Perception of the parasitic plant *Cuscuta reflexa*, as an invader, by the tomato *Solanum lycopersicum*.

Dissertation

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Contents

Abbreviations

AA	amino acid
CERK1	chitin elicitor receptor kinase 1
CrCrip21	*Cuscuta reflexa* cysteine-rich peptide
CrGRP	*Cuscuta reflexa* glycine-rich repeat protein
CuF	*Cuscuta* factor
CuRe1	*Cuscuta* receptor 1
DAMP	damage-associated molecular pattern
ESI-TOF	electrospray ionisation time-of-flight
FPLC	fast protein liquid chromatography
GRP	Glycine-rich peptide
HPLC	high performance liquid chromatography
HR	hypersensitive response
LC-MS	liquid chromatography-mass spectrometry
LORE	lipooligosaccharide-specific reduced elicitation
LRR	leucine-rich repeat
LysM	lysin motif
MAMP	microbe-associated molecular pattern
MAPKinases	mitogen-activated protein kinases
MS	mass spectrometry
NBS-LRR	nucleotide-binding site/leucine-rich repeat
PAMP	pathogen-associated molecular pattern
ParAMP	parasite-associated molecular pattern
PRR	pathogen recognition receptor
RLK	receptor like kinase
RLP	receptor like protein
ROS	reactive oxygen species
SCX	strong cation exchange
SERK1	somatic embryogenesis receptor-like kinase 1
SOBIR1	suppressor of brassinosteroid insensitive 1 associated kinase interacting receptor kinase1
SPE	solid phase extraction
TIC	total ion chromatogram
WAK1	wall-associated kinase 1

Zusammenfassung

Pflanzen der Gattung Teufelszwirn (*Cuscuta*) gehören zur Familie der Windengewächse (Convolvulaceae) und leben als obligate holoparasitische Pflanzen, die den Spross ihrer Wirtspflanzen infizieren. Aufgrund des breiten Wirtsspektrums und infizieren sie nahezu alle Dikotyledonen bis auf wenige Ausnahmen. Zum Beispiel kann die Tomate (*Solanum lycopersicum*) eine Cuscuta Infektion aktiv mit Hilfe einer äußerlich sichtbaren Hypersensitiven Reaktion (HR) abwehren. Außerdem reagiert sie auf Behandlung mit *Cuscuta reflexa* Extrakten in einer ähnlichen Weise wie bei der Detektion von mikrobiellen Pathogenen, z. B. mit der Produktion von Ethylen und reaktiven Sauerstoffspezies (ROS). Mittels rekombinanten Kreuzungslinien aus der resistenten *Solanum lycopersicum* und der anfälligen *Solanum pennellii* konnten wir den verantwortlichen Bereich im Tomaten Genom eingrenzen. Die detaillierte Untersuchung einzelner Kandidaten Gene führte zur Identifikation des Rezeptorproteins Cuscuta Rezeptor 1 (CuRe1), das eine extrazelluläre LRR-Domäne (engl: leucine-rich repeat) besitzt. CuRe1 ist in der Lage eine molekulare Struktur von *C. reflexa* zu erkennen und die Immunantwort in der Tomate auszulösen, sowie Resistenz in transgenen, CuRe1-expremierenden Pflanzen zu vermitteln.

Eine vorläufige Charakterisierung des molekularen Musters aus *Cuscuta* deutete auf ein Peptid mit sekundärer Modifikation hin. Um diese Parasiten-assoziierte molekulare Struktur zu isolieren und zu identifizieren wurden Extrakte aus *C. reflexa* hergestellt, chromatographisch auf gereinigt (SPE, FPLC, HPLC) und mittels Massenspektrometrie (MS) analysiert. Nach MS-Analyse fanden wir diverse 2-3 kDa schwere Peptide in verschiedenen Fraktionen des *C. reflexa* Extraktes, die mit der Aktivierung von CuRe1 in Korrelation gebracht werden konnten. Nach weiterführender massenspekrometrischer Analyse und partieller Peptidsequenzierung fanden wir heraus, dass diese kurzen Peptide Abbauprodukte desselben Proteins waren, einem Klasse II Glycin-reichem Protein (CrGRP, engl: *C. reflexa* glycine-rich protein). Nach Bestätigung des parasitären GRPs als Abwehr-auslösendes Protein, konnten wir das Epitop auf eine 21 Aminosäuren lange Sequenz mit sechs Cysteinen eingrenzen. Sowohl die synthetisierten Peptide als auch das heterolog exprimierte CrGRP waren in der Lage eine CuRe1 abhängige Abwehrreaktionen auszulösen. Diese Resultate bestätigen das CrGRP als die molekulare Struktur, welche von CuRe1 erkannt wird und die Tomatenpflanze befähigt *Cuscuta* als ein Pathogen zu erkennen. Bemerkenswerterweise ist

die Proteinklasse der GRPs unter Pflanzen, Mikroben und Tieren weit verbreitet. Selbst Tomaten besitzen diese Typ II GRPs mit vergleichbaren Motiven mit sechs Cysteinen. Dennoch reagiert CuRe1 nicht auf diese Homologe aus Tomate. In zukünftigen Versuchen soll geklärt werden warum dieses Motiv des GRPs als spezifisches Ziel für die Cuscuta Erkennung dient

Summary

Cuscuta spp. are assigned to the family of the Morning glories (Convolvulaceae) and live as obligate holoparasitic plants, which infect the shoot of the host plants. They have a broad host spectrum and infect nearly all dicot plants with a few notable exceptions. For example, tomato (*Solanum lycopersicum*) is able to fend off the parasite's attack with an active defense. It responds to extracts of *C. reflexa* in a similar manner as known for the detection of microbe-associated molecular patterns (MAMPs) (e.g. increased ethylene biosynthesis and the production of reactive oxygen species (ROS)). Using recombinant inbred lines between the resistant *S. lycopersicum* and the susceptible *S. pennellii* allowed for mapping of the corresponding locus within the tomato genome, responsible for the response against *Cuscuta*. Further mapping led to the identification of the leucine-rich repeat receptor like protein (LRR-RLP) CuRe1 (*Cuscuta* Receptor 1). CuRe1 perceives a molecular pattern from *C. reflexa* and subsequently induces the defense responses described above. Initial characterization indicated for a proteinaceous defense trigger with potential secondary modifications. To identify this parasite associated molecular pattern (ParAMP) we used chromatographic purification techniques (SPE, FPLC, HPLC) and mass spectrometry (MS). After purification and subsequent analyses, we found multiple 2-3 kDa peptides in different fractions of the *C. reflexa* extract that could be correlated with the CuRe1 activation. After in-depth MS analyses and partial peptide sequencing, we found that these peptides are degradation products of the same precursor protein, a class II *C. reflexa* glycine-rich protein (CrGRP). We could specify the epitope within the protein to a 21 aa long peptide with six cysteine residues. Synthesized peptides as well as heterologously expressed CrGRP were able to induce CuRe1-dependent defense responses. These findings demonstrate CrGRP as the ParAMP which is specifically recognized by CuRe1 and enables tomato to detect *C. reflexa* as a pathogenic invader. Notably, GRPs are widespread among plants, microbes and animals. Tomato itself possesses this type of GRP with a comparable six Cysteine motif. Nevertheless, these tomato homologs do not activate CuRe1. Future work will address the question why this motif serves as a specific target for *Cuscuta* perception.

List of Publications

Plants under stress by parasitic plants

Volker Hegenauer, Max Körner, Markus Albert

Current Opinion in Plant Biology. 2017 April 29; 38: 34-41. doi: 10.1016/j.pbi.2017.04.006

Detection of the plant parasite *Cuscuta reflexa* by a tomato cell surface receptor

Volker Hegenauer, Ursula Fürst, Bettina Kaiser, Matthew Smoker, Cyril Zipfel, Georg Felix, Mark Stahl, Markus Albert

Science. 2016 Jul 29;353(6298):478-81. doi: 10.1126/science.aaf3919.

Growth Assay for the Stem Parasitic Plants of the Genus Cuscuta

Volker Hegenauer, Max Körner, Max Welz, Markus Albert

Bio-Protocol. 2017 Apr 20; 7(8). doi:10.21769/BioProtoc.2243

Parasitic Cuscuta factor(s) and the detection by tomato initiates plant defense

Ursula Fürst, Volker Hegenauer, Bettina Kaiser, Max Körner, Max Welz, Markus Albert

Commun Integr Biol. 2016 Oct 10;9(6):e1244590. doi: 10.1080/19420889.2016.1244590. eCollection 2016.

The tomato receptor CuRe1 senses a cell wall protein to identify *Cuscuta* as a pathogen

Volker Hegenauer, Peter Slaby, Max Körner, Julien-Alexander Bruckmüller, Ronja Burggraf, Isabell Albert, Bettina Kaiser, Birgit Löffelhardt, Irina Droste-Borel, Jan Sklenar, Frank L. H. Menke, Boris Maček, Aashish Ranjan, Neelima Sinha, Thorsten Nürnberger, Georg Felix, Kirsten Krause, Mark Stahl and Markus Albert

Nat Commun. 2020 Oct 20;11(1):5299. doi: 10.1038/s41467-020-19147-4.

Introduction

As with all organisms, plants live under constant threat from pathogens. Plants need to defend themselves against a great variety of pathogens including herbivores, viruses and microbes, such as bacteria, fungi and oomycetes. Moreover, plants have to deal with attacks by parasitic plants which pose an additional threat and can heavily infest whole habitats of host plants. To resist all kinds of attacks, plants have evolved a set of passive and active defense mechanisms. Passive defense strategies comprise physical barriers and accumulated secondary metabolites that prevent from a pathogen invasion. To actively fend off pathogens, plants rely on a powerful innate immune system that enables the plant to specifically detect invaders and to switch on defense responses leading to resistance against pathogens.

Parasitic plants, a threat to other plants.

Approximately 4500 plant species live a parasitic life style. Interestingly, these parasitic plant species belong to 28 different angiosperm plant families, indicating independent evolution (Yoshida, Cui et al. 2016). Parasitic plants are classified into facultative or obligate parasites, while some are free to parasitize other plants other parasites totally depend on parasitism to complete their life cycle. For some parasites, their host is the sole source of nutrients and carbohydrates (holoparasites), while others can use additional sources (hemiparasites) (e.g. nutrient uptake by roots or energy fixation by their own photosynthesis). Finally, parasitic plants can be classified as root- or shoot parasites, depending on where the infection takes place. Although parasitism in plants evolved independently at least 12 times (Westwood, Yoder et al. 2010), all parasitic plants infect their host with haustoria, a specialized multicellular invasive structure which connects to the host´s vasculature, to withdraw nutrients and water from the host (Yoshida, Cui et al. 2016). Depletion of nutrients and water from the host leads to reduced seed and biomass production and in some cases a reduced lifespan of the host.

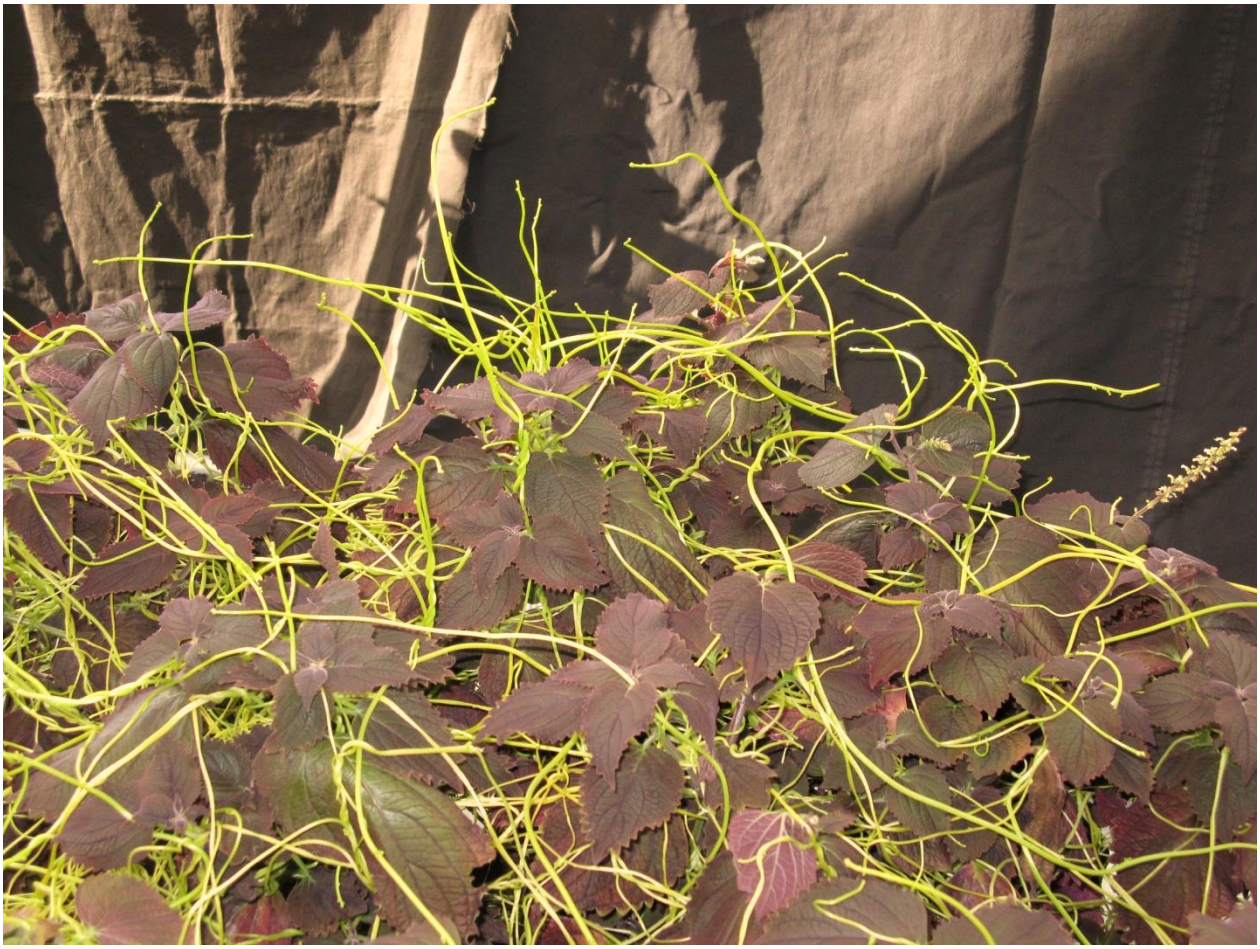

Fig. 1. Cuscuta reflexa growing on Coleus

Cuscuta and the interaction with susceptible host plants

Cuscuta spp., which belong to the family of Convolvulaceae, are obligate stem holoparasites, which means they fully depend on a host as the sole source of nutrients, carbohydrates, and water. *Cuscuta* spp. cause severe crop damage; for instance, *Cuscuta pentagona* alone is reported to affect 25 crops and lead to crop losses in 55 countries (Lanini 2005).

Seeds of Cuscuta spp. can persist in the soil for over 10 years (Lanini 2005), and it is thus problematic to fully get rid of the parasite once established in a field. After germination seedlings only have a few days to infect a susceptible host before dying from starvation. Young *Cuscuta* seedlings seem to find their host by detecting volatiles like terpenoids which are released by a potential host (Runion et al 2006).

