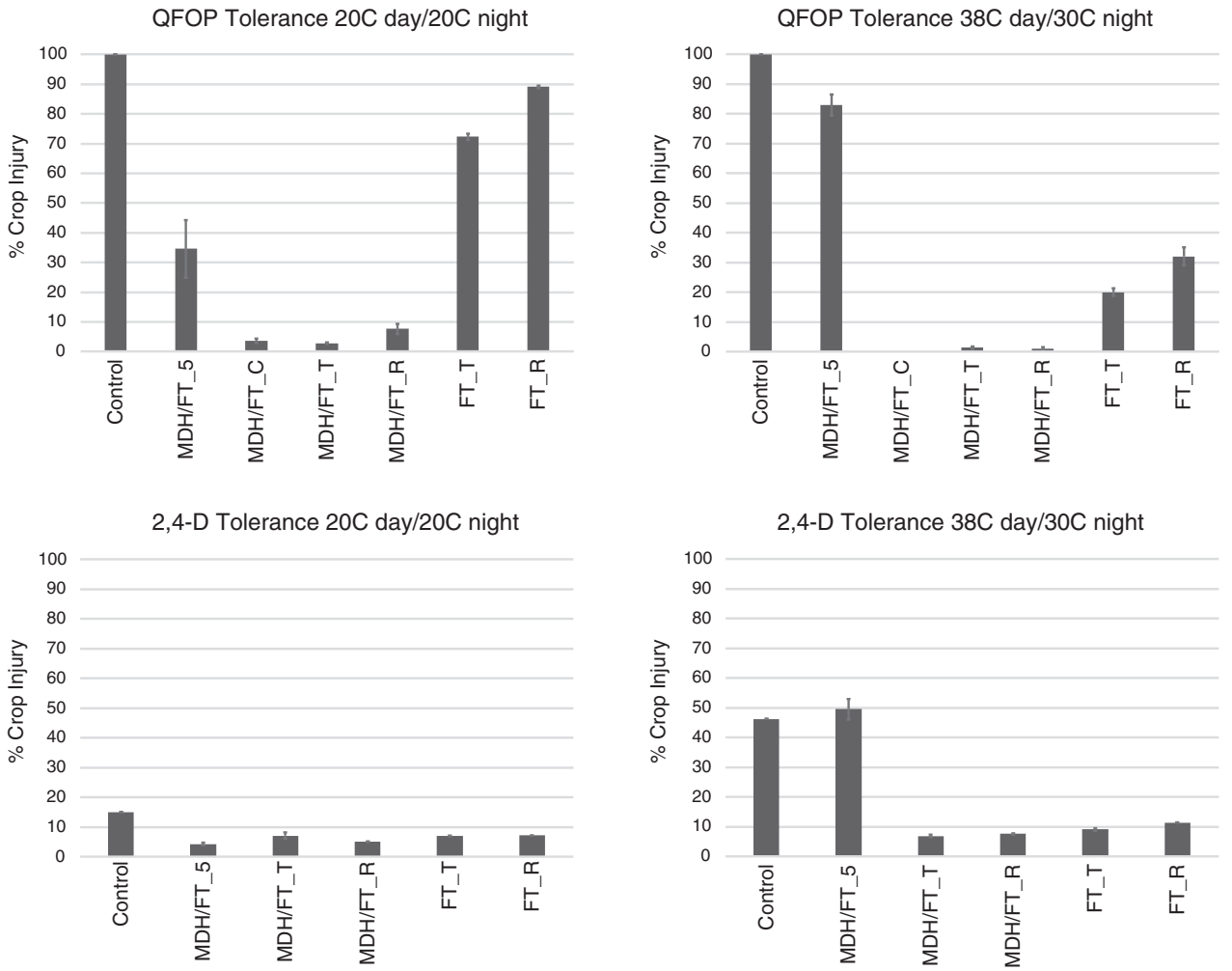

Figure 4. In-plant testing of FT enzymes in controlled environments. Controlled environment assays demonstrated differences in temperature stability of FT enzymes in maize plants. Plants were screened in non-stressed and heat-stressed conditions. Plants were grown to approximately V2 growth stage (early vegetative) and then moved to one of the two temperature regimes. After 2 days of acclimation, the plants were treated with either QFOP (4X, 360 g ha^{-1}) or 2,4-D (4X, 4500 g ha^{-1}) applications and rated for percentage injury 1 week after spray. Mean injury scores for eight replications shown with SEM indicated.

