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3 **Main Manuscript for**

4 **A Fungal Natural Product that Targets Cellulose Synthase Complex** 5 **and Inhibits Plant Cellulose Biosynthesis**

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25 **Author Contributions:** Z.W., L.L., Y.T. and S.E.J. designed the research, interpreted data, and
26 wrote the manuscript; Z.W. performed most experiments. L.L. performed the NGS analysis,
27 confocal analysis and generated multi-drugs resistant mutants and wrote the manuscript. W.H.
28 and Z.S. isolated MDD and related compounds. X.C. and P.X. performed some confocal analysis.
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35 Main Text

36 Figures 1 to 6

37 **Abstract**

38 Cellulose, a primary component of plant cell walls, is synthesized by cellulose synthase
39 complexes (CSCs) at the plasma membrane. Targeting this process with cellulose biosynthesis

inhibitors (CBIs) has significantly advanced our understanding of plant cell wall formation and provided valuable compounds for herbicide development. Here, we identified a fungal natural product, 8-methyldichlorodiaporthin (MDD), as a broad-spectrum plant CBI. Structure-activity relationship analyses demonstrate that methylation modifications on the isocoumarin ring and chlorination of side chain are crucial for MDD-induced growth inhibition. A chemical forward genetic screen in *Arabidopsis thaliana* revealed two semi-dominant CESA1 mutations, A903T and H1024Y, that confer insensitivity to MDD. Both mutations locate to transmembrane domains of CESA1, and we show that MDD depletes CSCs from the plasma membrane and reduces cellulose content. Further genetic analyses indicate that the *cesa1^{mdt1-1}* A903T mutant also confers resistance to CBIs quinoxiphen and C17, but not to CBIs isoxaben, indaziflam, or ES20. Stacking additional point mutations conferring resistance to other CBIs, *cesa6^{jxr2-1}* R1064W, and *cesa6^{es20-r3}* G935E into the *cesa1^{mdt1-1}* A903T background yields multiple-drugs resistant lines that maintain normal growth. These findings establish MDD, as a novel, natural CBI that targets CESA1, thereby extending our understanding of CSC regulation and developing multi-drugs resistant crop varieties. These findings offer new perspectives for weed management and plant biotechnology.

Significance Statement

Cellulose, a fundamental structural component of plant cell walls, is synthesized by cellulose synthase complexes (CSCs) and represents a critical herbicide target. While synthetic cellulose biosynthesis inhibitors (CBIs) like isoxaben and quinoxiphen have elucidated CSC function and aided in weed control, natural CBIs remain largely undiscovered. Here, we identify 8-methyldichlorodiaporthin (MDD), a fungal-derived isocoumarin, as a natural CBI that inhibits plant growth by targeting CESA1 and depleting CSCs from the plasma membrane. Genetic screens reveal MDD-resistant *cesa1* mutations, combining these with other CBI-resistant alleles yields multi-herbicide resistant plants that can grow normally. This research enhances our understanding of cellulose biosynthesis, aiding the discovery of bioactive compounds, and paving the way for multi-herbicide resistant crops with agricultural benefits.

Main Text

Introduction

Cellulose microfibrils, crystalline polymers of β -1,4-D-glucose, play a vital role in providing structural support and rigidity to plant cell walls. Within the plasma membrane, multiple monomeric cellulose synthases (CESAs) form a larger rosette-like structure known as the cellulose synthase complex (CSC) and are responsible for cellulose production (1-4). In *Arabidopsis*, the CSC that synthesizes cellulose in the primary cell wall consists of CESA1, CESA3, and either CESA6 or a CESA6-like subunit (CESA2, CESA5, or CESA9), whereas the CSC in the secondary cell wall is composed of CESA4, CESA7, and CESA8 (5-8).

Cellulose biosynthesis inhibitors (CBIs) are chemical compounds that disrupt the synthesis of cellulose in plants, thereby impacting their growth and development. These inhibitors can affect different stages of cellulose production, including the activity of cellulose synthases and the assembly of CSC. As a result, CBIs inhibit plant growth, cause cell swelling and/or affect CSC subcellular localization (9-11). Isoxaben, flupoxam, triazofenamide, C17, and quinoxiphen are five well-known CBIs that remove CSCs from the plasma membrane, hindering root growth and cellulose synthesis. All of these compounds have also been demonstrated to have inhibitory effects on a range of plant species, with isoxaben being widely used as an herbicide to control broadleaf weeds (6, 12-20). P4B was recently identified as a novel CBI through a chemical screen for plant growth inhibitors (21). Certain single amino acid mutations in the CESA3 and CESA6 proteins, such as *cesa3^{jxr1-1}* G998D, *cesa3^{jxr1-2}* T942I, *cesa6^{jxr2-1}* R1064W, confer resistance to isoxaben (19, 22). Similarly, mutations in CESA1 and CESA3, like *cesa1^{1B}* A1023T,

cesa1^{18A1} S307L and *cesa3^{8P} S1037F* can enhance resistance to C17 (17). A substitution of alanine with valine at position 903 in CESA1 (*cesa1^{aegus} A903V*) provides resistance to quinoxiphen (20), whereas a leucine-to-phenylalanine mutation at position 883 in CESA3 (*cesa3^{br1} L883F*) confers resistance to P4B (21). Interestingly, most mutations that confer resistance to these CBIs are located in transmembrane regions of CESA proteins (9, 18, 21, 23). The *cesa1^{aegus} A903V* and *cesa3^{ixr1-2} T942I* mutations increase the velocity of CSC complex reaction, but reduce cellulose microfibril crystallinity and crystalline cellulose content, leading to a more amorphous and mobile cellulose-like structure (20).

In contrast, Endosidin20 (ES20), another CBI, inhibits cellulose biosynthesis by directly targeting the catalytic site of CESA6. Mutants with point mutations in the central catalytic domain of CESA6 exhibited reduced sensitivity to ES20 (24). Indaxiflam, another CBI, increases the number of CSCs at the plasma membrane but still inhibits cellulose production (25). Morlin, which interferes with microtubule dynamics, indirectly affects the movement paths of CSCs at the plasma membrane (26). Notably, mutants that confer resistance to specific CBIs do not typically exhibit cross-resistance to other types (12, 18, 27-29). The diverse modes of action of CBIs have enhanced our understanding of cellulose biosynthesis and are useful for weed control in agriculture.

Bioactive natural products, produced by nearly all organisms but particularly by microbes, are significant small molecules due to their diverse biological properties and potential applications in medicine, pharmaceuticals, and agriculture (30-33). In microbial genomes, biosynthetic genes are typically found in biosynthetic gene clusters (BGCs). Advances in genomics and metabolomics have enabled the discovery of new compounds and their biological targets, (34-36). For example, expressing a relatively well-conserved fungal BGC found in diverse fungi in the heterologous *Aspergillus nidulans* host led to the successful production of isocoumarin compounds 8-methyldichlorodiaporthin (MDD) and dichlorodiaporthin (DD) (37). These are polyketide derived compounds that have undergone significant modifications, including *gem*-dichlorination, methylation and C-C bond cleavage. While these compounds were previously isolated and rediscovered through genome mining efforts (37, 38), the biological activity and target remained elusive. Synthetic isocoumarin compounds have been shown to be potential herbicides (22). Given the ecological niche of fungi in plant-fungal interactions, we hypothesized that MDD and/or DD could have potential roles in inhibiting key plant pathways and could become leads for herbicides.