Upon a tactile stimulus, *Cuscuta* winds around the hosts stem counterclockwise from bottom to the top. Within 24 hours *Cuscuta* spp. start building prehaustoria (adhesive discs). It secrets adhesive substances (pectins and other polysaccharides) while the inner parts of the prehaustoria (endophyte) start to penetrate the host tissue. Searching hyphae are formed which grow towards the host vascular system. The searching hyphea build interspecies plasmodesmata connecting to the host´s phloem transfer cells. Upon reaching the host xylem, the hyphae cells differentiate into xylem cells to form a xylem bridge between Cuscuta and the host.(Dörr 1972, Yoshida, Cui et al. 2016). After the haustorial connection between host and parasite is established, the parasite starts to withdraw nutrients and water for its own growth. The parasite proceeds winding around the host and establishing more haustorial connections. Ultimately the withdrawal of nutrients from the hosts leads to reduced growth, lower seed production and early die-off. In the agricultural context this translates to crop loss.

Fig. 2 Flowering Cuscuta reflexa (picture by Eric Melzer).

Resistant hosts

Even though *Cuscuta* has a broad host spectrum, not all plants are susceptible to *Cuscuta* infection. Very often this is due to anatomical constraints e.g. monocots are (with few exceptions) not susceptible to *Cuscuta* infection due to the organization of their vascular bundles (Dawson 1994). Additionally, there are a few examples of plants which are capable to actively fend-off the parasite, notably members of the Malvaceae (Capderon, Fer et al. 1985). The cultivated tomato (*Solanum lycopersicum*) is also capable of actively defending against Cuscuta infection (Ihl, Tutakhil et al. 1988, Albert, Werner et al. 2004). Tomato fends off *Cuscuta* by a HR like response, which hinders the haustorial intrusion and subsequently leads to the starvation of *Cuscuta*. This kind of response seems to be similar to the already known plant defense against pathogenic bacteria, fungi and oomycetes (Boller and Felix 2009).

Pathogen recognition, a prerequisite for defense.

As already introduced, plants fend off microbial pathogens via their innate immune system. Recognition of pathogens by pattern recognition receptors (PRRs) is the essential first step of a successful immune response (Nurnberger and Scheel 2001). They sense conserved molecular structures of microbial invaders, so-called microbe-associated molecular patterns (MAMPs). MAMPs are molecules that are very often specific for certain clades of pathogens, such as flagellin for bacteria or chitin for fungi. Additionally, damage-associated molecular patterns (DAMPs) can serve as signals for danger. DAMPs are host derived molecules that are released due to the damage of host cells or cell walls due to pathogen infection (e.g. systemin, eATP, AtPEP, OGA) (D'Ovidio, Mattei et al. 2004, Yu, Feng et al. 2017).

Molecular patterns – either MAMPs or DAMPs – can be perceived by cell surface PRRs, such as receptor-like kinases (RLKs) and receptor-like proteins (RLPs) or by cytosolic receptors mostly belonging to the NBS-LRR type. Membrane-bound PRRs, have different types of extracellular domains to detect diverse classes of MAMPs/DAMPs. Peptides are mainly perceived by leucine-rich repeats (LRR), while sugar motifs are rather perceived by receptors with LysM domains, e.g. Chitin perceived by CERK1 (Miya, Albert et al. 2007).

LRR-RLPs, unlike LRR-RLKs, do not have an integral intracellular kinase domain; they depend on the adaptor kinase SOBIR1 (Suppressor of Brassinosteroid insensitive 1 associated kinase interacting receptor kinase1) (Liebrand, van den Berg et al. 2013) to transduce the signal into the cell. Both LRR-RLKs and LRR-RLPs depend on the recruitment of co-receptors of the SERK-type (somatic embryogenesis receptor kinase) upon ligand binding (Liebrand, van den Burg et al. 2014). Pathogen recognition by PAMP perception triggers typical plant immune responses including extracellular alkalization, ethylene production, ROS production, the expression of defense related genes and phosphorylation of mitogen activated protein kinases (MAPKinases) (Yu, Feng et al. 2017). All these plant responses can be detected within the first three hours. In addition to the fast immune responses, long-term responses can also be observed such as callose deposition, seedling growth inhibition, hypersensitive responses and necrosis (Yu, Feng et al. 2017).

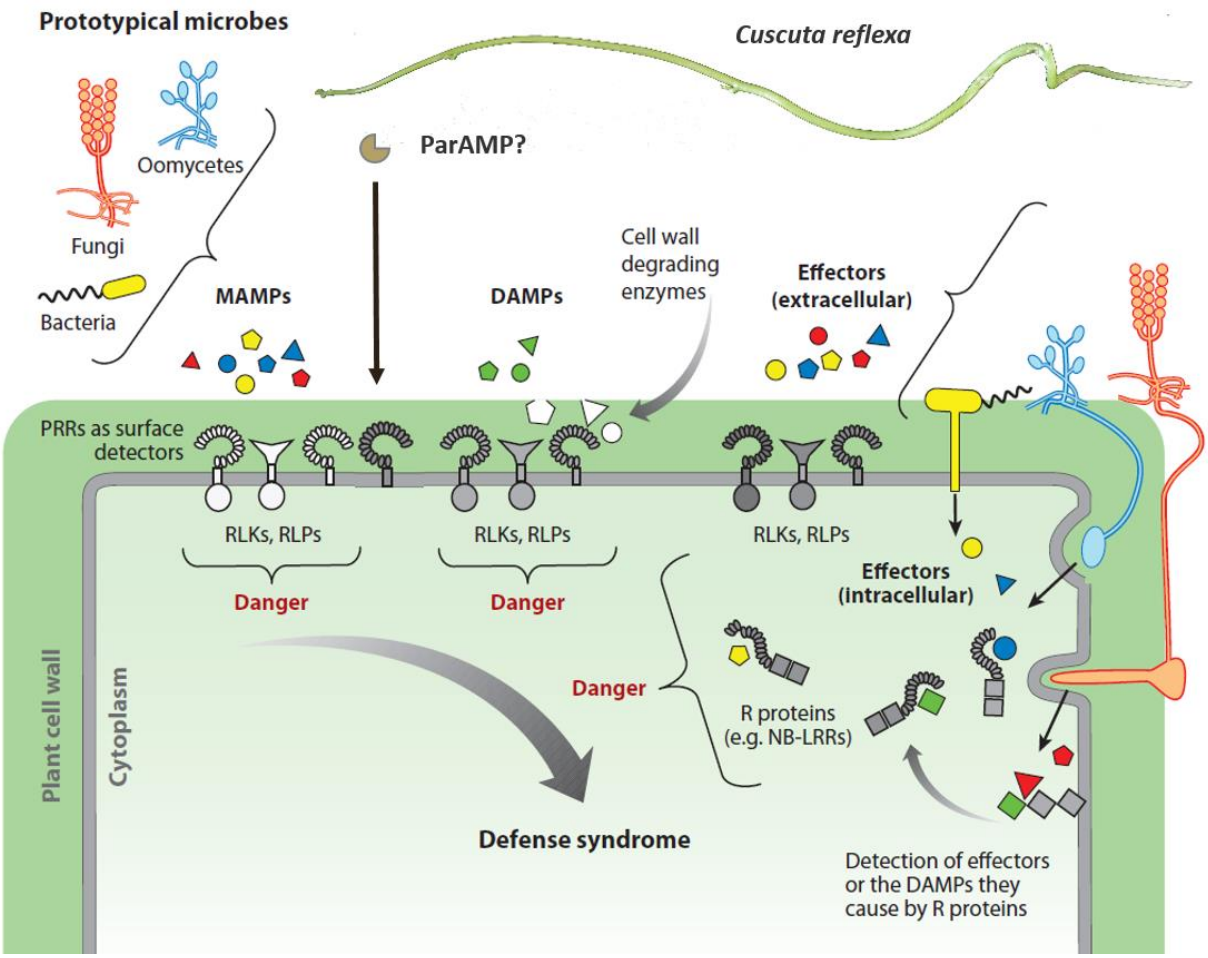

Fig. 3. Schematic depiction of plant innate immunity in regard to the detection of PAMPs, DAMPs and effectors by their respective receptors (RLK, RLP or NB-LRR) modified from (Boller and Felix 2009). Inclusion of Cuscuta indicates our assumption, that the defense against Cuscuta belongs to the plants innate immunity and that a parasite associated molecular pattern (ParAMP) is perceived by a cell surface receptor comparable to other PAMP receptors.

Aims

Tomato must be able to detect *Cuscuta* as an alien invader to fend it off. Mechanistically there could be a molecular pattern marking *Cuscuta* as a pathogen. This pattern must be recognized by a receptor to trigger defense responses. Consequently, there are two major questions to answer. First, is there is a PRR in *S. lycopersicum* to detect *Cuscuta*? Secondly, what kind of molecular pattern derived from Cuscuta activates this receptor? Plant innate immune receptors and their ligands are well described for plant-microbe interactions (Boller and Felix 2009), however *Cuscuta*, since it also belongs to the order of Solanales, is closely related to tomato! Therefore, there must exist a molecular pattern uniquely defining *Cuscuta* in contrast to tomato.

At the beginning of this project the resistance of tomato against *Cuscuta* was well characterized. It had been shown that tomato responds to treatment with extracts of various *Cuscuta* spp., with ethylene and ROS production as parts of its defense response. The release of these substances is measurable in well-established bio-assays (Kaiser 2010). Right before the start of this thesis work, a candidate gene of tomato, encoding for the receptor protein *Cuscuta* receptor 1 (CuRe1), was identified by B. Kaiser, U. Fürst (et al.). In this thesis work CuRe1 and its role in *Cuscuta* perception were characterized. In a biological context, it was of interest to test the contribution of CuRe1 to resistance against Cuscuta infection in tomato. To test for this, an assay had to be established to quantify the success of *Cuscuta* infection in a statistically evaluable and reproducible manner.

While the first part of this project focused on the resistant host tomato and CuRe1, the second part concentrated on the parasite. What is the assumed molecular signature of *C. reflexa* that is perceived by CuRe1 and triggers the tomato defense response? To identify this molecule, a preexisting purification protocol had to be improved by increasing the sample amount as well as extraction and purification efficiency. The *Cuscuta* factor could then be purified by high pressure liquid chromatography (HPLC) and finally identified by mass spectrometry. In parallel characteristics of the molecule like occurrence in distinct *Cuscuta* tissues, chemical and enzymatic stability should be examined.

Results and Discussion

Resistance against *Cuscuta* infection

Cuscuta spp. are parasitic plants with a broad host spectrum. While an infection with *Cuscuta* remains unrecognized by most host plants, *Solanum lycopersicum* is able to detect the infection and to defend itself with an immune response similar to the defense against microbial pathogens. This immune response includes the production of ethylene and ROS as well as a visible HR like response.

In this work, we provide substantial knowledge of how this perception and defense of tomato against *C. reflexa* works on a molecular level. Since *S. lycopersicum* is resistant against *C. reflexa* infection while *S. pennellii* is not, we could use 51 introgression lines of *S. lycopersicum* x *S. pennellii* (Eshed and Zamir 1995, Chitwood, Kumar et al. 2013) to screen for the loss of immune responsiveness against *C. reflexa*. We successfully mapped one resistance trait to chromosome 8. Its substitution does not lead to a complete loss of resistance but does result in a complete loss of the ethylene and ROS production as a response to treatment with *C. reflexa* extracts. On chromosome 8, we identified the gene encoding *Cuscuta* receptor 1 (CuRe1) as a critical component of tomato´s defense strategy. We could show that the LRR-RLP CuRe1, a LRR-RLP, belongs to a class of immune receptors which were already known to perceive MAMPs deriving from fungi, bacteria and oomycetes. CuRe1 requires interaction with SlSOBIR1, the same adaptor kinase as previously described for other LRR-RLPs (Liebrand, van den Berg et al. 2013, Albert, Böhm et al. 2015). We hypothesize that CuRe1 also shares the same downstream signaling as known for other LRR-RLPs.

From the parasite´s side, we identified the *C. reflexa* associated molecular pattern, a secreted glycine-rich protein (GRP). We showed that a ~21 aa long cysteine rich peptide (Crip) within the protein is sufficient to induce CuRe1-dependent defense responses in host plants.

Properties of the *Cuscuta* receptor

Expression of CuRe1 in non-responsive, susceptible *N. benthamiana* enabled it to respond with ethylene and ROS production, similar to *S. lycopersicum*, when treated with *C. reflexa* extracts ((Hegenauer, Furst et al. 2016) Fig. 3A,B). Using a quantitative infection assay to measure the success of *C. reflexa* parasitism (Hegenauer, Welz et al. 2017) we found CuRe1 transformation into *N. benthamiana* could significantly reduce the parasite´s ability to feed on the transformed plant ((Hegenauer, Furst et al. 2016), Fig. 3E,F). We also observed browning of the *N. benthamiana* tissue where haustoria tried to penetrate in a CuRe1 dependent manner ((Hegenauer, Furst et al. 2016)supplemental fig. S10 E,F). However, this effect was observed in the hypodermal tissues, but not in the epidermis as usually occurring in tomato.

Purification and identification of the *Cuscuta* factor

We found, that the CuRe1 dependent responses could be triggered by extracts deriving from various *Cuscuta* species but not in other members of the Convolvulaceae family (e.g. *Calystegia*) or other parasitic plants like *Rhinantus* (Orabanchaceae) ((Hegenauer, Furst et al. 2016) Fig. 1E). *Cuscuta* factor is present in all parts of the plant ((Hegenauer, Furst et al. 2016) Fig S2A). It is a heat stable peptide, but sensitive to treatment with NH_4OH (pH 12; 50°C), which, by a yet unknown reaction that changes the epitope, leads to a irretrievable loss of receptor perceptibility ((Hegenauer, Furst et al. 2016) Fig. 1C,D).

To identify the Cuscuta factor that triggers CuRe1-mediated immune response, we purified it from lyophilized *C. reflexa* as described in (Hegenauer, Furst et al. 2016). In total, ten liters of crude extract were produced, from which the Cuscuta factor was isolated and enriched using reversed phase and cation exchange HPLC.

The purest fractions were analyzed by mass spectrometry. From this approach, we could correlate the CuRe1 dependent ethylene induction to several different masses within these fractions (Table 1).

Table 1. Masses of molecules found in C. reflexa extract. CuF LC-MS-masses correlated to CuRe1 dependent ethylene response, sorted by their elution from the strong cation exchange column. Eluting material was pooled in pool 1 (91-170 mM KCl), pool 2 (190-270 mM KCl) and pool 3 (310-360 mM KCl) elution fractions and separately purified with C18 reversed phase chromatography before LC-MS analysis, Peptide masses given in Daltons.

CuF elution from SCX column with x mM KCl		
pool 1	pool 2	pool 3
91-170 mM KCl	190-270 mM KCl	310-360 mM KCl
2206.78 Da	2205.78 Da	
2263.82 Da	2262.80 Da	
2392.86 Da	2391.87 Da	2390.91 Da
2449.88 Da	2448.90 Da	2447.92 Da
		2461.98 Da
		2519.00 Da
		2981.22 Da
		3038.26 Da
		3095.31 Da
		3152.33 Da
		3209.38 Da

Remarkably all of the identified masses shared a few common properties. The masses co-eluted together with 57 Da lighter masses. At higher salt concentrations, the molecules eluted in 1-2 Dalton lighter variants from the SCX column (Table 1). Therefore, it was likely that the Cuscuta factor inducing CuRe1 response was indeed a mixture of molecules with at least 11 different masses, which were also present in structural isoforms.

The identified masses were then analyzed by LC-MS/MS. Since no *C. reflexa* proteome database was available for peptide mass fingerprinting the fragmentation results where sequenced *de novo*.