crystalized in a homotetramer asymmetric unit that contained a classical dioxygenase active pocket including a cobalt atom (replacing an iron atom found in the biologically active enzyme) coordinated by two histidine residues and one aspartic acid residue (Fig. 5 and see the supporting information, Fig. S1(a–d)). The substrates QFOP and α-KG were found to dock in the active pocket in an expected orientation for dioxygenase enzymes. A flexible loop caps the active pocket and was only observed to be well-ordered in crystal structure analysis when the enzyme contained a cobalt atom and both α-KG and a herbicide substrate.

Crystal structure analysis of the FT_5 enzyme was attempted to get a better understanding of the temperature instability. However, FT_5 was found to crystalize poorly, likely due to the instability of the enzyme (Fig. S1(e)). The enzyme could not be co-crystalized with the metal atom or substrates, and the flexible loop that was observed to become well-ordered in the crystal structures of other more stable FT variants upon substrate binding remained disordered in the FT_5 crystals. This disordered nature of the FT_5 enzyme is consistent with the inherent instability observed at higher temperatures in the enzyme assays and supports our hypothesis that the instability of FT_5 is a primary cause

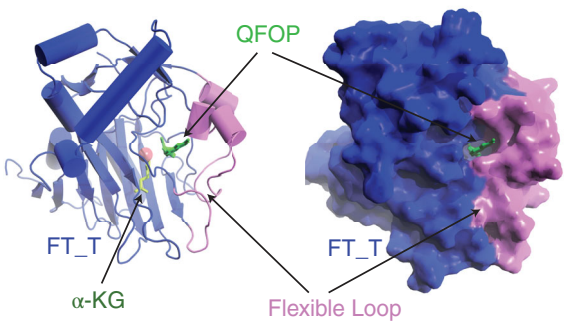

Figure 5. FT enzyme crystal structure. X-ray crystallography was used to solve the structure of FT enzymes (FT_T shown in both ribbons and space fill views). The binding of the co-substrates α-KG and QFOP (shown in light and dark green) resulted in an ordering of loops surrounding the active pocket (highlighted in purple) that also contained a metal ion. Cobalt is used to replace the iron atom during crystallization. The view as a monomer is shown for clarity.

of reduced tolerance at higher temperatures in plants. Comparison of crystal structures of the engineered variants and RdpA helped enable additional engineering.

Table 1. Characterization of selected FT enzyme variants

			RdpA	FT_R	FT_T	FT_Tv3	FT_Tv7
		Buffer	43	55	58	57	57
	Protein melting	Buffer (Fe and α-KG)	53	58	62	62	61
FOP	Quizalofop	V_{max}	2.76 (0.11)	1.60 (0.05)	1.62 (0.05)	1.51 (0.05)	1.53 (0.05)
		K_m	0.09 (0.01)	0.09 (0.01)	0.12 (0.01)	0.12 (0.01)	0.14 (0.01)
		k_{cat}/K_m	1.04	0.61	0.46	0.43	0.37
Synthetic auxins	Dichlorprop	V_{max}	5.32 (0.09)	3.03 (0.06)	2.09 (0.03)	1.29 (0.02)	1.04 (0.01)
		K_m	0.06 (0.004)	0.06 (0.01)	0.03 (0.002)	0.04 (0.003)	0.03 (0.002)
	2,4-D	V_{max}	0.25 (0.01)	0.63 (0.02)	1.17 (0.01)	3.38 (0.05)	2.91 (0.04)
		K_m	0.13 (0.02)	0.05 (0.01)	0.03 (0.002)	0.08 (0.004)	0.07 (0.004)
		k_{cat}/K_m	0.06	0.43	1.33	1.44	1.42
	MCPA	V_{max}	0.20 (0.01)	ND	1.23 (0.02)	3.64 (0.07)	2.93 (0.06)
	Mecoprop	V_{max}	4.11 (0.15)	ND	3.04 (0.07)	1.78 (0.03)	1.28 (0.02)