Here, we discovered that MDD is a broad-spectrum plant growth inhibitor. Structure-activity relationship analysis revealed the critical roles of aforementioned chemical features, including both 6-OMe and 8-OMe groups, and the *gem*-dichloro group in effective root growth inhibition. A forward chemical genetic screen in *Arabidopsis thaliana* revealed that mutations in CESA1 (*cesa1^{mdt1-1} A903T* and *cesa1^{mdt1-2} H1024Y*) confer resistance to MDD. MDD inhibits cellulose biosynthesis and depletes CSCs from the plasma membrane. The *cesa1^{mdt1-1} A903T* mutant is resistant to an additional two CBIs, quinoxiphen and C17. Additionally, we have successfully developed mutants with resistance to four CBIs by combining *cesa1^{mdt1-1} A903T* with *cesa3^{ixr1-1} G998D* or *cesa6^{ixr2-1} R1064W* or *cesa6^{es20-r3} G935E*. Moreover, a triple mutant combining *cesa1^{mdt1-1} A903T*, *cesa3^{ixr1-1} G998D* and *cesa6^{es20-r3} G935E* shows resistance to five CBIs, MDD, quinoxiphen, C17, isoxaben and ES20. The bioactivity study of MDD not only expands herbicide options for weed control but also helps mitigate herbicide-driven resistance evolution in grasses by enabling the rotation or combination of alternative modes of action.

Results

MDD is a broad-spectrum plant growth inhibitor.

The herbicidal activity of MDD (Figure 1A) was discovered through an *Arabidopsis*-based screening of a collection of natural fungal compounds obtained from our genome mining efforts.

MDD, a known natural product, was rediscovered through biosynthesis in *A. nidulans* by heterologous reconstitution of the *dia* BGC originally found in *Aspergillus oryzae*. The defining structural features of MDD include an isocoumarin core derived from a nonreducing polyketide synthase; a *gem*-dichloro group derived from a flavin-dependent halogenase and two methoxy groups at C-6 and C-8 from SAM-dependent O-methyltransferase.

The presence of MDD resulted in significant inhibition of *Arabidopsis* wild-type (WT) Col-0 hypocotyl and root growth in a dose-dependent manner (Figure 1B-C and Fig. S1A-B). The concentration of MDD that caused a 50% reduction in hypocotyl and root growth (IC₅₀) was determined to be 3.62 μ M and 1.76 μ M, respectively (Figure 1B-C). When exposed to MDD, hypocotyl and root cells exhibited severe swelling (Figure 1D-E and Fig. S1C-D), indicated by a notable decrease in hypocotyl cell length and an increase in hypocotyl cell width (Figure 1D and Fig. S1C) and by an increase in root cell length and width (Figure 1E and Fig S1D). Transcriptomic profiling revealed that MDD downregulated genes were significantly enriched in core cell cycle processes, particularly mitotic cell cycle and cytokinesis, consistent with its growth-inhibitory effects and cellular swelling phenotype (Figure 1F). Additionally, MDD significantly inhibited the growth of various dicotyledonous and monocotyledonous plant species, including tobacco, tomato, and maize (Figure 1G), demonstrating that MDD is an effective broad-spectrum plant growth inhibitor.

To further investigate the structure-activity relationship of MDD-induced plant growth inhibition, *Arabidopsis* plants were treated with MDD and its structural analogues (Figure 2A). These MDD analogues (DD and MA2-9) are either on-pathway intermediates or shunt products, isolated during the heterologous biosynthesis experiments (22). Notably DD is the C-8 desmethyl version of MDD and is also a known natural product from previous isolation (37). Formation of DD instead of MDD is attributed to the lack of one methyltransferase in the corresponding BGCs compared to that of MDD (37). Based on the structure-activity relationship studies, only DD and MA-6 caused significant inhibition, reducing WT root growth by 40% and 50% respectively (Figure 2B-C). The variations in root length among plants treated with MDD, DD, and MA-2 highlight the critical roles of the 6-OMe and 8-OMe groups in effective root growth inhibition. Additionally, the unique *gem*-dichloro moiety on the isocoumarin C-3 alkyl chain plays a pivotal role in the observed biological activity, since nonchlorinated analogues MA-5, MA-6 and MA-7 exhibited weaker or negligible inhibitory effect. Among them only MA-6 showed the strongest growth inhibition, likely due to its longer side chain that mimics the size of the *gem*-dichloro group in MDD.

Resistance to MDD is conferred by semi-dominant CESA1 alleles.

To elucidate the mode of action of MDD in plant inhibition, a chemical forward genetic screen was conducted to identify mutants that are insensitive to MDD. Approximately 5,000 *Arabidopsis* WT seeds were mutagenized using ethyl methanesulfonate (EMS), and approximately 30,000 M2 seeds were screened for growth insensitivity to MDD. *mdai1-1* and *mdai1-2* (*MDD-insensitive*) mutants were isolated, exhibiting a loss of sensitivity to MDD (Figure 3A-B and Fig. S1A-B).

To map the *mdai* mutants, homozygous *mdai* mutants were backcrossed with WT to create a mapping population. Sixty F₂ plants that were insensitive to MDD were selected for Illumina sequencing. Single nucleotide polymorphism (SNP) frequency analysis identified a single causal region for both *mdai* mutants located at the end of chromosome 4 (Fig. S2A). Detailed analysis of mutations in each *mdai* mutant revealed that *CESA1* (*AT4G32410*) is the only gene with mutations in both mutants. The *mdai1-1* mutant carries a G-to-A mutation at nucleotide 2707, resulting in an Ala903Thr substitution in the fourth transmembrane domain (Figure 3C). The *mdai1-2* mutant has a C-to-T change at nucleotide 3070, causing a His1024Tyr substitution in the seventh transmembrane domain (Figure 3C). The mutated histidine at position 1024 is absolutely conserved and alanine at position 903 is highly conserved among primary cell wall CESAs (*CESA1*, 3, 6, 2, 5, 9). Additionally, both residues are flanked by highly conserved residues (Figure 3D).

When *mda1-1* and *mda1-2* were backcrossed with WT, the resulting F1 plants were partially resistant to MDD, indicating that the *mda* mutations are semi-dominant (Fig. S2B). To confirm that mutations in CESA1 are responsible for the MDD-insensitive phenotype, we performed a transgene complementation analysis. We introduced N-terminal YFP-tagged versions of CESA1 with the A903T or H1024Y mutations into heterozygous *cesa1* knockout mutants (*SAIL_278_E08*). This completely rescued both the lethality of the *cesa1* null mutant (Fig. S2C) and restored chemical sensitivity of plants to MDD (Figure 3A-B). However, the introduction of WT CESA1 did not restore sensitivity to MDD (Figure 3A-B). These findings indicate that the missense mutations in CESA1 are sufficient to confer tolerance to MDD.

MDD depletes CSCs from the plasma membrane and inhibits cellulose biosynthesis.