As shown for one typical candidate ((Hegenauer, Furst et al. 2016) Fig. S4, shown here in Fig. 2) the fragmentation of all candidate masses showed only few fragment peaks in their spectra. Most of the high mass peaks could be identified as Y-fragments, so we could calculate the first amino acids of the N-terminus (for this candidate KGN or GKN).

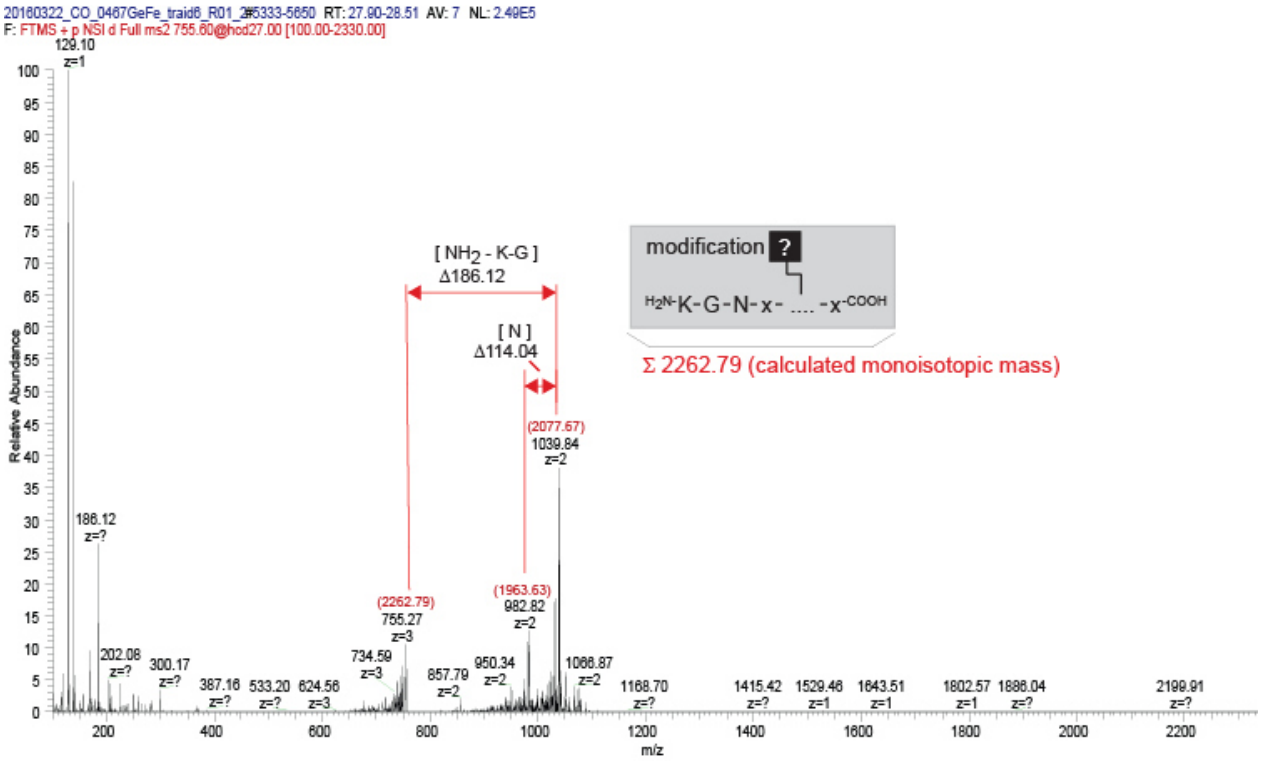

Fig. 4 ((Hegenauer, Furst et al. 2016) Fig. S4). "Fragment spectrum of the Cuscuta factor candidate mass 2262.79. Spectrum shows incomplete fragmentation of the Cuscuta factor. The inconclusive fragments did only allow for a limited sequence prediction of the N-terminus of the peptide (red arrows and lines). The peptide backbone in minimum consists of five amino acids and the molecular weight of 2262.79 indicates an upper size limit of ~22 amino acids if there are no sugar modifications or some lesser number of amino acids once sugar modifications are accounted for within the estimated molecular weight. Grey box: Model of the Cuscuta factor 2262.79 Da, N-terminally starting with the amino acids K-G-N, comprising an as yet unknown modification (small black box)."

As shown in table 1, we identified multiple CuRe1 activating *C. reflexa* peptides with different masses, eluting from the SCX column at different KCl concentrations. In pool 3 of the SCX elution (310-360 mM KCl) (Table 1), nine masses could be correlated to the induction of CuRe1 dependent ethylene response ((Hegenauer, Slaby et al. 2020)Fig. S1: pool 3 LC-MS correlation run). Notably, the five largest species differ in mass relative to each other in 57 Da increments, suggesting sequential loss of a glycine. In the example presented in Fig. 5, we focused on the heaviest (3209.38 Da) of these five candidates. Its fragmentation spectrum showed more Y-fragment peaks compared to the previous candidate mass shown in Fig. 4. In the fragmentation spectrum, the lighter candidates, including the 2263 Da and 2448 Da species, occurred as Y-fragments. With this information, N-terminal sequence of the peptide could be deduced for the first 15 amino acids. Two additional masses (2519 Da and 2461 Da) could be correlated.

Their fragmentation spectra looked similar but the fragment masses were 70.1 Da heavier than the masses originating from fragmentation of the 2448.9 Da and 2391.9 Da candidates. The mass difference of 70.1 Da resembles the mass of an alanine residue. The fact that all observed Y-fragments showed this difference together with the assumption that the candidates originate from the same protein suggest that an additional alanine at the C-terminus is likely.

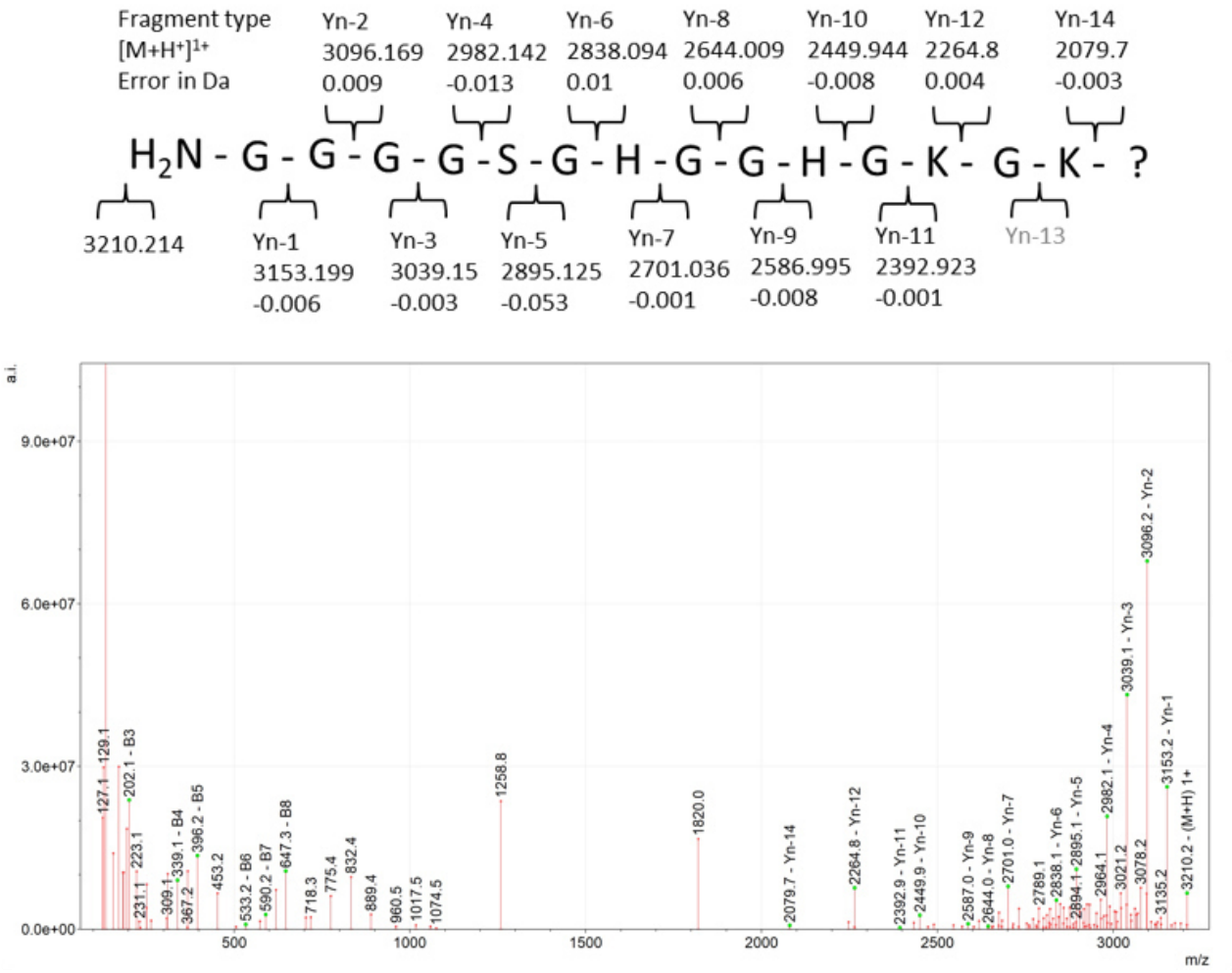

Fig. 5 ((Hegenauer, Slaby et al. 2020) Fig. S2). Sequencing of the CuF candidate mass 3209.38 Da. Sequence obtained from the de-convoluted fragmentation spectrum underneath. Green dots indicating fragment masses identified as Y fragments at high mass end of the spectrum.

Analysis of the eleven correlated masses based on the assumption that they are degradation products of the same precursor protein led to the identification of 16 AA with a 15 AA consecutive sequence. The immunogenic peptide, however, remained unknown. To identify the CuRe1-detected parasitic protein, the peptide sequence was compared to a translated transcriptome database of *C. reflexa* (collaborators research group Prof. Krause, Tromsø, NW).

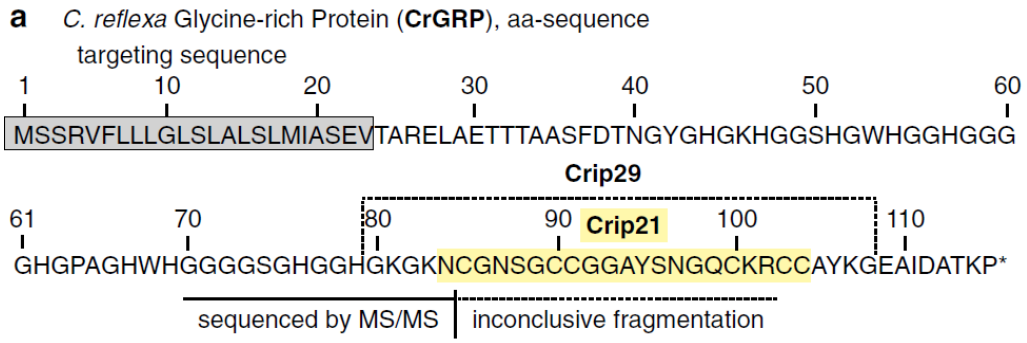

Fig. 6 ((Hegenauer, Slaby et al. 2020), Fig 2A) AA sequence of the identified Cuscuta glycine-rich protein. The MS-derived peptide sequence, see Fig. 5, is indicated below.

The peptide motif matched a Glycine-rich protein (GRP, Fig. 6) belonging to the family of class II GRPs (Mangeon, Junqueira et al. 2010). The 116 aa protein contains an N-terminal export signal and a Cysteine rich region near the C-terminus (Fig. 6;(Hegenauer, Slaby et al. 2020), Fig. 2A). Treatment of CuRe1 expressing *N. benthamiana* with heterologously expressed and purified CrGRP triggered ethylene response. To identify the CuRe1 binding epitope of CrGRP, we tested synthesized peptides of the presumed epitope. The responsive peptides were able to trigger ethylene production in a CuRe1 dependent manner with an EC50 of about 1-10 nM ((Hegenauer, Slaby et al. 2020), Fig. S6). The shortest functional peptide was 21 aa long ((Hegenauer, Slaby et al. 2020), Fig. S4) and was named CrCrip21 (*Cuscuta reflexa* Cysteine-rich peptide 21 AA). Replacement of AA by Ala or Ser showed that especially the cysteines are critical for the receptor activation ((Hegenauer, Slaby et al. 2020), Table S1).

Epitope and Peptides

The tested active peptides showed similar characteristics in MS analyses to the purified Cuscuta factor. In LC-MS analysis 2 or 4 Da lighter peptides eluted differentially from the C18 column as compared to the native peptide Fig. 7. The lighter masses are likely the result of one or two internal disulfide bridges. Because the epitope contains six cysteines there are multiple possible intramolecular disulfide bonds which would cause internal ring formations. This could lead to different isoforms all giving slightly different elution patterns from the C18 reversed phase columns. The absence of the 2 or 4 Da lighter peptide isoforms in the DTT pre-treated fraction in

Fig. 7 also supports this hypothesis. This may also explain the occurrence of Cuscuta factor candidates slightly different masses in Table 1.

Structural isoforms due to disulfide bridge formation could occur during the Cuscuta factor extraction. These could eventually be separated by SCX chromatography and pooled into different fractions.

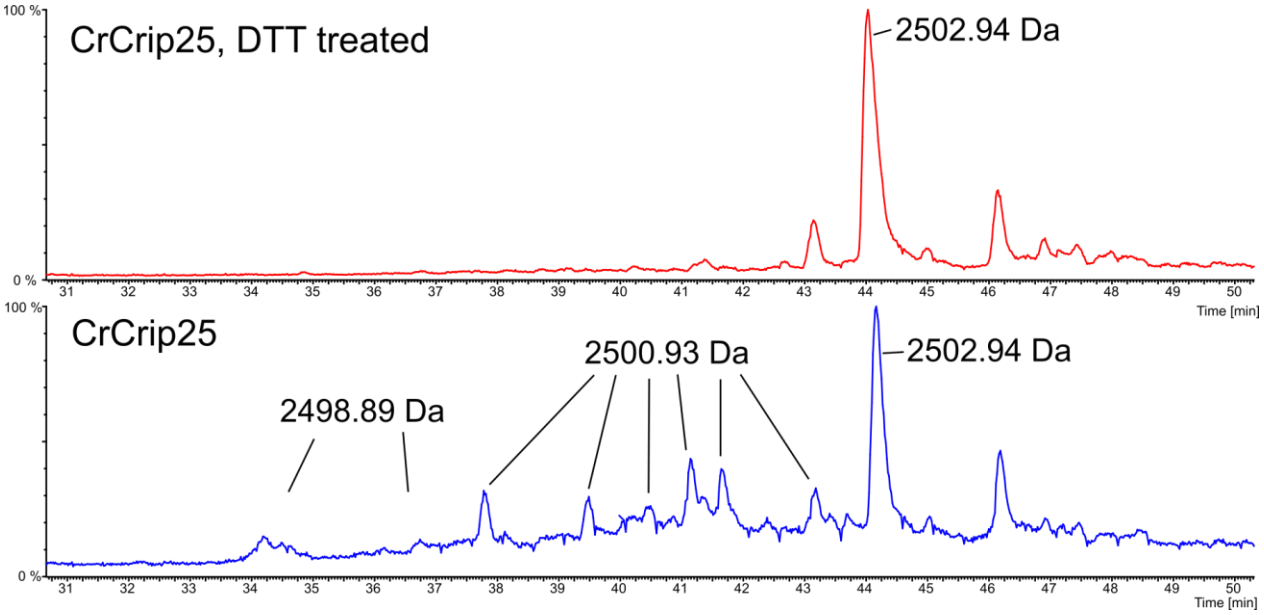

Fig. 7 TIC of synthetic CrCrip25 eluted from a C18 column measured with ESI-TOF MS. (Red) DTT-treated CrCrip25, the peptide eluted in one peak with the estimated monoisotopic mass of 2502.94 Da. (Blue) Mock treated CrCrip25, elution at least eight distinct peaks of either 2498.89 Da or 2500.93 Da masses additional to the monoisotopic mass of CrCrip25 were observed.