Lead FT variants and RdpA as a control are characterized for enzyme temperature stability and enzyme kinetics with FOP and synthetic auxin herbicides. Protein melting is recorded as the temperature (°C) in which the protein is 50% denatured in buffer alone or with supplemental Fe(II) and α-KG. Enzyme kinetic parameters are recorded for representative FOP and synthetic auxin herbicides with V_{max} (μmol mg^{-1} min^{-1}), K_m (mM) and k_{cat}/K_m (M^{-1} min^{-1}) shown. The standard error is shown in parenthesis. ND, not determined.

3.4 Temperature stable FT variants are identified

To engineer variants with improved temperature stability, structure-based design was applied in addition to the engineering approaches described above. Structure-based design leveraged a focus on the loops identified above and computational design. High-throughput screens were modified to include elevated temperatures and data-driven design enabled combinations of positive variants.

The temperature stable FT_C variant was identified with enhanced temperature stability *in vitro*. FT_C had an approximately 20 °C increase in protein melting temperature over FT_5 and maintained enzymatic activity towards QFOP (data not shown). Therefore, the FT_C variant was used as a base variant for enzyme engineering with the goal of maintaining good temperature stability while improving enzyme activity, particularly for 2,4-D as a substrate. We hypothesized that optimizing 2,4-D activity would be essential for building a 2,4-D tolerance trait in soybeans, a crop that is more sensitive to this herbicide relative to maize.

The new engineered FT variants, FT_T and FT_R, showed enhanced temperature stability with improved enzymatic activity (Table 1). Activity assays with QFOP and 2,4-D as substrates were completed with variants FT_T and FT_R (with RdpA as a control) in a range of reaction temperatures. In these assays, RdpA lost >50% activity towards QFOP at 40 °C while both FT_T and FT_R maintained >50% activity at 40 °C relative to control reactions at 25 °C (data not shown). Activity assays were also completed in which the FT_T and FT_R variants were preheated at selected temperatures (25–95 °C) before returning to 25 °C for an activity assay with QFOP. In these assays, both variants maintained approximately 50% activity with a 45 °C preheat (relative to the 25 °C preheat), further demonstrating robust temperature stability over a broad temperature range (Fig. S2). The enzymes were irreversibility inhibited at temperatures above 75 °C in this assay, consistent with the observed protein melting temperatures of approximately 60 °C (Table 1). In addition, enzymatic analysis using HPLC and mass spectrometry to confirm the reaction products demonstrated that FT_T metabolized QFOP and 2,4-D in the same manner as RdpA (Fig. S3).

3.5 FT variants with optimized 2,4-D activity are identified

FT_T showed a distinct improvement in activity towards 2,4-D relative to RdpA in the *in vitro* assays (Table 1). However, we were interested in additional engineering to determine if 2,4-D activity could be further optimized to enhance activity and crop tolerance, especially for soybean. Additional enzyme engineering, using FT_T as a base scaffold with computational protein design focused on substrate binding, resulted in the creation of variants, including FT_Tv3 and FT_Tv7. These variants had enhanced enzymatic activity when tested with 2,4-D as a substrate (Table 1), activity levels similar to SdpA, a dioxygenase characterized as having stronger activity on 2,4-D relative to RdpA (data not shown;[6,9]).