CESA1 encodes a cellulose synthase isomer, which colocalizes with CESA3 and CESA6 to the plasma membrane and form CSC involved in microfibril production (1). We examined the localization and dynamics of CSC complexes under normal conditions and following MDD treatment. In ethanol treated hypocotyl epidermal cells, GFP-CESA3 was prominently associated with the plasma membrane. However, when treated with MDD for 5 hrs, the GFP-CESA3 signals at the plasma membrane were significantly reduced (Figure 4A-B), suggesting that MDD depletes CSC complexes from the plasma membrane. Consistent with this observation, MDD treatment decreased the crystalline cellulose content in light-grown seedlings (Figure 4C). Both *cesa1^{mda1-1}* A903T and *cesa1^{mda1-2}* H1024Y mutants exhibited slightly lower cellulose content as compared to wild type Col-0, but were insensitive to MDD inhibition (Figure 4C). Additionally, treatments with MDD greatly reduced the velocity of labeled CSC complexes (Figure 4D). Moreover, we monitored the delivery of GFP-CESA3 to the plasma membrane by fluorescence recovery after photobleaching (FRAP). The rate of CSC plasma-localization was significantly reduced when plants treated with MDD compared to ethanol-treated hypocotyl epidermis (Figure 4E). These results collectively indicate that MDD depletes CSCs from the plasma membrane and inhibits their role in cellulose biosynthesis.

MDD sensitivity remains unaltered by CSC complex quantity or regulatory mechanisms at the plasma membrane.

CSCs are assembled in the Golgi and subsequently transported to the plasma membrane with the assistance of vesicle trafficking proteins and the trans-Golgi network (39). CESA INTERACTIVE PROTEIN 1 (CSI1), also known as POM-POM2 (POM2), links the CSC to cortical microtubules, guiding the movement of the CSC (40, 41). PATROL1 (PTL1) interacts with CSI1/POM2 and exocyst subunits to facilitate the delivery of CSC vesicles to the plasma membrane (42). In contrast, the SHOU4 protein regulates cellulose synthesis by limiting CSC exocytosis, and increased abundance of CESA proteins were observed at the plasma membrane in the *shou4* mutant background (43). To investigate whether MDD impacts these CSC-mis-accumulation mutants at the plasma membrane, we planted the *csi1-3* (SALK_138584), *patrol1-2* (SALK_018676C), and *shou4-3* (GK793F10) T-DNA insertion lines on ½ MS plate with MDD. However, none of these mutants were resistant to MDD (Fig. S3A-B), suggesting that MDD may also affect the functionality or operational efficiency of the CSCs, despite its pronounced effect on their abundance at the plasma membrane.

MDD inhibits cellulose biosynthesis using the same mode of action as quinoxiphen and C17 but operates differently than isoxaben, indaziflam, and ES20

The chemical structures of CBIs, including isoxaben, indaziflam, C17, and ES20 are quite distinct (Figure 1A and Fig. S4), yet all these compounds can inhibit cellulose biosynthesis (12, 17, 24, 25). To explore whether other known CBIs share the same mechanism of action, we examined the toxicity of MDD on *Arabidopsis* mutants resistant to established CBIs. Isoxaben-resistant mutants *cesa3^{ixr1-1}* G998D, *cesa3^{ixr1-2}* T942I, *cesa6^{ixr2-1}* R1064V have mutations in the C-terminal transmembrane regions of the corresponding CESAs, where the *mda* mutations are found.

However, none of these mutants showed resistance to MDD (Fig. S5A). ES20 inhibits cellulose biosynthesis by targeting the catalytic domain of cellulose synthase, and most mutations that reduce sensitivity to ES20 are located in the central cytoplasmic domain. None of the tested ES20-resistant (*es20r*) mutants exhibited resistance to MDD (Fig. S5B). Interestingly, two C17-resistant mutants (*cesa1^{9R} G1013R* and *cesa1^{18A1} S307L*) showed partial insensitivity to MDD (Fig. S5C). The mutated glycine at position 1013 and serine at 307 are absolutely conserved and flanked by several highly conserved residues (Fig. S5D).

We also tested whether the *mddi* mutants exhibit altered sensitivity to other CBIs such as isoxaben, quinoxiphen, C17, and ES20. When treated with 10 nM isoxaben, or 1 μ M ES20, the *mddi* mutants were inhibited to a similar level as the WT plants (Fig. S6A-B). In contrast, the *cesa1^{mddi1-1} A903T* mutant showed complete resistance to 5 μ M quinoxiphen and 100 nM C17, while the *cesa1^{mddi1-2} H1024Y* mutant was completely resistant to 5 μ M quinoxiphen but remained susceptible to 100 nM C17 (Figure 5A-B). Notably, *cesa1^{mddi1-1} A903T* contains a mutation at the same residue as *cesa1^{aegaeus} A903V*, which is the only reported quinoxiphen-insensitive mutant (20). The insensitivity of *cesa1^{mddi1-1} A903T* to quinoxiphen and C17 also suggest that these two CBIs may use the same mode of action. Indeed, C17 insensitive mutants (*cesa1^{9Q} L872F*, *cesa1^{14U} G1013E*, *cesa1^{9R} G1013R* and *cesa1^{18A1} S307L*) showed partial resistant to quinoxiphen (Fig. S7A-B). The differing sensitivities of the *mddi* mutants to these CBIs suggest that MDD may share common characteristics in affecting cellulose synthesis with quinoxiphen and C17, but not with isoxaben, indaziflam, or ES20.

Generation of multiple CBIs-resistant mutants.

The *cesa1^{mddi1-1} A903T* mutant exhibited resistance to multiple CBIs, including quinoxiphen, C17, and MDD (Figure 3A-B and Figure 5A-B). To further enhance plant resistance, we attempted to stack the isoxaben-resistant mutation *cesa3^{ixr1-1} G998D* or *cesa6^{ixr2-1} R1064W* with *cesa1^{mddi1-1} A903T*. The resulting double mutants, *cesa1^{mddi1-1} A903T cesa3^{ixr1-1} G998D* or *cesa1^{mddi1-1} A903T cesa6^{ixr2-1} R1064W*, were able to tolerate combination treatments that included 10 μ M MDD, 10 nM isoxaben, 5 μ M quinoxiphen, and 50 nM C17 (Fig. S8A-B). When we reduced the concentrations of the inhibitors by 75%, the single mutants remained sensitive to the CBIs, while the double mutants maintained complete resistance (Fig. S8A-B). We further expanded the resistance by incorporating the ES20-resistant mutant *cesa6^{es20-r3} G935E* with *cesa1^{mddi1-1} A903T*. Similarly, the resultant double mutant was also resistant to combinations of 10 μ M MDD, 1 μ M ES20, 5 μ M quinoxiphen, and 50 nM C17, even at a reduced concentration (Fig. S9A-B). Remarkably, a triple mutant composed of *cesa1^{mddi1-1} A903T*, *cesa3^{ixr1-1} G998D* and *cesa6^{es20-r3} G935E* achieved resistance to five distinct CBIs simultaneously, despite showing a slight inhibition, potentially due to higher concentrations of solvent (Figure 6A-B). To mitigate solvent side effects and decrease the effective herbicide concentration, we applied one-fifth of the original herbicide concentrations. Despite this reduction, the triple mutant continued to exhibit resistance to the five CBIs, while all single mutants remained susceptible (Figure 6A-B). Importantly, no noticeable plant growth inhibition was observed with the lower concentrations of solvent (Figure 6A-B). Although *cesa* null mutants were not viable, all CBI-resistant single and multiple mutants displayed wild-type morphology under our growth conditions (Figure 6C-D), demonstrating that stacking these mutations confer multiple CBIs resistance without compromising basal function of CSC complex and plant development.