Cuscuta's GRP is the hallmark that is perceived by tomatoes immune system

The CrGRP belongs to the class II GRPs which contain an export signal. Class II GRPs are described as cell wall proteins and their cysteine-rich domain (CD) predicted to mediate it´s to the cell wall (Domingo, Sauri et al. 1999, Ringli, Keller et al. 2001). It is not yet clear what the endogenous function of CrGRP in *Cuscuta* is or whether it is involved in the infection process. CrGRP can be released from purified *Cuscuta* cell wall by treatment with pectinase ((Hegenauer, Slaby et al. 2020), Fig. 1D). It is present in the whole *Cuscuta* shoot and not solely at the infection site ((Hegenauer, Furst et al. 2016), Fig. S2). The epitope perceived by CuRe1 contains a conserved cysteine-rich domain (CD) which is common among this protein class. GRPs, as well as the CD motif, are widely present in the plant kingdom, including tomato, *A. thaliana* and *N. tabacum*

(Domingo, Sauri et al. 1999). Also, from our pectinase based extraction approaches we assume that CrGRP derives from *Cuscutas* cell walls.

This indicates for a comparable role of the CrGRP as described for *Nt*TLRP, function as a protoxylem associated cell wall protein (Domingo, Sauri et al. 1999). From a defense-strategic point of view, CrGRP gives an accessible and highly abundant target to be sensed by tomato´s immune system. During the phase of haustorial growth, CrGRP could be mobilized due to cell wall growth and degradation by parasitic cell-wall degrading and rearranging enzymes(Vaughn 2002, Vaughn 2003, Albert, Werner et al. 2004, Johnsen, Striberny et al. 2015). Nevertheless, it remains unknown why the CrGRP epitope CrCrip21 is different from the homologs of tomato or how conserved it is among parasitic and nonparasitic plants. The tomato peptides of GRP homologs were not able to induce a CuRe1-dependent defense response ((Hegenauer, Slaby et al. 2020), Fig. S6), and thus we exclude an endogenous function as trigger of defense or stress related responses in tomato. However, it cannot be excluded that the tomato GRPs have other functions and may stimulate other signaling pathways via alternative receptors.

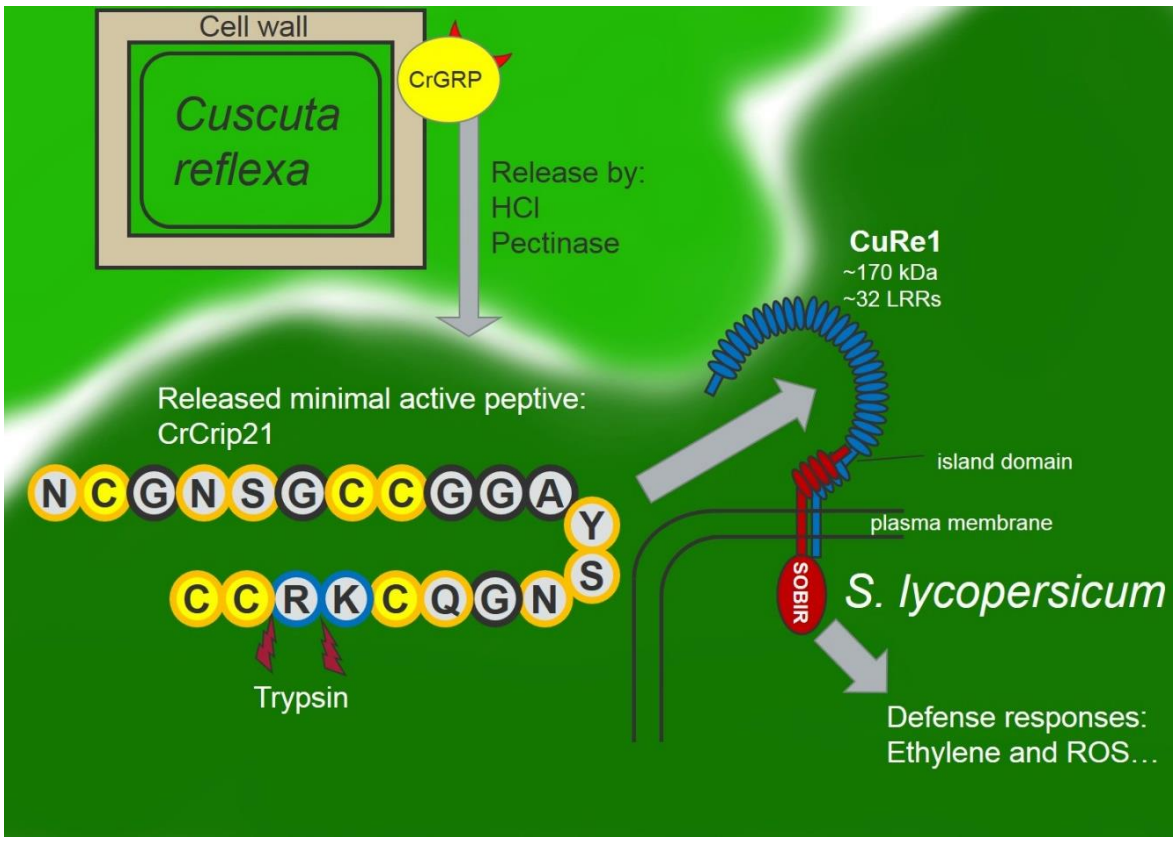

Fig. 8 Working model for the perception of C. reflexa by S. lycopersicum. The LRR-RLP CuRe1 perceives the 21 AA minimal motif of the C. reflexa glycine-rich protein (CrGRP) which is most likely cell wall associated. Perception of CrGRP, or fragments containing at least the CrCriP21 minimal motif by CuRe1 will subsequently trigger the immune response of the plant, including ethylene and ROS production.

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Appendix

Plants under stress by parasitic plants

Volker Hegenauer, Max Körner, Markus Albert

Current Opinion in Plant Biology. 2017 April 29; 38: 34-41. doi: 10.1016/j.pbi.2017.04.006

Detection of the plant parasite Cuscuta reflexa by a tomato cell surface receptor

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Science. 2016 Jul 29;353(6298):478-81. doi: 10.1126/science.aaf3919.

+ supplemental data

Growth Assay for the Stem Parasitic Plants of the Genus Cuscuta

Volker Hegenauer, Max Körner, Max Welz, Markus Albert

Bio-Protocol. 2017 Apr 20; 7(8). doi:10.21769/BioProtoc.2243

Parasitic Cuscuta factor(s) and the detection by tomato initiates plant defense

Ursula Fürst, Volker Hegenauer, Bettina Kaiser, Max Körner, Max Welz, Markus Albert

Commun Integr Biol. 2016 Oct 10;9(6):e1244590. doi: 10.1080/19420889.2016.1244590. eCollection 2016.

The tomato receptor CuRe1 senses a cell wall protein to identify Cuscuta as a pathogen

Volker Hegenauer, Peter Slaby, Max Körner, Julien-Alexander Bruckmüller, Ronja Burggraf, Isabell Albert, Bettina Kaiser, Birgit Löffelhardt, Irina Droste-Borel, Jan Sklenar, Frank L. H. Menke, Boris Maček, Aashish Ranjan, Neelima Sinha, Thorsten Nürnberger, Georg Felix, Kirsten Krause, Mark Stahl and Markus Albert

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+ supplemental data

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Current Opinion in Plant Biology

Plants under stress by parasitic plants

Volker Hegenauer, Max Körner and Markus Albert

In addition to other biotic stresses, parasitic plants pose an additional threat to plants and cause crop losses, worldwide. Plant parasites directly connect to the vasculature of host plants thereby stealing water, nutrients, and carbohydrates consequently leading to tremendously reduced biomass and losses in seed yields of the infected host plants. Initial steps to understand the molecular resistance mechanisms and the successes in ancient and recent breeding efforts will provide fundamental knowledge to further generate crop plants that will resist attacks by plant parasites.

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Introduction

To resist biotic invaders, including microbes or herbivorous arthropods, plants have evolved diverse tactics, such as passive physical or chemical barriers or an innate immune system, which they can use to detect their enemies as 'non-self' and activate to fend off the invaders. In addition to microbial invaders, parasitic plants are a major threat to crops, worldwide. Each year, plant parasites cause estimated crop yield losses of up to US $1 billion, and farm losses are estimated to negatively impact the food supply of over 100 million people annually, and rising [1,2]. Among the flowering plants, there are approximately 4000 known parasitic plant species in more than 20 plant families, indicating that the step from an autotrophic to an heterotrophic lifestyle has evolved independently several times during the radiation of angiosperms [3]. Depending on the targeted host plant organs, parasitic plants are categorized as root parasites and shoot parasites; furthermore, regarding their degree of host dependency they are classified as holoparasites or hemiparasites. Only a few of the latter can be facultative and, in principle, can complete their lifecycle without a host plant. During the evolution of plant parasitism, autotrophy has been stepwise lost, and it has occurred on several independent occasions in parasitic flowering plants [4,5,6•]. Within the family of the Orobanchaceae nearly all forms of root-parasitism can be found, from facultative hemiparasitic (*Rhinanthus* spp.) to obligate holoparasitic (*Orobanche* spp.) or hemiparasitic (*Striga* spp.) member species [3].

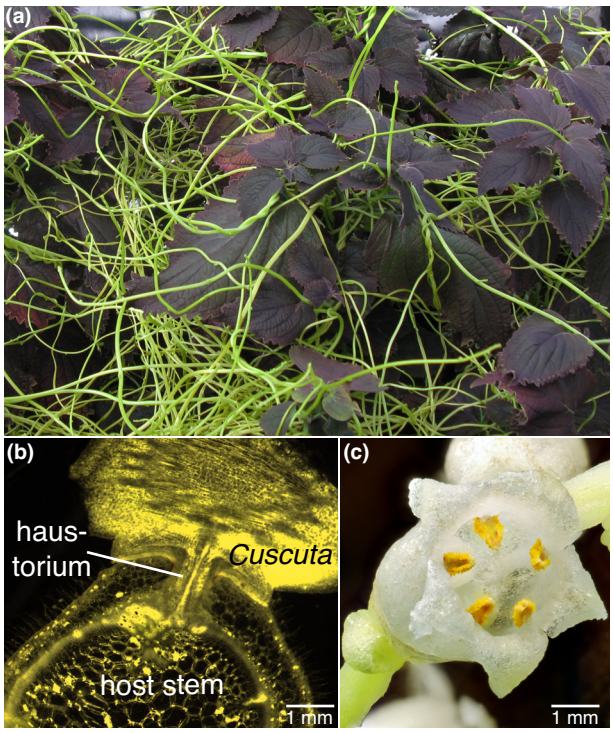
Well-known and agriculturally-relevant genera include *Striga* (witchweed) and *Orobanche* (broomrape) from the family of Orobanchaceae and *Cuscuta* spp. (dodders) (Figure 1a) from the family of Convolvulaceae. While *Striga* and *Orobanche* can severely affect crop yields in drier and warmer areas of Africa and Asia [2,7•], *Cuscuta* spp. thrives in regions with a warm and more humid climate where the highest *Cuscuta*-dependent crop yield losses occur [8,9]. Plants of the genus *Cuscuta* comprise approximately 200 known species which all live without roots as obligate stem holoparasites with broad host spectra for exclusively herbaceous plants [3,8–10]. *Cuscuta* species can be found on all continents [11], and, in respect to agriculture, the most important *Cuscuta* species are *Cuscuta pentagona* and *Cuscuta campestris*. Severe crop loss due to *Cuscuta* has been reported for 25 crop species in 55 countries, including soybeans, coffee, clover, and rape [12].

Establishing a plant–plant connection

All parasitic plant species share one common feature, the connection to the host plant vasculature, which is the ability to exhaust water, nutrients, and/or carbohydrates. Thus, parasitic plants have evolved a specific multicellular organ, the haustorium (Figure 1b) (from the Latin, *haurire*, which means 'to drink'). In haustorium development, the penetration of host tissues and the connection to the parasite's vasculature represent an essential step in the parasite's lifecycle despite its taxonomic divergence. The following section will briefly highlight the most important steps involved in haustorium development and host penetration, for more details please refer to Refs. [3,8,13•,14].

Before a haustorium is formed, the parasite seeds have to germinate and the seedlings must find a suitable host. Seeds of the obligate root parasites of the Orobanchaceae family (*Orobanche*, *Phelipanche*, *Striga*) are highly dormant, and they require strigolactones secreted by the host roots as signals to induce the parasite's germination [15,16], after perception by the strigolactone receptor(s), a family of related α/β-hydrolases that specifically detect

Figure 1



(a) *Cuscuta reflexa* overgrows the susceptible host plant *Coleus blumei*. **(b)** Cross section of a penetrating *C. reflexa* haustorium. **(c)** *C. reflexa* flower, detail (photograph by Eric Melzer).

strigolactone(s) in picomolar ranges [17•,18•,19•]. This induced germination, which occurs exclusively in proximity to a host root, makes sense because the parasites have to infect hosts within a short time frame due to limited resources. Furthermore, haustorium-inducing factors, mostly belonging to the class of soluble phenolic acids or flavonoids, are important for initiating the formation of a haustorium [20–22,23•] at the radicle tip, which attaches to the host root and begins the establishment of the connection between the host root and the parasite [13•,24].

Germinating seedlings of the stem-parasitic *Cuscuta* spp. do not depend on 'germination signals'; rather, they are able to sense plant volatiles as chemo-attractants for a direct growth toward their hosts [25•]. In *Cuscuta* spp., the first physical contact with a potential host initiates an attachment phase in which the parasitic epidermal and parenchymal cells begin to differentiate into a secondary meristem and develop prehaustoria [26], also known as adhesive discs. Important signals that initiate and control this attachment process and the prehaustoria formation of *Cuscuta* spp. include tactile signals, osmotic potentials, and phytohormones, such as cytokinine and auxin [8,27]. During this attachment phase, host cells in proximity to the *Cuscuta* haustoria respond with an increase in cytosolic calcium, which is detectable in plants expressing aequorin as a calcium reporter [28,29]. The host cells become enlarged, and the induction of several genes can be observed that encode the proteins associated with cell wall growth, rearrangement, and restructuring [30–32]. Genes or transcripts of proteins that play an important role in rearranging the cell wall, such as expansins, Arabinoglactan proteins, or Xyloglucan-endotransglycosylases, respectively, get upregulated during infections by both root parasites and shoot parasites, and they can be found within the transcriptome profiles of penetrated host tissues as well as in penetrating parasitic haustoria. RNA-seq studies conducted over the past years have shown significant amounts of transcripts related to up-regulated or downregulated genes, indicating that many cellular processes seem to be established and rearranged during plant-plant interactions; for further details, please refer to [33–37].