The FT variants were further characterized with FOP and auxin family herbicide chemistries (Table 1). The FT variants displayed lower activity with QFOP relative to RdpA. However, as discussed below, FT variants' FOP activity was suitable for robust in-plant tolerance, but 2,4-D activity needed to be optimized. The optimized FT variants showed enhanced 2,4-D activity with FT_Tv3 and FT_Tv7 showing a greater than 10-fold V_{max} improvement over RdpA. The 2,4-D and MCPA chemistries have two-carbon side chains that are cleaved by FT, while dichlorprop and mecoprop have three-carbon side chains identical to the FOP chemistries. The FT_Tv3 and FT_Tv7 variants have been optimized with a modified substrate preference based on the cleaved side chain, relative to RdpA. During structural analysis, several unsuccessful attempts were made to co-crystalize RdpA or FT_T with 2,4-D. FT_Tv7 was successfully co-crystalized with 2,4-D, suggesting that this variant binds 2,4-D in its active pocket better than RdpA or FT_T (Fig. S1(f)).

3.6 In-plant controlled environment and field testing demonstrates robust performance of FT variants

In-plant testing was used to further characterize selected variants. In maize, the temperature-stable variants displayed improved heat tolerance in controlled growth chamber experiments with low

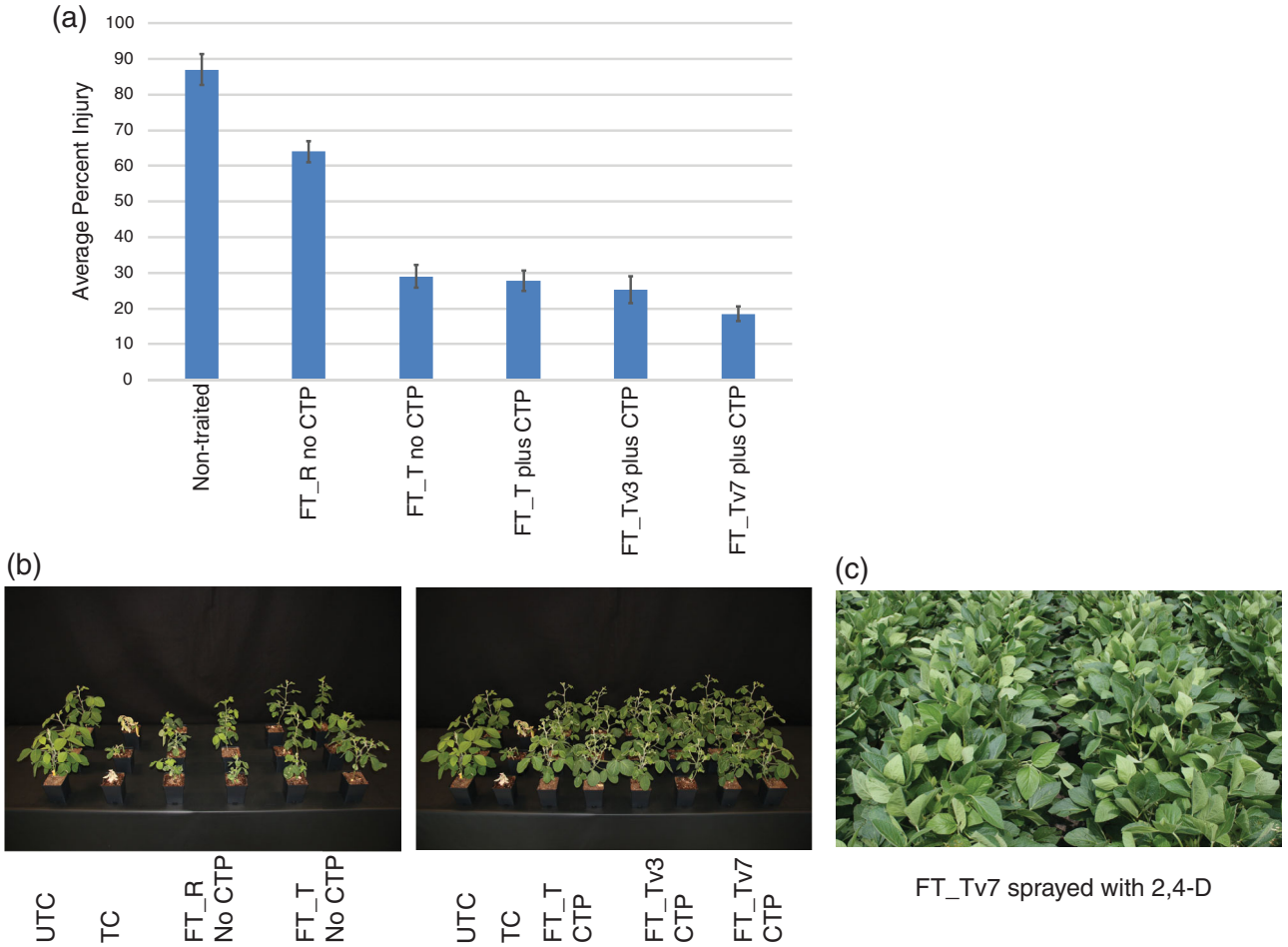

Figure 6. FT enzymes enable robust 2,4-D tolerance in soybeans. (a) Greenhouse 2,4-D spray assays in soybean demonstrated tolerance to 2,4-D in soybeans expressing FT_T, FT_Tv3 or FT_Tv7, with or without a CTP relative to control plants. Soybean lines expressing the indicated FT variant, or control plants, were treated with 2,4-D (1680 g ha^{-1}). Error bars indicate SEM. (b) Images of representative plants from (a). UTC, untreated control plants; TC, treated control plants. Control plants are in single rows of three plants; experimental plants are in double rows of three plants each. Treated control plants show extreme stunting, tissue necrosis and chlorosis. FT_R plants show some stunting and chlorosis. FT_T, FT_Tv3 and FT_Tv7 plants only show limited epinasty/malformation of upper stems and leaves. (c) Field view of FT_Tv7 expressing soybean plants following two applications (V3 and R1 growth stages) of 2,4-D (2240 g ha^{-1}) demonstrating 2,4-D tolerance.