Discussion

Bioactive natural products have long served as valuable resources for discovering novel herbicidal agents. CBIs are of particular interest as herbicide due to their ability to disrupt the formation of plant cell walls, thereby impairing plant growth. In this study, we identified MDD as a novel, natural CBI that inhibits plant cellulose synthesis by targeting the transmembrane domains of CESA1. MDD suppresses plant growth in both dicot and monocot species, suggesting broad-spectrum applicability. Furthermore, the generation of multiple-drugs resistant mutants through

stacking mutations in CESA subunits demonstrates a promising strategy for improving crop resistance to CBIs while informing fundamental mechanisms of cellulose biosynthesis.

Thaxtomin A, a phytotoxin produced by the plant-pathogenic bacterium *Streptomyces scabies*, is one of the most well-characterized natural CBI (44). It disrupts cellulose synthesis in expanding plant cells, leading to characteristic cell swelling and root growth defects. This toxin is directly responsible for causing potato scab disease (45, 46), highlighting its strong impact on plant microbe interactions. Similarly, MDD is a natural product, produced by *Aspergillus oryzae* and *Hamigera fusca*, with homologous BGCs conserved across various fungi species (37, 38). Further exploration of MDD's role in these fungi, particularly in plant-microbe interactions, could provide valuable insights into its ecological function. Structurally, thaxtomin A differs significantly from synthetic CBIs like isoxaben, quinoxiphen, or C17, yet it exerts similar phenotypic effects, including inhibition of cellulose deposition and perturbation of cell wall integrity disruption. Investigating the structural diversity of these natural and synthetic CBIs could pave the way for designing novel and more effective cellulose biosynthesis inhibitors for agricultural and biotechnological applications.

The observation that MDD depletes CSC complexes from the plasma membrane suggests that it might interfere with their normal delivery or maintenance, potentially by disrupting CSC trafficking or destabilizing the complexes themselves. However, the absence of resistance to MDD in mutants lacking key vesicle trafficking regulators—such as CSI1, PATROL1, and SHOU4—indicates that the quantity of CSCs at the plasma membrane is not the sole factor affecting sensitivity to MDD. It is possible that MDD alters membrane composition or integrity, or may influence other, yet unidentified, trafficking or anchoring processes. Interestingly, MDD, quinoxiphen, and C17 insensitive mutants exhibit a degree of cross-resistance, but not others, suggesting that some mutation sites in their insensitive mutants may serve as herbicide binding sites. However, due to the structural diversity of these herbicides, not all mutations for one herbicide necessarily behave the same way for another. As a result, these herbicides may disrupt the functionality or efficiency of CSCs, even while significantly affecting their abundance at the plasma membrane. This understanding emphasizes the complexity of the action of CBIs and highlights the need for further exploration into how MDD and other CBIs affects CSC functionality beyond simply altering their quantity at the plasma membrane.

The multiple drug-resistant mutants created here remained morphologically normal while exhibiting robust resistance to multiple CBIs—including isoxaben, ES20, MDD, quinoxiphen, and C17—at concentrations that severely inhibit WT plants. From a weed management perspective, engineering crops with resistance to multiple CBIs is highly advantageous. Given the lack of cross-resistance between different inhibitor-resistant mutants, employing a rotation or combination strategy with various CBIs can enhance weed control efficacy while mitigating the risk of herbicide-resistant weed emergence, a pressing issue in modern agriculture. Therefore, expanding the range of available herbicides—particularly those that target distinct residues within cellulose synthases—offers greater flexibility and sustainability in integrated weed management programs.

Materials and Methods

Plant materials and growth conditions

All *Arabidopsis* plants used in this paper are Col-0 ecotype. *N. benthamiana*, *S. lycopersicum*, *Z. mays* and *Arabidopsis* plants are grown under standard condition with 16 h light/8 h dark at 22 °C. The CBI resistant lines used in this study were reported previously (17, 19, 22, 47). The T-DNA insertion lines used in this study is *cesa1* heterozygous mutant (CS812877; *SAIL_278_E08*), *csi1-4* (48), *patrol1-2* (48) and *shou4-3*(49). Agrobacterium (AGL0 strain)-mediated floral dipping was used to generate all the transgenic plants. *pCESA1:YFP-CESA1*, *pCESA1:YFP-CESA1A903T* and *pCESA1:YFP-CESA1H024Y* constructs were transformed into *cesa1*

heterozygous mutant. 16 transgenic T1 plants were selected on ½ MS medium containing hygromycin. Two T2 homozygous transgenic plants were tested their sensitivity ½ MS plates with 10 µM MDD.

Compound isolation

Compound isolation was described previously (22). Briefly, the spores of *A. nidulans* transformed with corresponding plasmids were inoculated into 4 L CD-ST agar media and grown for 4 days at 28°C. The culture was extracted with 2 L ethyl acetate for two times and the solvent was evaporated to dryness under vacuum to obtain the crude extract. The crude extract was applied to normal-phase silica flash chromatography on a CombiFlash instrument with a gradient of ethyl acetate-hexanes (15:85 to 95:5). The fractions containing MDD were concentrated and subjected to reverse-phase C18 flash chromatography on a CombiFlash instrument with a gradient of CH₃CN-H₂O (40:60 to 60:40, 0.1% FA) to yield 31 mg of MDD (7.75 mg/L).

RNA-seq

One week old of Col-0 seedlings under ethanol or MDD treatment were collected and ground into a fine powder with liquid nitrogen. Total RNA was extracted using the Direct-zol RNA Miniprep kit (Zymo) according to the manufacturer's instructions. One microgram of total RNA was used to prepare the libraries for RNA sequencing (RNA-seq) following the TruSeq Stranded mRNA kit (Illumina), and the libraries were sequenced on NovaSeq X Plus instruments.

EMS Mutagenesis, mutant Screening and mapping

To obtain a mutagenized Arabidopsis population, approximately 5,000 Col-0 seeds were mutagenized with ethyl methanesulfonate (EMS) for 14 h. Roughly 30,000 M2 plants representing ~2,000 M1 families were grown on ½ MS medium with 10 µM MDD. To map *mdai* mutants, homozygous *mdai* mutants were backcrossed with Col-0 to generate a mapping population. Sixty F2 plants insensitive to MDD were selected for Illumina sequencing. Single nucleotide polymorphism (SNP) frequency was calculated.

Plasmid construction

For *pCESA1:YFP-CESA1*, *pCESA1:YFP-CESA1A903T* and *pCESA1:YFP-CESA1H024Y*, the genomic DNA sequences were first cloned into pENTR/D-TOPO vectors (Invitrogen) and then to the destination vector pEG302-GW by LR reaction (LR Clonase II, Invitrogen). Primers used in this study are presented in Supplementary Table 1.

Structural Modeling of the CESA1

The general topology of CESA6 was predicted using the UniProt (<https://www.uniprot.org>) and PredictProtein (<https://www.predictprotein.org>) servers, and the cartoon was drawn using the Protter program (<http://wlab.ethz.ch/protter/start/>).