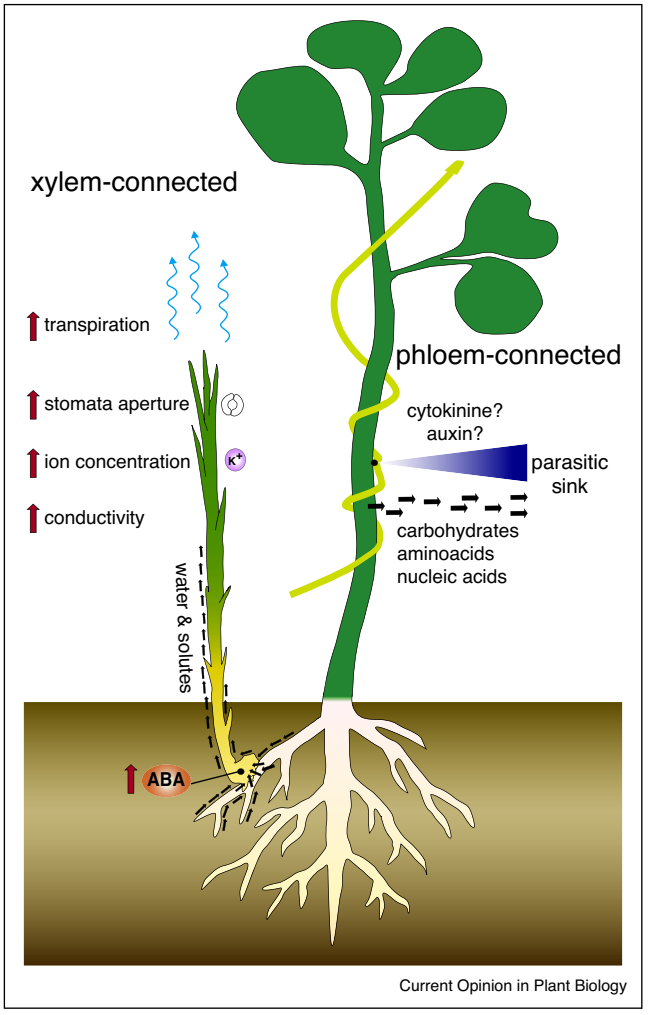
The RNA-seq data support the idea that parasitic haustorium penetration seems to be a very well-orchestrated process, probably including several stepwise-induced developmental programs. Auxin seems to play an essential role in the haustorium development of both root parasites and shoot parasites [27,38••], and increased auxin levels can be measured at haustoria penetration sites. Very recently it has been shown that YUCCA, a flavin monooxygenase in auxin biosynthesis Bcl-2-related protein, gets up-regulated in the haustorium of the facultative parasite *Phtheirospermum japonicum* (Orobanchaceae) during infection, and it was further demonstrated that this auxin biosynthesis-related enzyme is required to mediate and support a susceptible host parasite interaction [38••]. Importantly, during penetration the host cells are not destroyed by the haustorium; rather, they are moved to the side by the ingrowing haustorium, which tries to reach the veins [13•]. Cells at the tip of the invading haustoria of both *Cuscuta* spp. and Orobanchaceae form 'searching hyphae', which try to reach the phloem or xylem cells of the host plant's vascular bundles. In the case of *Cuscuta*-infections, after contact with a sieve cell, the searching hyphae grow around the cell, whereby the parasitic cell surface, which interacts with the host sieve cell, is enlarged [8,26,39–41]. The root parasites of the Orobanchaceae develop analog structures resembling pollen tubes [13•]. Infections by both root parasites and shoot parasites lead to the formation of chimeric cell walls interspersed with interspecific plasmodesmata, which organize a cytoplasmic syncytium between the parasite and the host [26,40,42,43]. To form a connection to the xylem, parasitic *Cuscuta* and the host cells of the xylem parenchyma begin synchronized development, fusing to build a continuous xylem tube from the host to the parasite [40].

With the haustoria as functional connections to the xylem and/or phloem of the host plant, the parasitic plant is supplied with water, nutrients, and carbohydrates [13•,44•,45,46]. In addition to water and nutrients, *Cuscuta* and its host plants efficiently exchange all kinds of macromolecules, such as proteins and RNA, as well as viruses, in a bidirectional manner [47••,48,49••,50,51]. After the plant parasite establishes its connection to the host, infection is established and parasite can grow to complete its lifecycle [3,13•].

Increased stresses in hosts due to parasitic plant infestations

Reproduction is an important feature for parasitic plants, which also requires flowering (Figure 1c) and seed growth in a manner that is similar to the process found in autotrophic plants. In comparison to microbial invaders, plant parasites have much longer vegetative growth periods, mostly as long as their host plants, and their flowering and seed growth are often synchronized with the host plant [8]. This means that plant parasites should keep their hosts alive as long as possible since they are completely dependent on the host's nutrients until they successfully reproduce. For that matter, both partners seem to arrange a kind of 'trade agreement' that—at least in the first few weeks of infection—allows both partners to survive and grow. Both hemiparasites and holoparasites have to exhaust water and solutes from the host. Therefore, plant parasites seem to have a higher xylem conductance than their hosts, and the stomata of the parasites remain wider and, sometimes, continuously open (day and night) in comparison to their hosts [52,53] (Figure 2). While this mechanism cannot be fully explained, in the case of *Striga hermonthica* it seems to relate to the modulatory effects of high potassium concentrations that accumulate in the parasite's leaves as a result of high transpiration rates [53]. Studies on the interaction between the facultative root hemiparasite, *Rhinanthus minor*, with barley (*Hordeum vulgare*) revealed that water uptake, water deposition, and transpiration in *Rhinanthus* were dramatically increased after attachment to the barley host. Most of the water was extracted as xylem sap from the host thereby exhausting about 20% of the total water originally taken up by the host [52]. This is mainly due to the constantly opened parasitic stomata and the strongly increased hydraulic conductance in the seminal roots of *Rhinanthus* in comparison to the roots of the infected host. The latter phenomenon can be explained by the dramatic changes in abscisic acid (ABA) flow, metabolism, and deposition on a per plant basis in *Rhinanthus*. After attachment, the ABA biosynthesis in *Rhinanthus* was increased 12-fold, resulting in a 14-fold increase of ABA flow in the xylem [54]. The overall changes in growth-related water deposition in both the host and parasite leads to a decreased shoot growth, but favored root growth in the host barley and a strongly increased shoot growth in the hemiparasite [52].

Figure 2



Parasitic strategies of nutrient theft; (**left**) both hemiparasitic and holoparasitic plants connect to the host xylem (here *Striga* spp. infecting a host plant root, left) and attract and redirect the water and solute fluxes by increasing the hydraulic conductivity. Thereby, the increase (red arrows) in the level of abscisic acid (ABA) in the parasite's seminal roots (*i.e.*, *Rhinanthus* spp., *Striga* spp.) is important as is the increased stomata opening and ion accumulation (K^+) within the leaves of the parasitic plant. (**right**) Exclusively, holoparasites (*i.e.*, *Cuscuta* spp. lime green) or phloem-feeders present as attractive carbohydrate sinks, probably by the use of an auxin and/or cytokinine gradient. Hence, the parasites are able to exhaust the carbohydrates as well as water, amino acids, nucleic acids, and other macromolecules, such as proteins, which all may take the phloem route via the interspecific cytoplasmic syncytium often observed in phloem–phloem connections. Overall, the theft of these essential nutrients leads to tremendous losses of biomass, seed yield, and fitness in host plants.

These findings clearly indicate that plant parasitic infections cause drought stress in their hosts; increased host root growth especially points to a long-term mechanism known to compensate for this type of stress. If water is no limit, the hosts might deal relatively well with this

problem. However, many hemiparasites, such as *Striga* spp., are a threat in arid and semiarid regions where water is the major limiting factor; thus, infections by plant parasites lead to drastic crop loss.

Xylem-feeding hemiparasites and holoparasites also exhaust mineral nutrients from the host [55]. A few studies have clearly demonstrated the nitrogen and phosphorus uptake by parasitic plants [44•,56,57]. Amongst others, in these works it has been shown that even the fixed nitrogen is mobilized within the infected host, *Lupinus albus*, to be then exhausted by *Cuscuta* spp., or that nitrogen is also taken up in the form of amino acids. In the case of phloem feeders, this nutrient uptake does not exclusively occur via the xylem connection; minerals are also exhausted via the phloem—at least in case of the holoparasites, such as *Cuscuta* spp. or some *Orobanche* spp. [44•,57]. Reduced nitrogen uptake also leads to reduced growth of *Orobanche foetida*, as was demonstrated by the use of susceptible and tolerant (resistant) faba bean strains bred in a Tunisian breeding program [58].

To obtain carbon (Figure 2), mainly in the form of sugars, the strategy of most parasitic plants relies on a yet unknown mechanism, represented as an attractive sink, probably mimicking a part of the infected host (*e.g.*, a young leaf, a growing shoot, or a developing fruit). As a plant, a parasite possesses all the physiological requirements, such as phytohormones, to accomplish this. Interestingly, even facultative and/or obligate hemiparasites, such as *Rhinanthus* spp. or *Striga* spp., seem to obtain 10–30% of their carbohydrates from the host plant [59], even though they only have a xylem-connection and they do not have a direct phloem. It was further observed that *S. hermonthica* reduces photosynthesis in *Sorghum* during infection, although it exclusively has contact with the xylem [60]. Holoparasites, for example, *Orobanche* ssp. or *Cuscuta* spp., obtain 100% of their carbohydrates exclusively from the host plants, while some species of the latter genus possess residual or 'cryptic' photosynthesis, which seems to contribute less than 1% to the total parasitic carbohydrates [61•,62]. Studies on *Cuscuta reflexa* parasitizing a variety of hosts and *Orobanche cernua* parasitizing tobacco (*Nicotiana benthamiana*) have demonstrated that almost all the carbon for the parasite plant is taken up from the host phloem. Interestingly, infection by both *Cuscuta* and *Orobanche* might represent an additionally generated sink, and it induces an increase in host photosynthesis of approximately 20–40% [45,46,57]. However, up to 80% of the photosynthesis products are taken up by *Cuscuta*, meaning a net loss of the fixed carbon for the infected host. To represent itself as a sink, an auxin gradient may play a supposable role since its increased biosynthesis has been observed during plant–plant interactions [27,38••] right at the infection sites, and, importantly, it is known to act as a phytohormone to indicate a sink [63]. This mimicking allows the parasite to redirect and draw the flow of nutrients, solutes, and carbohydrates. Consequently, this tapping leads to tremendously reduced biomass and seed yield in the hosts, as usually observed and recently described and quantified for faba beans infected by *Orobanche crenata* [64•].

Getting parasites rejected: perspectives for the future

In general, the theft of nutrients, water, and carbohydrates by parasitic plants causes major stresses in host plants, and it results in reduced plant growth, diminished biomass, premature flowering, and untimely seed ripening. However, the early haustorium penetration phase should also cause stress in the host since it includes the wounding of tissue as well as mechanical pressure. Especially, cell wall degradation products released due to parasitic hydrolytic enzymes may serve as damage-associated molecular patterns that host plants can detect as danger signals [65,66]. To date, no damage-associated molecular pattern (DAMP) has been reported to induce host plant defense in the case of plant–plant interaction, and the wounding of hosts mostly seems undetected perhaps due to the parasite's yet unknown suppression strategies [10,67]. In general, it would be a significant benefit for host plants to sense attacks and to prevent plant parasitic infestations as early as possible. Apparently, most host plants lack active defense systems to ward off other plants, which seem to go unrecognized. Perhaps because of their close phylogenetic relationship, it is difficult for host plants to detect parasitic plants as 'non-self'; thus, defense mechanisms are inefficient for dealing with plant parasites. However, some host plants are resistant to plant parasites. A few of the known details about the molecular mechanisms of these resistant plants are delineated below.

The detection or rejection of species of the Orobanchaceae family, such as *S. hermonthica*, for example, occurs at different infectious stages [68], and it is mainly categorized as pre-attachment and post-attachment resistance. Post-attachment resistance occurs after haustorium penetration, leading to a failed vasculature connection. Pre-attachment resistance occurs within the parasite's germination or during the early attachment phase [2]. The resistance, especially against *Striga* spp. or *Orobanche* spp. [69], is complex and probably relies on quantitative resistance traits, as supported by the findings of high variation within and between *Striga* ecotypes, which makes complete host plant resistance rare. However, recent breeding efforts have shown great potential for striking back against *Striga* [70,71••,72] or *Orobanche* [69], although the detailed molecular mechanisms behind the resistance are rarely understood. Overall, the situation of host plant resistance to parasitic *Striga* spp. or *Orobanche* spp. is similar to cereal resistance against fungal rust infections, where many resistance genes, mainly encoding nucleotide-binding site leucine-rich repeat

(NBS-LRR) proteins, are required to confer resistance (recently reviewed in Ref. [73]). Indeed, gene encoding such as a cytosolic immune receptor protein, has been identified in cowpea; the coiled-coil nucleotide binding site leucine-rich repeat (CC-NNB-LRR) domain, encoded by the resistance gene *RSG3-301*, is important in conferring resistance against the root parasite *Striga gesnerioides* [74••]. These types of cytosolic receptors are essential parts of effector-triggered immunity (ETI) that detects the virulence effectors usually produced by microbial pathogens, and they are known to induce strong resistance-related phenotypes often including hypersensitive response or programmed cell death in the infected tissues of host plants [75].

Resistance was also observed against *Cuscuta* spp.; as one notable exception, cultivated tomato (*Solanum lycopersicum*) exhibits a form of hypersensitive response (HR) to attempted penetration exclusively by *C. reflexa* haustoria, resulting in resistance against this parasite. This tomato-specific HR was observed decades ago [76], but the mechanisms behind it have not yet been fully explained. The modified tissue of tomato plants, which builds an insurmountable barrier for the parasite's haustorium, mainly consists of phenolic compounds and aliphatic di-OH-fatty acids [10,77,78], and it is evocative of the suberin formed in plants after wounding [79]. Interestingly, few other *Cuscuta* species, for example *C. pentagona*, can successfully penetrate tomato plants although slight HR-symptoms are visible. This incomplete defense can be observed mainly for younger tomato plants, and it seems to involve jasmonic acid (JA) and salicylic acid (SA) [80]. Such a species-specific defense also points to a mechanism known as gene-for-gene interaction [81], which has been described for the interaction between tomato plants and the fungal pathogen, *Cladosporium fulvum*, where the avirulence proteins of various fungus strains are recognized by diverse resistance receptors present in different tomato cultivars [82,83].

In a recent study [84••], the cell surface receptor *Cuscuta* Receptor 1 (CuRe1) of tomato plants has been identified as the critical receptor to detect a plant parasite-associated molecular pattern, a secondarily modified peptide with a molecular weight (MW) of ~2 kDa, which was called the '*Cuscuta* factor'. Tomato *CuRe1* is sufficient to confer defense-related responsiveness, such as the production of ethylene or reactive oxygen species (ROS), specifically for treatments with this *Cuscuta* factor; it further increases resistance to parasitic *C. reflexa* infections when heterologously expressed in otherwise susceptible host plants [84••]. Taken together, these immune responses are similar to those usually activated in response to microbe-associated molecular patterns (MAMPS) or pathogen-associated molecular patterns (PAMPs) [65,85]. However, CuRe1 alone is not sufficient to induce a strong HR in transgenic, *CuRe1*-expressing *N. benthamiana*, usually visible on the tomato stem at the penetration sites of the parasitic haustorium; this indicates that CuRe1-mediated resistance is just a small part of a complex tomato strategy ending in complete resistance against *C. reflexa* [84••,86,87].