injury ratings for both the non-stressed and heat-stressed treatments (Fig. 4). This observation confirms that the *in vitro* temperature stability of these enzyme variants directly translated to tolerance stability in plants.

Field plots with maize expressing the indicated variants were sprayed with QFOP and 2,4-D herbicides. Both QFOP and 2,4-D were tested at rates up to 16-fold over recommended agricultural field rates with repeated applications at several growth stages. In the treatments with QFOP, the control plants were completely killed while the temperature-stable variants (FT_C, FT_T and FT_R) showed little injury (Fig. 3(a) and (b)). FT_5 showed injury that increased at higher herbicide rates, demonstrating incomplete tolerance. In treatments with 2,4-D, a similar trend as with QFOP was observed (Fig. 3(c) and (d)). Maize is naturally partially tolerant to 2,4-D so complete plant death was not observed, but typical 2,4-D injury, including malformation, lodging and stunting in the control plants, was observed. Plants expressing the temperature-stable variants displayed excellent tolerance to 2,4-D, while plants expressing the FT_5 variant demonstrated incomplete tolerance at high 2,4-D rates. This demonstrated that the temperature-stable variants provided excellent tolerance to both QFOP and 2,4-D herbicide applications under field conditions in maize. Maize plants were also sprayed with clethodim, a herbicide with the same mode of action as QFOP, but a different chemical family (cyclohexanedione, DIM). As expected, no tolerance was observed for clethodim-treated FT_T expressing maize plants (data not shown). Therefore, DIM herbicides can control volunteer maize plants expressing FT in a maize-soybean crop rotation.