Crystalline Cellulose Content Measurement

WT and *mdai* Arabidopsis seeds were sown on ½ MS plates with ethanol or 10 µM MDD for 7 days. Seedlings were washed with double-distilled water three times to remove seed coats and any residue from the growth medium and then ground into a fine powder in liquid nitrogen. The powder was extracted twice with 80% ethanol, once with 100% ethanol, once with 1:1 (v/v) methanol: CHCl₃, and once with acetone. The resulting insoluble cell wall fraction was dried in a fume hood for 2 d and weighed. Cellulose content was measured by the Updegraff method (22). Briefly, the cell wall material was hydrolyzed with Updegraff reagent (acetic acid: nitric acid: water, 8:1:2 [v/v/v]) to yield crystalline cellulose. The residual pellet obtained after the hydrolysis was rinsed twice with 10 volumes of water to remove acetic and nitric acids. Air dry the pellet to

remove excess water. Crystalline cellulose was hydrolyzed to Glc using 72% (v/v) sulfuric acid. Glc concentration was measured via a colorimetric method by developing color in Anthrone reagent (freshly prepared 2 mg/mL anthrone in concentrated sulfuric acid) and reading OD625 plate reader.

Live-cell imaging with spinning-disk confocal microscopy

Confocal microscopy experiments were performed using the LSM 980 confocal microscope or Evident FV4000 confocal microscopy. The root samples were collected from 1-week-old seedlings grown on ½ MS plates with ethanol or 10 µM MDD under standard condition with 16 h light/8 h dark at 22 °C. The hypocotyl samples were collected from 4-day-old seedlings grown on ½ MS plates with ethanol or 10 µM MDD under dark condition. Propidium iodide were used to stain cells. To examine the localization of the CSCs at the plasma membrane, the seedlings of GFP-CESA3 grown on ½ MS plates for 4 days were treated with ethanol or 100 µM MDD for 5 hr. GFP fluorescence was excited with a 488-nm laser and the emissions were collected using a 500-nm to 580-nm laser filter. For time series observation of GFP-CESA3, the pictures were taken every 10 s per picture for 4 min in total. For the FRAP assay, images were collected using the Evident FV4000 confocal microscopy. Photobleaching was performed using a 488-nm laser line at 30% power with a speed of 200.0 µs/pixel. Photos were taken before, right after bleaching, and followed by recovery for 5 min.

Image analysis

Image analysis was performed using Fiji/ImageJ software. For CESA particle dynamic analyses, 5-min time-lapse series with 5-s intervals were collected. Average intensity projections were generated to identify the trajectories of the CSC particles. Image drift was corrected by the StackReg plugin. The moving rate of CESA3 was qualified by using Kymograph analysis as described (Bringmann et al. 2012). In brief, after each group of frames were stacked. Kymographs were made using the multiple Kymograph plugin for ImageJ (http://www.embl.de/eamnet/html/body_kymograph.html), and then, particle velocities were calculated from the slopes of kymographs.

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Data and materials availability

All the high-throughput sequencing data generated in this study is accessible at the Genome Sequence Archive database in National Genomics Data Center (<https://ngdc.cncb.ac.cn/>) under the accession PRJCA055487.

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Figures

Fig. 1. MDD is a broad-spectrum plant growth inhibitor.

- A. Chemical structure of MDD.
- B. Representative image and quantification of hypocotyl growth of 5-day-old dark-grown Arabidopsis seedlings grown on ½ MS medium supplemented with Ethanol or increasing concentrations of MDD. Bars = 1.0 cm.
- C. Representative image and quantification of root growth of 7-day-old light-grown Arabidopsis seedlings grown on ½ MS medium supplemented with Ethanol or increasing concentrations of MDD. Bars = 1.0 cm.
- D. Representative image of hypocotyl cells of 4-day-old dark-grown Arabidopsis with Ethanol or 10 µM MDD treatment. Bars = 50 µm
- E. Representative image of root cells of 4-day-old light-grown Arabidopsis with Ethanol or 10 µM MDD treatment. Bars = 50 µm.
- F. Top 10 enriched GO terms of downregulated genes of MDD treated WT plants.
- G. Representative image of 14-day-old light-grown *Nicotiana Benthamiana*, *Solanum lycopersicum* and *Zea mays*.

Fig. 2. Methylation patterns on the isocoumarin ring and chlorination of side chain are crucial for MDD-induced growth inhibition.

- A. Chemical structures of MDD, DD and 8 analogs (MA-2-9).
- B. Representative image of 7-day-old Arabidopsis light-grown seedlings grown on ½ MS medium supplemented with Ethanol or 10 µM of MDD and indicated analogs. Bars = 1.0 cm.
- C. Quantification of root growth of 7-day-old light-grown Arabidopsis seedlings grown on ½ MS medium supplemented with Ethanol or 10 µM of MDD and indicated analogs. Statistical significance is indicated by different letters ($p < 0.01$). Error bars represent means $\pm$ standard deviations ($n = 3$).

Fig. 3. Mutations in CESA1 cause reduced sensitivity to MDD.

- A. Representative image and quantification of hypocotyl growth of 5-day-old dark-grown Arabidopsis seedlings grown on ½ MS medium supplemented with 10 µM MDD. Bars = 1.0 cm. Statistical significance is indicated by an asterisk ($p < 0.01$). Error bars represent means $\pm$ standard deviations ($n = 3$).
- B. Representative image and quantification of root growth of 7-day-old light-grown Arabidopsis seedlings grown on ½ MS medium supplemented with 10 µM MDD. Bars = 1.0 cm. Statistical significance is indicated by asterisks ($p < 0.01$). Error bars represent means $\pm$ standard deviations ($n = 3$).
- C. Predicted topology of CESA1. Locations of the mutated amino acids that cause reduced sensitivity to MDD are labeled with red color.
- D. Sequence logo assessment of residues in the *mddi1-1*, *mddi1-2* mutation regions of primary cell wall CESA proteins illustrates the location and conservation of the mutated alanine and histidine residues in CESA1.

Fig. 4. MDD depletes CSCs from the plasma membrane and inhibits cellulose biosynthesis.

- A. Representative single frame and time-projected images (4 min 24 frames) of CESA3-GFP in 5 hrs Ethanol- or 100 µM MDD-treated hypocotyl cells. Scale bar = 10 µm.
- B. Quantification of plasma membrane-localized GFP-CESA3 in hypocotyl cells after ethanol or 100 µM MDD treatment for 5 hrs. Error bars represent means $\pm$ standard deviations ($n = 80$ cells in each treatment).

C. Glucose content of 7-day-old light-grown Arabidopsis seedlings without and with 10 μ M MDD. Statistical significance is indicated by asterisk ($p < 0.01$). Error bars represent means $\pm$ standard deviations ($n = 3$).
D. Histogram with the frequencies of GFP-CESA3 particle velocity in hypocotyl cells after ethanol or 100 μ M MDD treatment for 5 hrs. Data in the chart represent mean $\pm$ standard deviation.
E. Representative FRAP images of GFP-CESA3 in hypocotyl cell plasma membranes when plants were treated with ethanol or 100 μ M MDD for 5 hrs.