Conclusion

Parasitic plants pose an increasing danger to cultivated crops, worldwide. Chemical control by herbicide applications to such plants is very limited, if not impossible, since most treatments negatively impact the crop hosts as well. Therefore, the breeding of resistant crops together with an understanding of the molecular mechanisms of a host plant's resistance to parasitic plants could provide a highly promising basis to prevent larger epidemics and crop losses caused by parasitic plants.

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The authors discovered that tomato responds to a small peptide factor occurring in *Cuscuta* spp. with immune responses typically activated after perception of microbe-associated molecular patterns. In tomato, they identified the cell surface receptor-like protein CUSCUTA RECEPTOR 1 (CuRe1) as essential for the perception of this parasite-associated molecular pattern. CuRe1 is sufficient to confer responsiveness to the -*Cuscuta* factor and increased resistance to parasitic *C. reflexa* when heterologously expressed in otherwise susceptible host plants. These findings revealed that plants recognize parasitic plants in a manner similar to perception of microbial pathogens.

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then still impair women's chances to pursue a career in quantitative science (or philosophy), but only at the early stages of the curriculum, before or just as they enter the pipeline that leads to a Ph.D. or a professorial position.

Nevertheless, there is no compelling evidence of hiring discrimination against individuals who have already decided against social norms to pursue an academic or a teaching career in a field where their own gender is in the minority. This result has three consequences for policy. First, active policies aimed at counteracting stereotypes and discrimination should probably focus on students at early ages, before educational choices are made. Second, nonblind evaluation and hiring should be favored over blind-evaluation in order to reduce gender imbalances across academic fields. In particular, policies that impose anonymous curricula vitae in the first stage of academic hiring are likely to have effects opposite to those expected. Third, many women may shy away from male-dominated fields at early ages because they believe that they would suffer from discrimination. Advertising that they have at least as good—or even better—opportunities as their male counterparts at the levels of secondary school teaching and professorial recruiting could encourage talented young women to study in those fields.

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SUPPLEMENTARY MATERIALS

www.sciencemag.org/content/353/6298/474/suppl/DC1
Materials and Methods
Supplementary Text
Figs. S1 to S3
Tables S1 to S14
References (*33*–*38*)

13 March 2016; accepted 29 June 2016
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PARASITIC PLANTS

Detection of the plant parasite *Cuscuta reflexa* by a tomato cell surface receptor

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Parasitic plants are a constraint on agriculture worldwide. *Cuscuta reflexa* is a stem holoparasite that infests most dicotyledonous plants. One exception is tomato, which is resistant to *C. reflexa*. We discovered that tomato responds to a small peptide factor occurring in *Cuscuta* spp. with immune responses typically activated after perception of microbe-associated molecular patterns. We identified the cell surface receptor-like protein CUSCUTA RECEPTOR 1 (CuRe1) as essential for the perception of this parasite-associated molecular pattern. CuRe1 is sufficient to confer responsiveness to the Cuscuta factor and increased resistance to parasitic *C. reflexa* when heterologously expressed in otherwise susceptible host plants. Our findings reveal that plants recognize parasitic plants in a manner similar to perception of microbial pathogens.

Along with microbial pathogens and herbivorous arthropods, parasitic plants represent an additional class of threat to crops (*1*). As many as 4000 species, belonging to more than 20 plant families, have been classified as parasitic plants; hence, the switch to a parasitic lifestyle occurred independently and on several occasions during evolution (*2*).

The plant genus *Cuscuta* (dodder) comprises about 200 species, all of which live as obligate stem holoparasites with broad host spectra (*3*–*6*). Germinating *Cuscuta* seedlings sense plant volatiles and direct their growth toward their host (*7*). Initial contact induces the formation of haustoria (*8*), specialized structures that attach, penetrate, and connect to the vascular bundles of their hosts. Once connected, *Cuscuta* parasites withdraw water, nutrients, and carbohydrates (*5*, *9*–*11*) from host plants, and also exchange macromolecules such as proteins and RNAs, as well as viruses, in a bidirectional manner (*12*–*17*).

Most susceptible plants lack efficient defense systems to ward off *C. reflexa*. However, the cultivated tomato (*Solanum lycopersicum*) is resistant to *C. reflexa* and exhibits a hypersensitive response to attempted penetration by *C. reflexa* haustoria (*18*–*21*) (Fig. 1A). We asked whether tomato might detect and respond to molecular signals associated with the parasitic plant in a

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manner comparable to the response of plants to microbe-associated molecular patterns (MAMPs). We tested extracts of *C. reflexa* for their ability to induce release of reactive oxygen species (ROS) and to trigger synthesis of the stress-related phytohormone ethylene. Indeed, *C. reflexa* extract triggered both responses in *S. lycopersicum* but not in susceptible plants, including the related Solanaceae *Nicotiana tabacum*, *N. benthamiana*, *S. tuberosum*, and the wild tomato species *S. pennellii* (Fig. 1B and fig. S1).

Initial characterization showed that the factor present in the *C. reflexa* extract is heat-stable at 95°C but is sensitive to treatment with proteases (Fig. 1C). Checking for putative secondary modifications, we observed that enzymatic de-N-glycosylation had no influence on its activity, whereas treatment with ammonia, a procedure known to remove ester-type modifications such as sugar side chains from peptide backbones (*22*), led to a loss of functionality (Fig. 1D). The Cuscuta factor appeared to be constitutively present in all parts of *C. reflexa*, including shoot tips, stems, haustoria, and, at lower levels, in flowers (fig. S2A), indicating that this factor is not produced only at certain developmental or infectious stages. Activity was apparently associated with the cell walls of the parasite, from which it could be released by acidic conditions (fig. S2B).

We observed induction of ethylene production in *S. lyopersicum* with extracts from six different *Cuscuta* species but not with extracts from *A. thaliana*, *N. benthamiana*, or *S. lycopersicum* (Fig. 1E). Also inactive were extracts from *Calystegia sepium* (hedge bindweed), a nonparasitic species of the Convolvulaceae related to *Cuscuta*, and *Rhinanthus alectorolophus*, a hemiparasitic flowering plant of the Orobanchaceae, which infects roots of many herbaceous plants (Fig. 1E). Thus, the active factor seems to be common to *Cuscuta* species but absent from plants outside this genus.

To purify and identify the Cuscuta factor, we established a purification scheme involving sequential separation steps (fig. S3A). Prepurified *C. reflexa* extract was first separated by cation exchange chromatography, where activity eluted as several peaks indicating heterogeneity with respect to charge (fig. S3B). Active fractions of "peak 2" (fig. S3B) were pooled and further purified by reversed-phase chromatography (RPC) on C18 material using different pH conditions. This activity further split into different peaks and fractions (fig. S3C); this indicates that the activity, rather than representing a single defined molecule, is associated with a range of physicochemically heterogeneous compounds present in the *Cuscuta* extract. Although this heterogeneity dispersed activity to numerous subfractions, we succeeded in purifying a single molecule with a molecular mass of 2262.79 Da that correlated with activity in elution from the final RPC used for liquid chromatography–mass spectrometry (MS) analysis (fig. S3D). However, we did not obtain conclusive fragmentation patterns from tandem MS/MS analysis of this molecule in several attempts (fig. S4). Apart from the low amount of this particular form of the Cuscuta factor, this might be attributable to the yet unidentified modification present on the peptide. Nonetheless, our data suggest that the Cuscuta factor is associated with a small, potentially modified (e.g., O-glycosylated) peptide that is characteristically present in extracts from *Cuscuta* spp.

We exploited the natural variation between susceptible *S. pennellii* (*23*) and resistant *S. lycopersicum* to identify the receptor for the Cuscuta factor. We used an introgression library of *S. lycopersicum* × *S. pennellii* (*24*) to map genomic regions essential for the differential response to the Cuscuta factor. The collection of 49 introgression lines (ILs) included chromosome fragments of *S. pennellii* covering ~98% of the tomato genome (*25*). Only line IL8-1 was unresponsive to the Cuscuta factor (Fig. 2A). Further mapping with sublines IL8-1-1 and IL8-1-5 (*24*) (Fig. 2B) identified a chromosome region termed bin d8-B (Fig. 2C) (*25*), which has 822 annotated genes. Only five of these genes are predicted to encode cell surface receptor–type proteins (*25*) that could perceive the Cuscuta factor. We individually expressed these candidate genes,

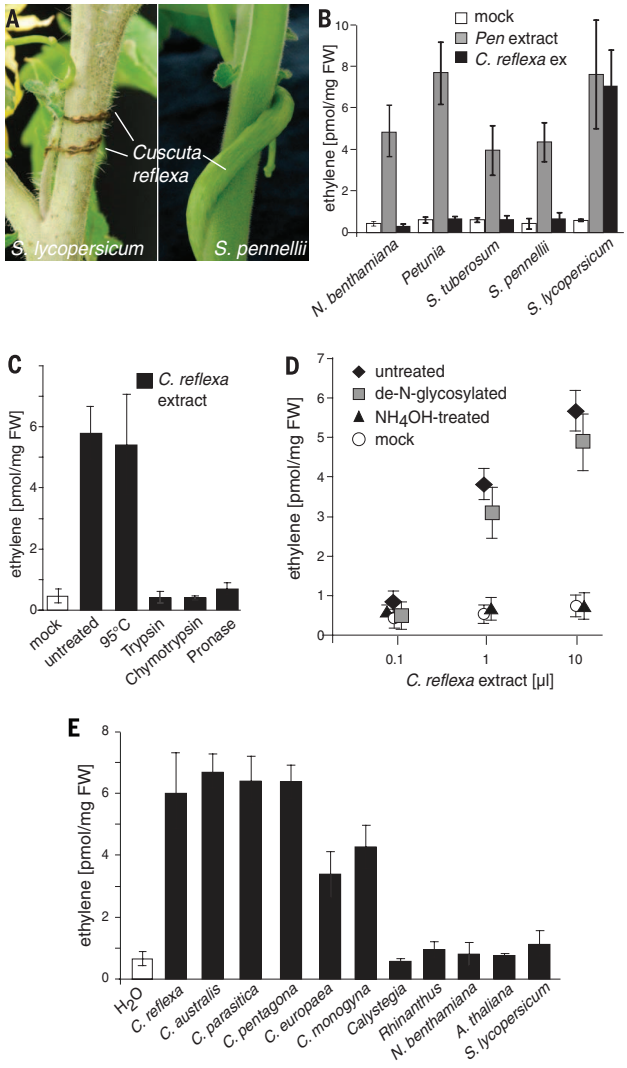

Fig. 1. *S. lycopersicum* (cultivated tomato) shows defense responses to *C. reflexa* and to extracts thereof. (**A**) Left: *C. reflexa* cannot form connections to *S. lycopersicum* and dies off. Right: *C. reflexa* on the susceptible host *S. pennellii*. Photos were taken ~14 days after parasite onset. (**B**) *C. reflexa* extract triggers ethylene biosynthesis in *S. lycopersicum* but not in other plant species. Bovine serum albumin (BSA) buffer in 25 mM MES buffer, pH 5.7 (0.01 mg/ml) was added as mock control; *Penicillium* extract (0.05 mg/ml) served as positive control (*38*). FW, fresh weight. (**C** and **D**) Characteristics of the Cuscuta factor present in the *C. reflexa* extract. (C) Ethylene biosynthesis of tomato leaf pieces to *C. reflexa* extract, to boiled extract (95°C, 30 min), or to extract pretreated with the proteases indicated. (D) Ethylene response to different doses of the Cuscuta factor after enzymatic de-N-glycosylation or to Cuscuta factor treated with 20% NH_4OH (45°C, 16 hours), respectively. (**E**) Ethylene response of tomato leaf pieces triggered by extracts of other *Cuscuta* species or by extracts of other plants. In (B) to (E), ethylene measurements show means of three technical replicates; error bars denote SD. All experiments were repeated more than three times.

which encode three leucine-rich repeat receptor-like proteins (LRR-RLPs) and two receptor-like kinases (RLKs), in *N. benthamiana*, a species lacking an endogenous detection system for the Cuscuta factor (Fig. 1B and fig. S1). Four of these candidates had no effect, but *N. benthamiana* leaves expressing Solyc08g016270 responded to the Cuscuta factor with increased ethylene biosynthesis (Fig. 2D) and an oxidative burst (Fig. 3A). Dose dependence of response (Fig. 3B) showed half-maximal stimulation with Cuscuta factor at an estimated concentration of <0.3 nM. Thus, the protein encoded by Solyc08g016270 is sufficient to confer sensitive responsiveness specific for the Cuscuta factor, and we termed it CuRe1 (Cuscuta receptor 1). To corroborate its function as a genuine receptor that directly interacts with the Cuscuta factor as a ligand, we tested whether immunoprecipitates of CuRe1 could specifically retain Cuscuta factor when incubated with *Cuscuta* extract. As controls, we used similar immunoprecipitates obtained from *N. benthamiana* leaves expressing the receptor kinase EFR (*26*) and the LRR-RLP AtRLP23 (*27*) from *Arabidopsis*. Cuscuta factor, assayed by the ethylene induction assay in tissue expressing CuRe1, was reproducibly detected in immunoprecipitates with CuRe1 but not with control receptors or empty beads (Fig. 3C).

Because activity in *Cuscuta* extracts separates into distinct subfractions during purification (fig. S3B), we tested these different forms of the Cuscuta factor for bioactivity in CuRe1-expressing *N. benthamiana* plants. Samples from all subfractions (fig. S3B) induced clear ethylene responses in a CuRe1-dependent manner, indicating that the Cuscuta factor, although heterogeneous in structure, triggers CuRe1 via a common active principle. Similarly, we confirmed that the extracts of other *Cuscuta* species (Fig. 1E) also induced ethylene biosynthesis via CuRe1 (fig. S5B).

CuRe1 encodes a typical LRR-RLP that comprises an N-terminal signal peptide for export via the endoplasmic reticulum, a large LRR ectodomain with 30 to 32 LRRs and 18 potential N-glycosylation sites, a single transmembrane helix, and a short cytoplasmic tail (fig. S6). Full-length *CuRe1* is represented in *S. lycopersicum* genomic DNA and cDNA but is absent from *S. pennellii* and from IL8-1-1 (fig. S7). This is in accordance with available genomic sequencing data showing that only truncated forms of *CuRe1*—annotated as Sopen08g00656 and Sopen08g006740—are present in *S. pennellii* (*28*). The closest relatives of *CuRe1* in *S. lycopersicum*, with amino acid sequence identities of 82% and 72%, respectively (Solyc08g016210 and Solyc08g016310), were found to be in close proximity to *CuRe1* on chromosome 8 but were unable to initiate ethylene production in response to the Cuscuta factor when transiently expressed in *N. benthamiana* (Fig. 2D). CuRe1-like sequences with similar amino acid sequence identities of ~70 to 80% can also be found in other Solanaceae but not in species outside this family. However, the CuRe1-related genes present in *N. benthamiana* and *S. tuberosum* seem not to be sufficient to confer responsiveness to the Cuscuta factor in these species (Fig. 1B and fig. S1).