Maize plants were transformed to express FT variants coupled with the MDH CTP or without a CTP. As discussed above, the in-plant assays focused on using FT enzymes coupled with a CTP based on the chloroplast localization of the herbicide target. The mode of action for 2,4-D is not known to target the chloroplasts, suggesting a CTP was unlikely to improve 2,4-D tolerance.[17,18] To test these hypotheses on sub-cellular targeting, maize plants were also generated expressing FT enzymes without a CTP. The CTP-targeted FT demonstrated robust tolerance to both QFOP and 2,4-D, while the non-targeted versions of the FT enzymes (no targeting peptide) showed robust 2,4-D tolerance with reduced QFOP tolerance (Fig. 4). In the high-temperature regime, rapid growth rates appeared to somewhat, but not fully, compensate for QFOP injury observed in the non-targeted FT expressing plants. In the field-based testing, greater injury was also observed at higher QFOP rates in plants expressing FT without a CTP than FT

with a CTP (Fig. 3(a)), further suggesting that proper sub-cellular targeting of the FT enzyme improves tolerance to FOP herbicides. In addition, we also noted some increase in FT enzyme expression when FT is coupled with a CTP, suggesting this may, in part, contribute to enhanced tolerance. However, in the treatments with 2,4-D, FT enzymes with or without a CTP showed comparable performance, suggesting that the 2,4-D chemistry can be effectively deactivated both within and outside the chloroplast to provide 2,4-D herbicide tolerance (Fig. 3(c)).

In maize, the FT variants provided robust tolerance to 2,4-D herbicide applications. However, we were also interested in determining if the improved 2,4-D activity characterized for some of the temperature-stable FT variants could provide tolerance in soybeans to 2,4-D (a more sensitive crop). Therefore, we transformed a commercially relevant soybean line, A3555, using *Agrobacterium*-mediated transformation into excised embryos with several different FT enzyme variants, both with and without a CTP. Soybean plants expressing these variants were treated with 2,4-D applications. Similar to what was observed in maize, the presence or absence of the CTP did not significantly change plant 2,4-D tolerance. FT_R, a variant with improved 2,4-D activity relative to RdpA (but less activity than FT_T, FT_Tv3 and FT_Tv7; Table 1), was unable to provide good 2,4-D tolerance in soybean, with injury rates above 50%. However, FT_T, FT_Tv3 and FT_Tv7 demonstrated 2,4-D tolerance in soybean with injury rates below 30% (Fig. 6(a) and (b)). FT_Tv7 also showed commercially relevant 2,4-D tolerance in field applications of 2,4-D (Fig. 6(c)). These data support the hypothesis that engineering FT variants with optimized 2,4-D activity was essential to providing full tolerance in crops, especially sensitive crops such as soybean.

4 DISCUSSION AND CONCLUSIONS

Successful weed management is essential for ensuring robust crop yields. Here we report the successful development of engineered FT enzymes that can provide herbicide tolerance in crops to two distinct families of herbicides. Weed resistance for many herbicide families (including FOP and synthetic auxins) is already present in agricultural production areas. Therefore, successful utilization of this trait, and any herbicide tolerance trait, requires a systems-based approach to weed management. This approach includes careful consideration of the weeds present, the biology and herbicide resistance of the weed populations, the agronomic and cultural practices available and the use of combinations of herbicides encompassing multiple modes of action. The FT herbicide tolerance trait can be paired with other herbicide tolerance traits and other selective herbicides to enable additional options for farmers in their holistic weed management systems.

Ever-expanding genomics and proteomics knowledge from a variety of sources, including microbes, provides a rich source of enzyme scaffolds that can be utilized in biotechnology to design optimized enzymes. These resources can be leveraged in the optimization of enzymes for agronomically relevant properties, such as temperature stability of the FT enzymes, which may be essential for robust performance in a field setting where plants are exposed to wide temperature ranges. Another optimization target is modulating enzymatic activity with substrates of interest. For example, the FT enzymes provide tolerance to members of two herbicide families, FOP (QFOP) and synthetic auxin (2,4-D) herbicides. In conclusion, a microbial enzyme scaffold served as a starting point for the engineering of FT enzyme variants that were optimized for both enzyme activity on preferred herbicide chemistries and for enzyme temperature stability. In-plant analysis demonstrated that both elements were essential to provide robust herbicide tolerance to two herbicide families in maize and soybean crops.

SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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