Fig. 5. MDD inhibits cellulose biosynthesis using the same mode of action as quinoxiphen and C17.

A. Representative image and quantification of Col-0, *cesa1^{mdt1-1}* A903T and *cesa1^{mdt1-2}* H1024Y root growth of 7-day-old light-grown Arabidopsis seedlings grown on $\frac{1}{2}$ MS medium supplemented with 5 μ M Quinoxiphen. Bars = 1.0 cm.
B. Representative image and quantification of Col-0, *cesa1^{18A1}* S307L, *cesa1^{14U}* G1013E, *cesa1^{mdt1-1}* A903T and *cesa1^{mdt1-2}* H1024Y root growth of 7-day-old light-grown Arabidopsis seedlings grown on $\frac{1}{2}$ MS medium supplemented with 100 nM C17. Bars = 1.0 cm.

Fig. 6. Stacking different mutations to generate multiple drug-resistant mutants.

A. Representative image of Col-0, *cesa3^{ixr1-1}* G998D, *cesa6^{es20-r3}* G935E, *cesa1^{mdt1-1}* A903T, *cesa3^{ixr1-1}* G998D *cesa1^{mdt1-1}* A903T, *cesa6^{es20-r3}* G935E *cesa1^{mdt1-1}* A903T and *cesa6^{es20-r3}* G935E *cesa1^{mdt1-1}* A903T *cesa3^{ixr1-1}* G998D hypocotyl growth of 5-day-old dark-grown Arabidopsis seedlings grown on $\frac{1}{2}$ MS medium supplemented with Ethanol/DMSO, a combination treatment with 10 μ M MDD/10 nM isoxaben/50 nM C17/1 μ M ES20/5 μ M Quinoxiphen, or a combination treatment with 1.25 μ M MDD/1.25 nM isoxaben/6.25 nM C17/250 nM ES20/400 nM Quinoxiphen. Bars = 1.0 cm.
B. Representative image of Col-0, *cesa3^{ixr1-1}* G998D, *cesa6^{es20-r3}* G935E, *cesa1^{mdt1-1}* A903T, *cesa3^{ixr1-1}* G998D *cesa1^{mdt1-1}* A903T, *cesa6^{es20-r3}* G935E *cesa1^{mdt1-1}* A903T and *cesa6^{es20-r3}* G935E *cesa1^{mdt1-1}* A903T *cesa3^{ixr1-1}* G998D root growth of 7-day-old light-grown Arabidopsis seedlings grown on $\frac{1}{2}$ MS medium supplemented with Ethanol/DMSO, a combination treatment with 10 μ M MDD/10 nM isoxaben/50 nM C17/1 μ M ES20/5 μ M Quinoxiphen, or a combination treatment with 1.25 μ M MDD/1.25 nM isoxaben/6.25 nM C17/250 nM ES20/400 nM Quinoxiphen. Bars = 1.0 cm.
C. Representative image of 4-week-old of Col-0, *cesa1^{mdt1-1}* A903T, *cesa3^{ixr1-1}* G998D, *cesa6^{ixr2-1}* R1064V, *cesa6^{es20-r3}* G935E, *cesa1^{mdt1-1}* A903T *cesa3^{ixr1-1}* G998D, *cesa1^{mdt1-1}* A903T *cesa6^{ixr2-1}* R1064V, *cesa1^{mdt1-1}* A903T *cesa6^{es20-r3}* G935E, and *cesa1^{mdt1-1}* A903T *cesa3^{ixr1-1}* G998D *cesa6^{es20-r3}* G935E Arabidopsis plants. Bars = 1.0 cm.
D. Fresh weight of 4-week-old of Col-0, *cesa3^{ixr1-1}* G998D, *cesa6^{es20-r3}* G935E, *cesa1^{mdt1-1}* A903T, *cesa3^{ixr1-1}* G998D *cesa1^{mdt1-1}* A903T, *cesa6^{es20-r3}* G935E *cesa1^{mdt1-1}* A903T and *cesa6^{es20-r3}* G935E *cesa1^{mdt1-1}* A903T *cesa3^{ixr1-1}* G998D Arabidopsis plants. Bars = 1.0 cm.

Fig 1

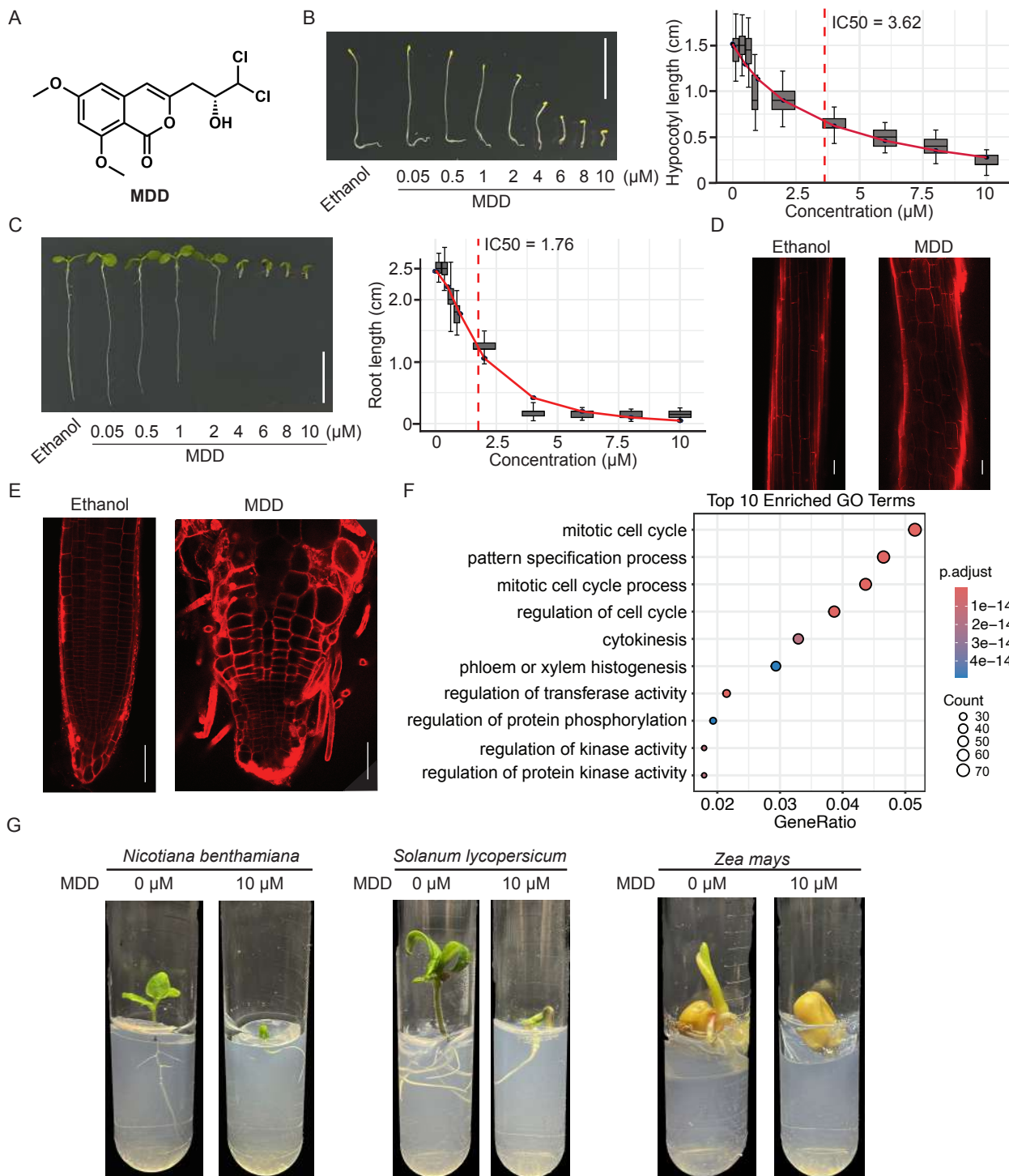
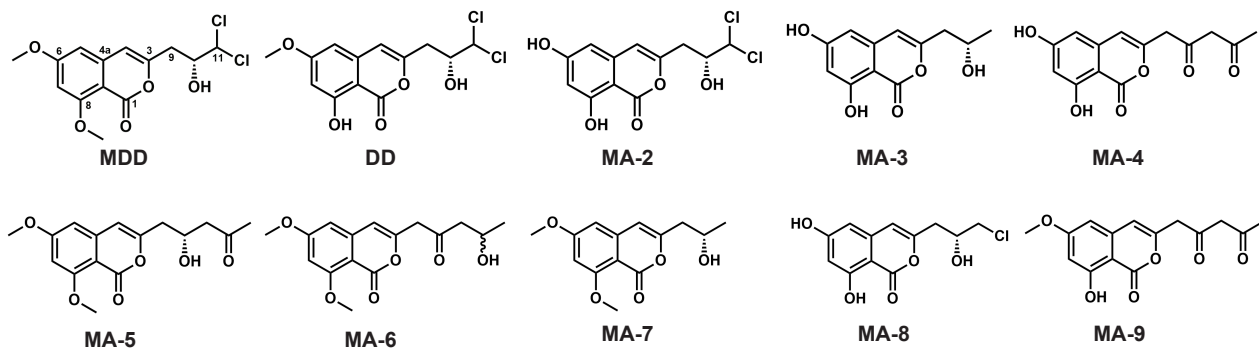
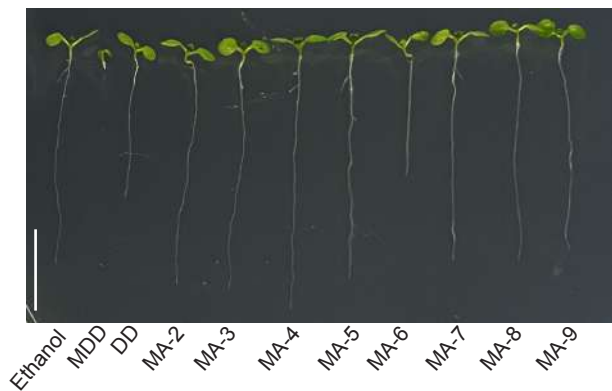