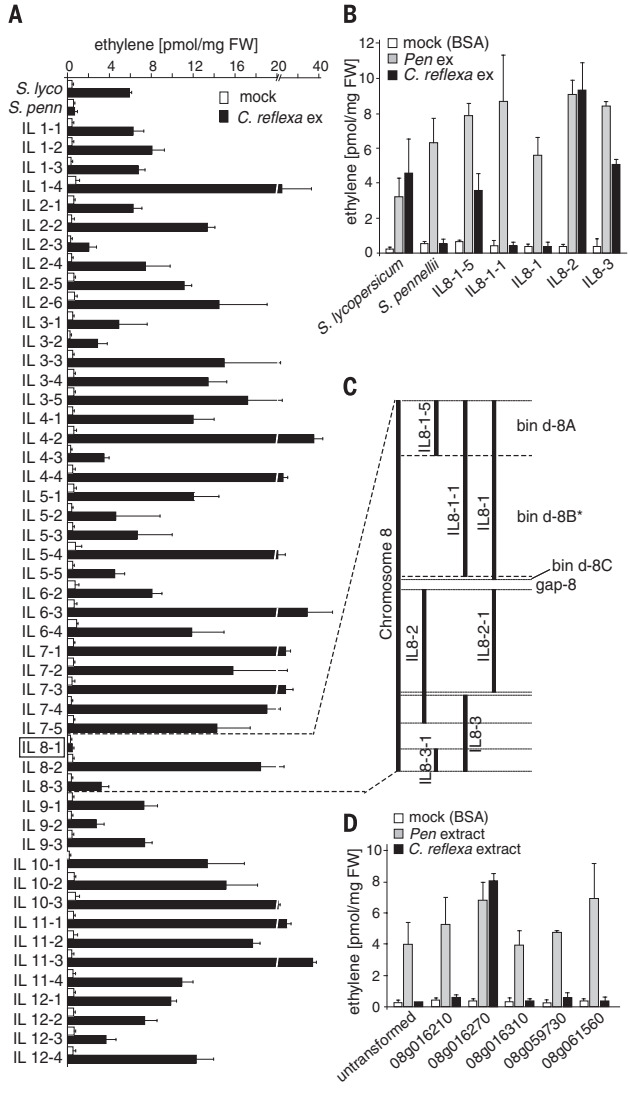

Fig. 2. Mapping of responsiveness to *C. reflexa* extracts in tomato. (**A**) Tomato introgression lines of *S. lycopersicum* × *S. pennellii* were screened for ethylene production in response to *C. reflexa* extract; BSA/buffer was used as mock control. (**B**) Ethylene response of additional ILs related to tomato chromosome 8. (**C**) Graphical scheme of the mapped chromosome region bin d8-B as modified from (*25*). (**D**) Receptor candidate genes encoded within bin d8-B were expressed in *N. benthamiana*. Ethylene production was measured after treatment with *C. reflexa* extract, *Penicillium* extract, or BSA as indicated. Data are means ± SD of $n = 3$ replicates.

Plant receptor-like proteins lack cytoplasmic kinase domains for signaling output and, in general, seem to depend on adaptor kinases of the SOBIR1 (suppressor of BAK1-interacting receptor kinase) type (*27*, *29*–*33*). Coimmunoprecipitation analysis with tagged versions of CuRe1 and SlSOBIR1 or SlSOBIR1-like from *S. lycopersicum* (tomato) showed constitutive interaction of CuRe1 with both of these adaptor kinases (Fig. 3D). As for other RLPs, such as tomato Cf-9 and Cf-4 or *A. thaliana* AtRLP23, AtRLP30, and ReMax/AtRLP1 (*27*, *29*, *30*, *33*, *34*), formation of the complex between CuRe1 and SlSOBIR1 occurred irrespective of the presence or absence of the Cuscuta factor as stimulus (Fig. 3D).

To check for the biological function of CuRe1, we stably transformed *CuRe1* constructs into *S. pennellii* and *N. benthamiana*, which are usually insensitive to the Cuscuta factor and susceptible to *C. reflexa* attack (Fig. 1B and fig. S1) (*23*, *35*). Transformed lines of *S. pennellii* and *N. benthamiana* plants gained responsiveness to Cuscuta factor (figs. S8 and S9) and exhibited increased resistance to *C. reflexa* infestation (Fig. 3, E and F). Thereby, the process of parasite ingrowth seems disturbed, as hypersensitive response symptoms were visible at haustoria penetration sites on the host (fig. S10). Thus, CuRe1 from tomato improves resistance to *C. reflexa* attack in both the closely related species *S. pennellii* and the more distant species *N. benthamiana*.

Full resistance of tomato against *C. reflexa* seems to require more than CuRe1 and perception

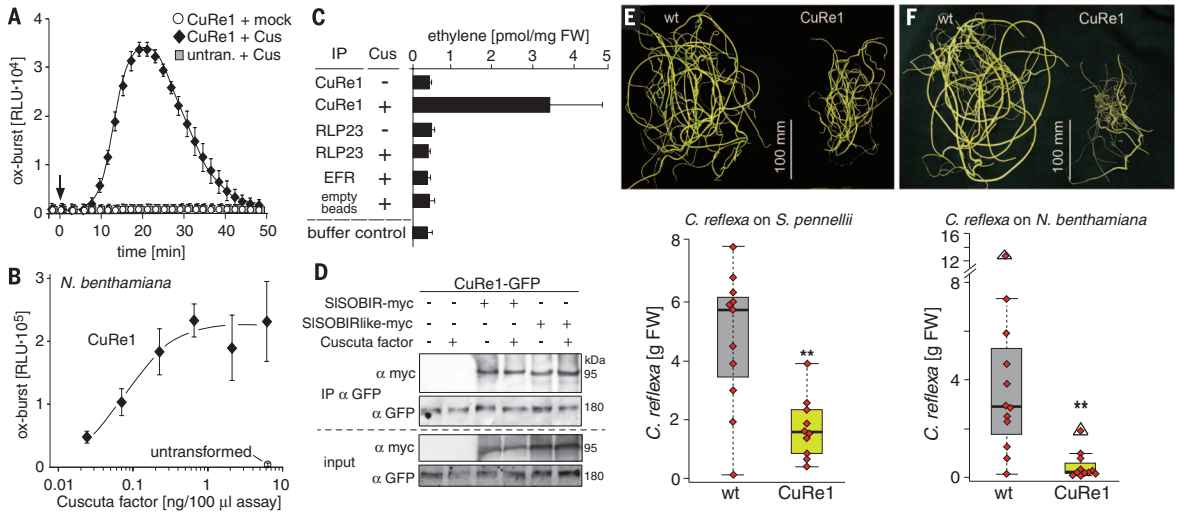

Fig. 3. CuRe1 exhibits properties as pattern recognition receptor for the Cuscuta factor. (**A**) *N. benthamiana* leaves expressing CuRe1 respond with rapid induction of an oxidative burst to treatment with the Cuscuta factor. Data are means ± SD of $n = 5$ replicates. RLU, relative light units. (**B**) Dose dependence of oxidative burst induction by purified Cuscuta factor. Assuming the preparation contains only Cuscuta factor (~2 kDa), this allows estimation of a half-maximal effective concentration $EC_{50} < 0.3$ nM. Values show maxima of oxidative burst (mean ± SD of three measurements). (**C**) Binding of Cuscuta factor (Cus) to immunoprecipitates (IP) of CuRe1; EFR, AtRLP23, or empty beads were used as controls. Ethylene production of *N. benthamiana* leaf tissue expressing CuRe1 and treated with the elutions derived from the receptor IPs indicated; data are means ± SD of $n = 3$ replicates. The experiment was independently repeated >3 times. (**D**) CuRe1 forms a complex with SOBIR1-type adaptor kinases. Immunoblots of SlSOBIR1-myc and SlSOBIR1-like–myc, coimmunoprecipitated with CuRe1; pulldown against the C-terminal green fluorescent protein tag present at CuRe1. Proteins were coexpressed in *N. benthamiana*, and samples were treated with Cuscuta factor (+; 1:100 diluted in water) or water alone (–) as control. (**E**) Growth of *C. reflexa* shoots on *S. pennellii* plants transformed with CuRe1 (T_1 generation) or nontransformed wild-type (wt) controls during 14 days of infestation with one *C. reflexa* shoot [15 cm in length, ~0.6 g FW] per host plant. Red diamonds represent weight of individual *C. reflexa* shoots. Box plots show median values of $n = 12$ replicates. $^{**}P_{adj} = 0.0015$ (Tukey honestly significant difference test). (**F**) Growth of *C. reflexa* shoots on *N. benthamiana* plants stably transformed with CuRe1 (homozygous T_2 generation) or nontransformed wild-type controls during 21 days of *C. reflexa* infestation. Experimental conditions and data evaluation were as in (E). Triangles mark outliers not included in analysis. $^{**}P \leq 0.005$ (Student t test). Data presented in (E) and (F) are representative of three independent repetitions, each with different lines of transformants.

of the Cuscuta factor alone. This is evident from the observation that the two introgression lines that lack CuRe1 (IL8-1 and IL8-1-1) (fig. S8) still showed HR symptoms and proved fully resistant to *C. reflexa* (fig. S11). Immunity against *C. reflexa* in tomato may be a process with layers additional to CuRe1, much like defense systems against microbial pathogens where MAMP-triggered immunity (MTI) and effector-triggered immunity (ETI) act as two perception layers of the plant immune system (*36*, *37*). Nonetheless, our data show that the MTI type of responses stimulated by the Cuscuta factor via the pattern recognition receptor CuRe1 significantly contribute to protection of host plants against *C. reflexa*. Identification of CuRe1 thus provides a starting point for studying and managing parasitic plant infestations.

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SUPPLEMENTARY MATERIALS

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Figs. S1 to S11
Materials and Methods
References (*39*–*43*)

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Science
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Detection of the plant parasite *Cuscuta reflexa* by a tomato cell surface receptor

Volker Hegenauer, Ursula Fürst, Bettina Kaiser, Matthew Smoker, Cyril Zipfel, Georg Felix, Mark Stahl and Markus Albert (July 28, 2016)

Science **353** (6298), 478-481. [doi: 10.1126/science.aaf3919]

Editor's Summary

Resistance is not, after all, futile

The parasitic plant known as dodder attaches to its hosts and sucks the life out of them. Oddly, the common tomato stands tall when under attack. Hegenauer *et al.* have leveraged that difference to identify part of the molecular defense system that protects tomato plants (see the Perspective by Ntoukakis and Gimenez-Ibanez). In a process analogous to defenses mounted against microbial infection, the host plant perceives a small-peptide signal from the parasitic plant and initiates defense responses. The candidate receptor isolated from the tomato plant provided partial protection when transferred to two other susceptible plant species.

Science, this issue p. 478; see also p. 442

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Supplementary Materials for

Detection of the plant parasite *Cuscuta reflexa* by a tomato cell surface receptor

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This PDF file includes:

Materials and Methods

DNA extraction and cloning of receptor candidates

Plants were grown under long day conditions (16 h day/8 h night) at 22 °C, in growth chambers or in a greenhouse. Genomic DNA was extracted from frozen tissue using the Plant DNA Preparation Kit (Jena Biosciences, Germany), and PCR was performed with gene specific primers for candidate genes (Solyc08g016270 (CuRe1): FW: ATGGGGAATATTAAGTTTTTG, REV: ACCAACATTCTTGTACCATCTAC; Solyc08g059730: FW: ATGGGGTCTTGGATTTCCC, REV: TCTTGGACCTGAGAGCCGAACAGC; Solyc08g061560: FW: ATGGCATCATTTTTACTCCAAAG, REV: GCCACTATTCTGGGATATGACC; Solyc08g016210: FW: ATGGGGAACGTTAAGTTTTTGTTG, REV: ATTAATCAACCTTCTACTCTTGATG; Solyc08g016310: FW: ATGGGGAACATTAAGTTTTTGTTG, REV: ACCAACATTCCTGAACCAGCTAC). The PCR products were cloned to the pENTR^{TM}/TEV/D-TOPO® vector (Invitrogen^{TM}). For directional cloning, a CACC tetra-nucleotide was added to the forward primers. Reverse primers without stop codon allowed for C-terminal fusion to GFP and myc tags after recombining via LR-reaction (LR-clonase® II Plus enzyme mix, Invitrogen^{TM}) into respective vectors (pB7FWG2.0, pK7FWG2.0, both with C-terminal GFP tag; plant systems biology, university of Gent). Total RNA was extracted from tomato plants (RNeasy Plant Mini Kit, Quiagen), and cDNA was synthesized by reverse transcription (First-Strand cDNA Synthesis Kit, GE Healthcare Life Sciences).

Plant transformation

For stable transformation, the 35S::CuRe1:GFP constructs (in vectors pB7FWG2.0 and pK7FWG2.0) were transformed into *N. benthamiana* leaves using *Agrobacterium tumefaciens* (strain GV3101). Stably transformed *S. pennellii* plants were regenerated from either leaf disc or cotyledon-derived calli using *Agrobacterium tumefaciens* strain Agl1 as described (T0 generation). For transient expression of 35S::CuRe1:GFP constructs, *A. tumefaciens* cultures (OD_{600} = 0.1 in 10 mM $MgCl_2$) were infiltrated into leaves of 4 weeks old *N. benthamiana* plants, according to the described protocol (*39*). About 24 h post infiltration, leaves were cut into small pieces (3 x 4 mm), floated at rt overnight on water in a Petri dish (*39*) and used the following day in ethylene or ROS-burst bioassays.

Plant response assays

Ethylene assays were performed as previously described (*39, 40*), using samples of 3 leaf pieces (~3 x 4 mm each) in 6 ml glass reaction tubes with 500 µl H_2O. Samples were treated with Cuscuta factor or other elicitors as indicated, sealed with rubber plugs and incubated for 3 h at rt on a shaker (130 rpm). Ethylene was measured by injecting 1 ml of the air phase into a gas chromatograph (Shimadzu) and analyzed as described (*40*). ROS-burst was performed as previously reported (*27, 39*), using 20 µM luminol (L-012, Wako Chemicals USA) and 5 µg/ml horseradish peroxidase (Applichem, Germany). Emitted light was detected with a multi-well luminometer (LBCentro 960, Berthold technologies, Germany) by collecting light signals for 1 s per well/sample.

Cuscuta growth assay

Cuscuta reflexa shoots of ~15 cm length (diameter 0.2–0.3 cm), including the growing tip, were cut and wrapped around wooden sticks. One day later (before formation of pre-haustoria), the weight of each shoot was determined (~0.6 g $\pm$ 0.2 g), and the shoots were wrapped around host plant stems. Fourteen or twenty-one days later, *C. reflexa* shoots were removed, and the fresh weight (FW) determined. Statistical analysis was performed using "R" (*41*); boxplots were generated with the add-on "ggplot2" (*41*).

Immunoprecipitation assays

For immunoprecipitation, leaves of *N. benthamiana* transiently transformed with 35S::CuRe1:GFP, alone or in combination with 35S::SlSOBIR/SOBIR-like:myc (*29*) for ~48 h, were treated with Cuscuta factor (1:100 in water) or water as control solution for 5 min, material was frozen in liquid nitrogen and ground to fine powder. Samples of 300 mg were solubilized and used for immunoprecipitation as reported (*42*) using α-GFP trap sepharose beads (ChromoTek, IZB Martinsried, Germany). Samples were separated by SDS-PAGE (8% Acrylamide gels) and transferred to nitrocellulose membrane. Western blots were probed using the α-GFP (Torrey Pines Biolabs) or α-myc (SIGMA) antibodies, diluted according to the instructions of the suppliers, and developed with secondary antibodies conjugated to alkaline phosphatase as described (*27, 43*).