Fig 2

A



B



C

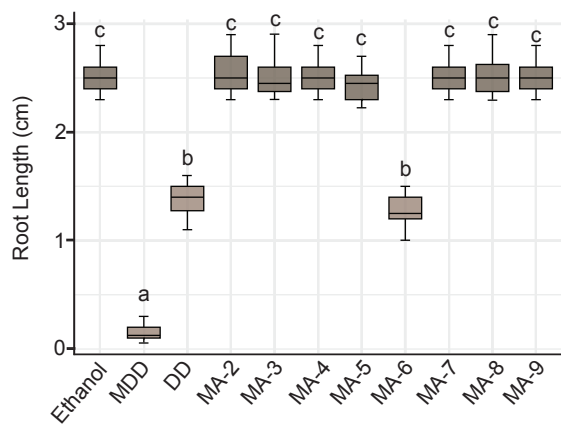


Fig 3

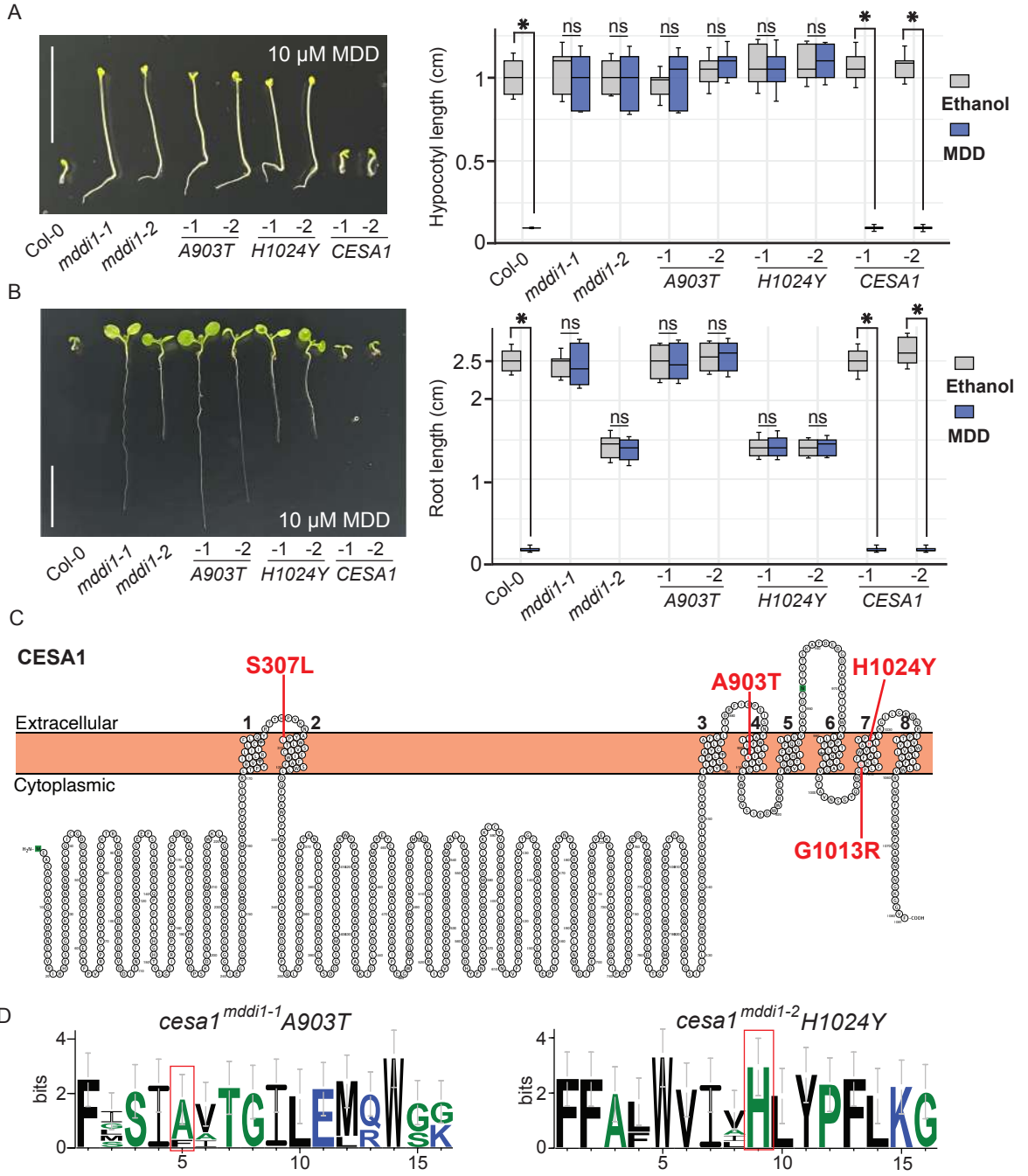


Fig 4

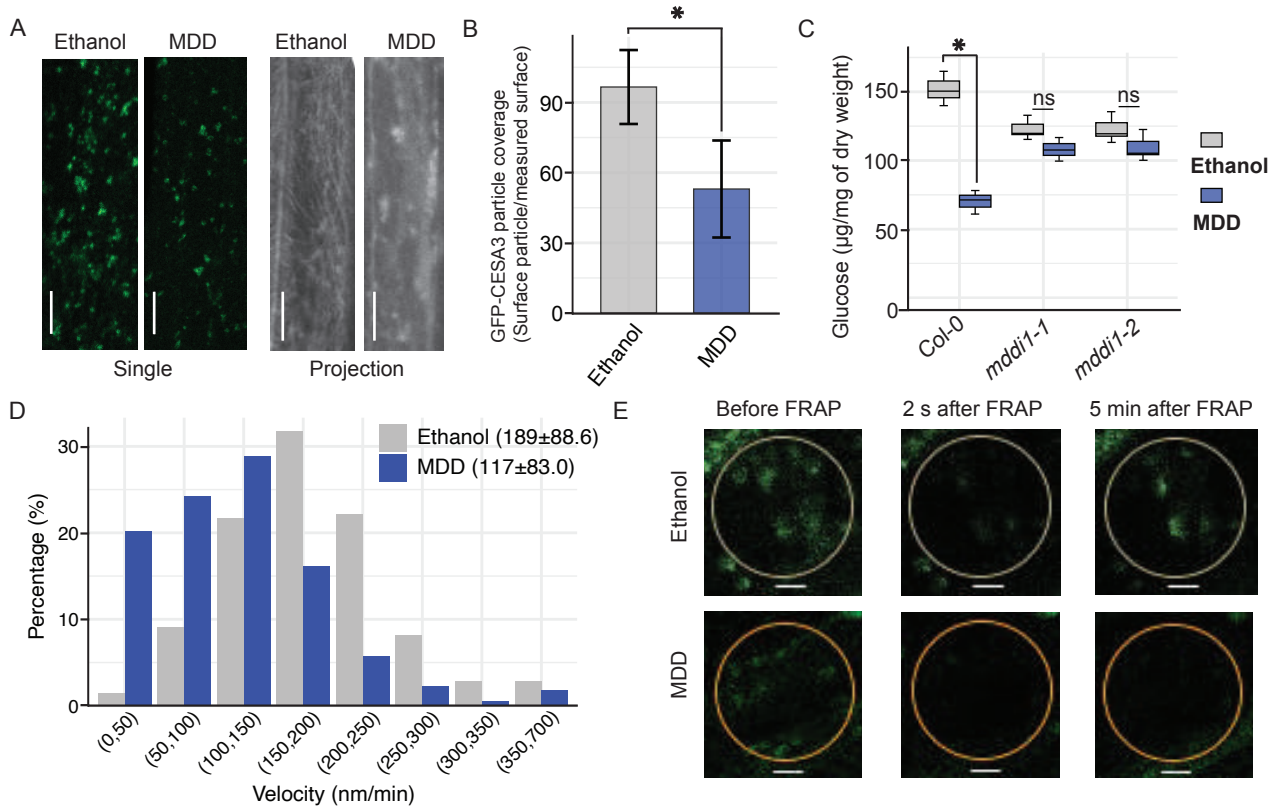


Fig 5

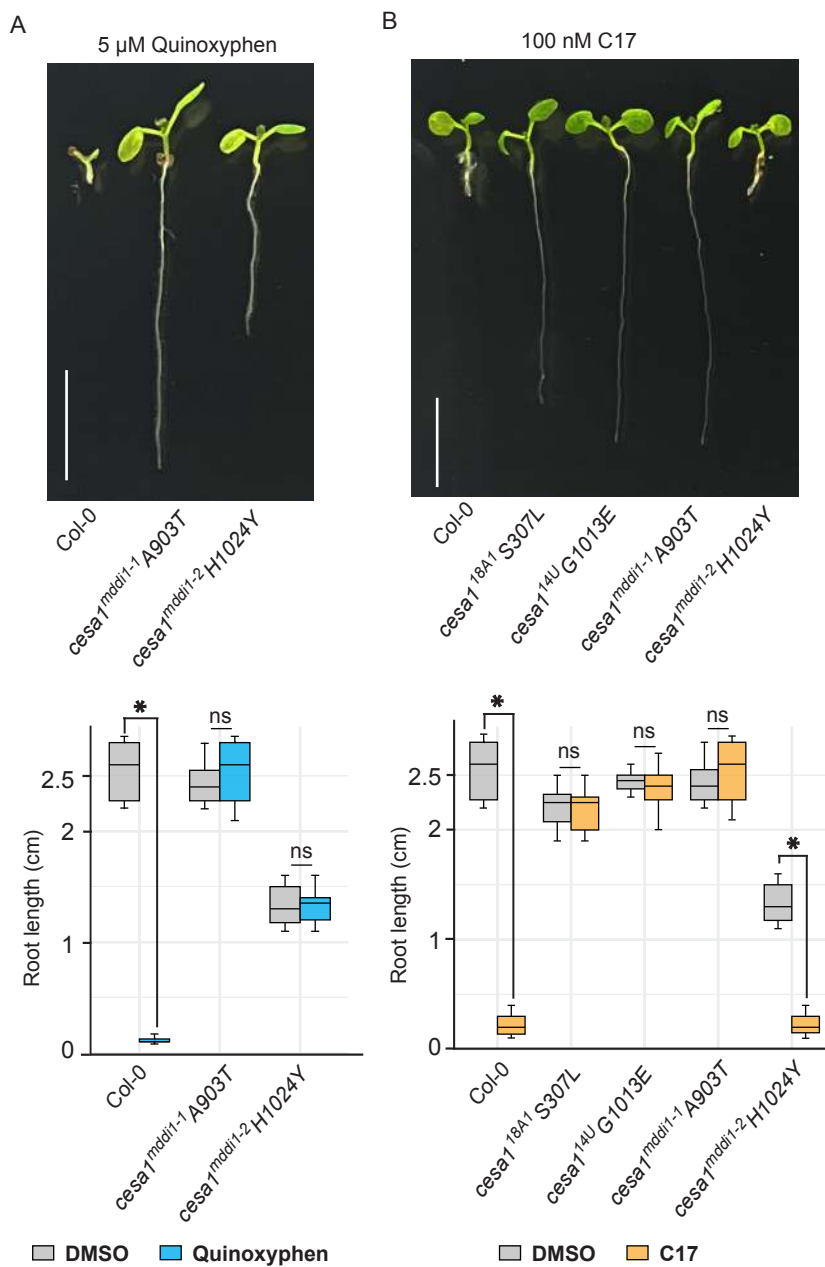


Fig 6