CuRe1 binding assay

Leaves of *N. benthamiana* plants expressing CuRe1 or the control receptors AtRLP23 or EFR, respectively, were harvested, membrane-bound proteins were solubilized and immunoprecipitation was carried out as described above using 50 µl α-GFP trap beads/sample. Beads were washed twice in solubilization buffer (150 mM NaCl, 25 mM TRIS pH 8.0; 1% NP40, 0.5% DOC) and equilibrated by washing 2x with binding buffer (25 mM MES pH 5.8, 50 mM NaCl, 2 mM $MgCl_2$). Cuscuta extract was added and samples were incubated on an over-head shaker (6 rpm) at 4°C. After 30 min, supernatant was discarded and samples were washed 5-6x with 1 ml binding buffer (4°C). Receptor-bound ligand was eluted by boiling samples (95°C) in 100 µl binding buffer, 5 min; per treatment, 5 µl supernatant (eluted ligand) was used in the ethylene bio assay.

Cuscuta extract preparation and purification

Cuscuta ssp. shoots were harvested, frozen in liquid nitrogen and freeze-dried for storage. Extraction was performed with 12 mM HCl at 60 °C for 16 h. Extract was filtered (0.22 µm MCEM Filters, Merck Millipore), loaded on a cation-exchange column (SP Trisacryl® M, Sigma; 25 mM MES pH 5.5) and eluted with 600 mM KCl. This fraction was desalted and further pre-purified by loading on a C18 reversed-phase column (Chromabond, bench-top, 20 mM ammoniumacetate/acetic acid, pH 4.5) and elution with 40% acetonitrile. This preparation, termed pre-purified Cuscuta extract, was sequentially (Fig. S3) separated on a SCX cation exchange column (GE healthcare) equilibrated with 25 mM Mes-KOH (pH 5.5), a first run on a C18 reversed phase column (ZORBAX Rx-C18, Agilent) with 20 mM ammoniumacetate/acetic acid (pH 4.5) and elution with a gradient of acetonitrile (0-20%) and a second run on the same column with 0.1 % acetic acid and elution with a gradient of acetonitrile (0-20%). Fractions with highest activity

from this second run were further separated on a reversed phase column (Waters, AQUITY C18 HSST3) equilibrated with 0.1% formic acid and eluted with a gradient of methanol (0-30%, 60 min). Eluate of this final step was analyzed by MS/MS (Waters Acquitiy UPLC - Synapt G2 LC/MS system with electrospray ionization; gradient) and for activity in the ethylene induction assay.

Fig. S1

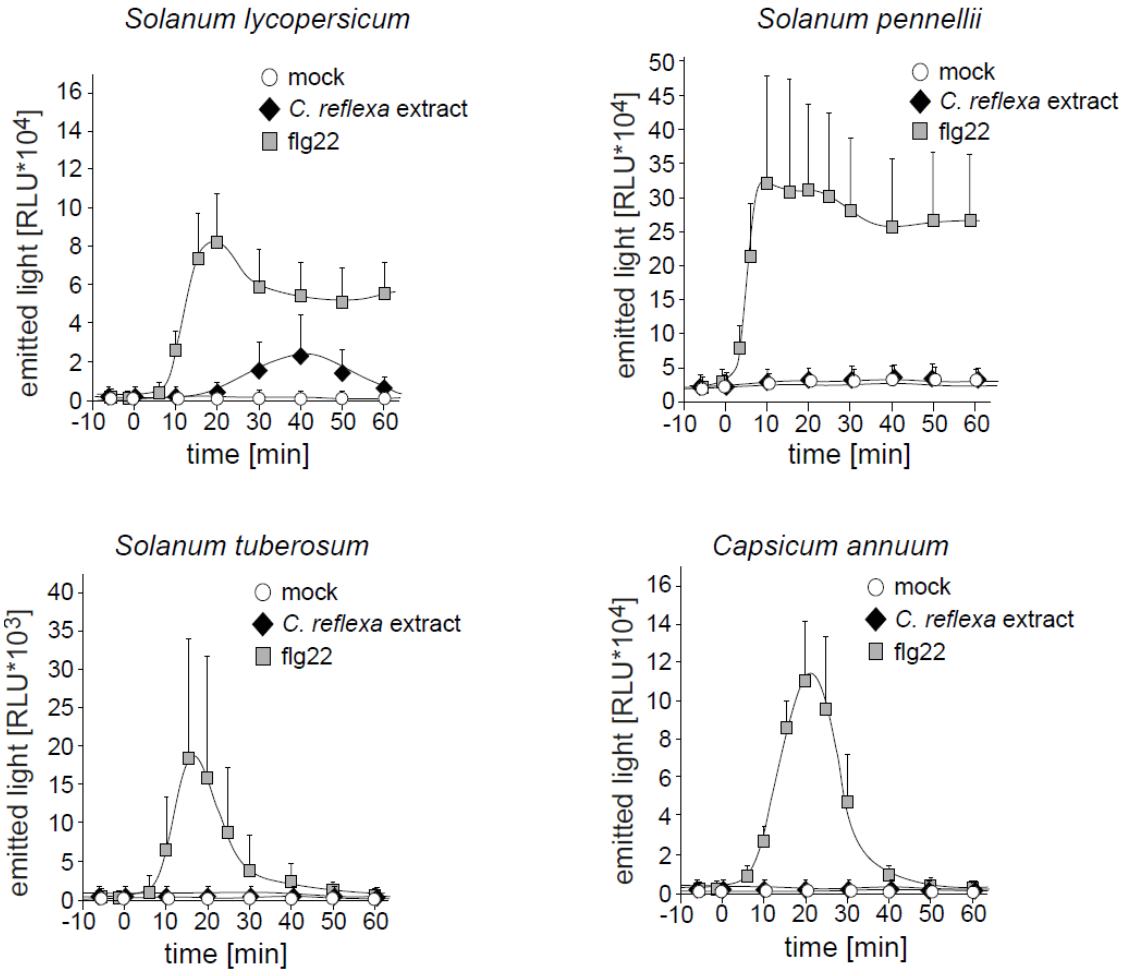

Fig. S1. ROS production in leaves of different plant species after treatment with *C. reflexa* extract, 100 nM flg22 as positive control or 0.01 mg/ml BSA as mock control. ROS production was monitored using a luminol-based assay, and emitted light was detected as relative light units (RLU) by a luminometer. Values represent means of 4 technical replicates; bars indicate standard deviations.

Fig. S2

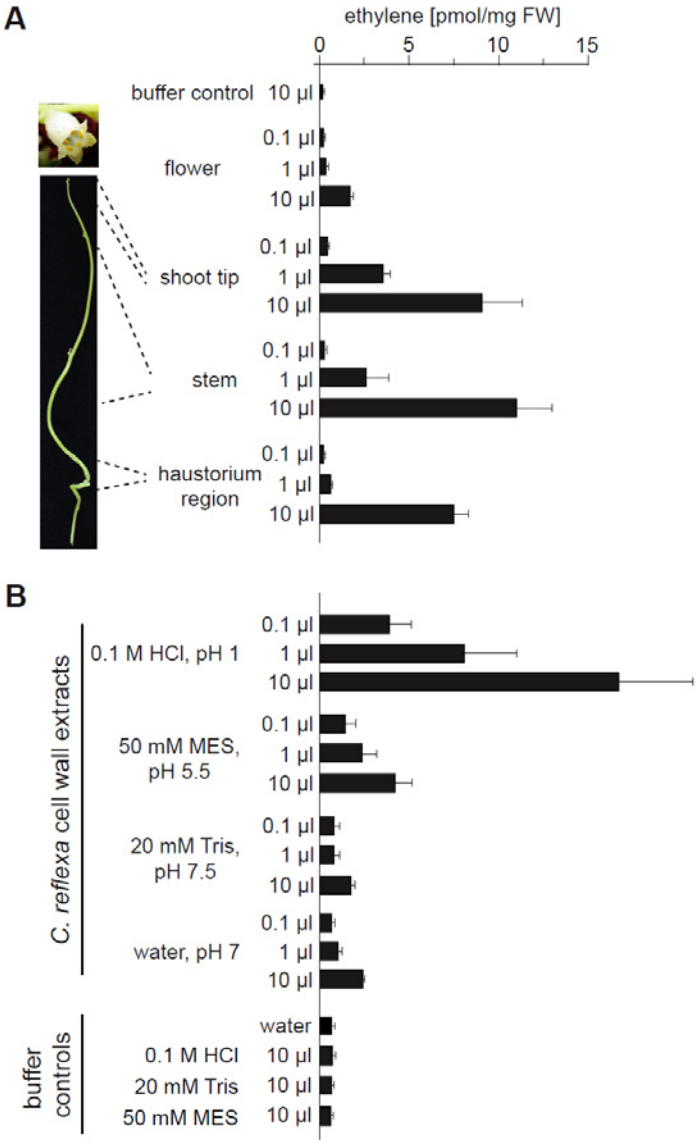

Fig. S2. (A) Cuscuta factor occurs in different parts of *C. reflexa* shoots. *C. reflexa* parts indicated were extracted (10 mM HCl, overnight at 50 °C) and assayed at doses of 0.1 µl, 1 µl or 10 µl per 500 µl sample volume for induction of ethylene response in tomato leaves. (B) Cuscuta factor appears associated with the cell walls of *C. reflexa* plants. For preparing cell walls of *C. reflexa*, freeze-dried Cuscuta shoots were ground to a fine powder that was sequentially washed with 70 % ethanol, chloroform/methanol (1:1 v/v) and acetone. After drying under vacuum, aliquots (70 mg) of this wall preparation were incubated for 4 h at r.t. in 1 ml of 0.1 M HCl (pH 1), 50 mM MES pH 5.5, 20 mM Tris/HCl (pH 8.0) or water (pH 7), respectively. Supernatants were adjusted to a pH of 5.5 and assayed at doses of 0.1 µl, 1 µl or 10 µl for induction of ethylene response in tomato leaves. Results indicate that Cuscuta factor seems associated with the cell walls of the Cuscuta plants. The activity is released under physiological conditions, pH 5.5 at r.t., albeit significantly more activity can be found after extraction under acidic conditions.

Fig. S3

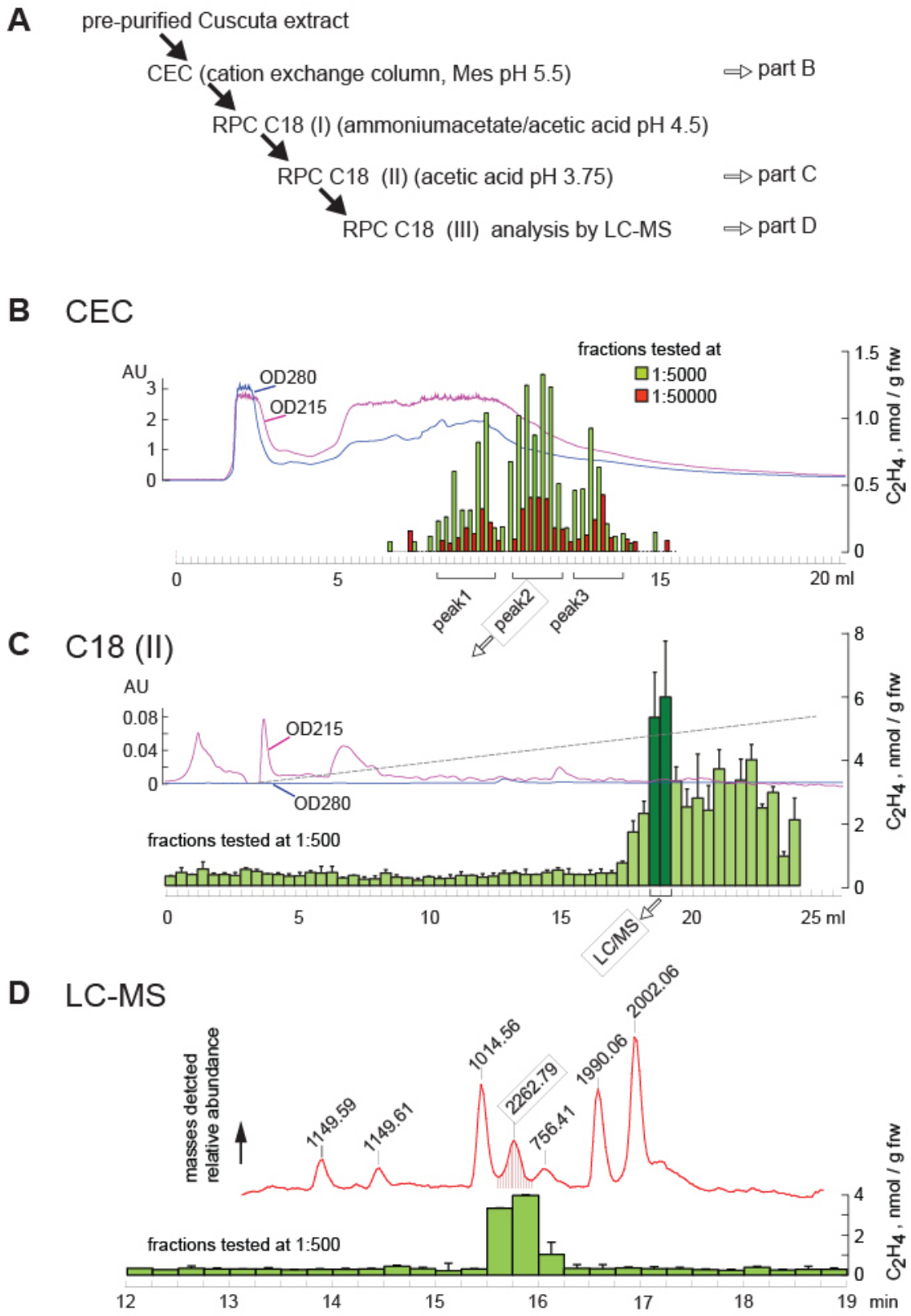
A
pre-purified Cuscuta extract
CEC (cation exchange column, Mes pH 5.5)
part B
RPC C18 (I) (ammoniumacetate/acetic acid pH 4.5)
RPC C18 (II) (acetic acid pH 3.75)
part C
RPC C18 (III) analysis by LC-MS
part D
B CEC
fractions tested at
1:5000
1:50000
AU
OD280
OD215
C2H4, nmol / g fw
20 ml
peak1
peak2
peak3
C C18 (II)
AU
OD215
OD280
fractions tested at 1:500
25 ml
LC/MS
D LC-MS
masses detcted
relative abundance
1149.59
1149.61
1014.56
2262.79
756.41
1990.06
2002.06
fractions tested at 1:500
min

Fig. S3. Purification of *C. reflexa* extract. (**A**) Purification scheme overview. (**B**) Pre-purified, *C. reflexa* extract was separated by cation-exchange chromatography (CEC) using a salt gradient (0–700 mM KCl) for elution. Fractions were tested in the ethylene bioassay with tomato leaf pieces at the dilutions indicated (bar diagram). Activity eluted as three broad peaks and pooled fractions of peak 2 were further purified by reversed-phase chromatography (RPC on a C18 column). (**C**) Second round of purification by RPC using acidic conditions (0.1% acetic acid) and elution with an acetonitrile gradient of 0–25% demonstrated further separation of activity into fractions with clear activity but low absorbance at OD_{280} and OD_{215}. Fractions (250 µl) were tested for induction of ethylene production (1 µl per 500 µl sample). Values and error bars show means and standard deviations of three replicates. (**D**) Total ion chromatogram (TIC); fractions with highest activity in (**C**) were analyzed by LC-MS using RPC on a C18 column with formic acid (0.1 %) and a methanol gradient for elution. Activity eluted from this column in two fractions containing a compound with a molecular weight of 2262.79 Da.

Fig. S4

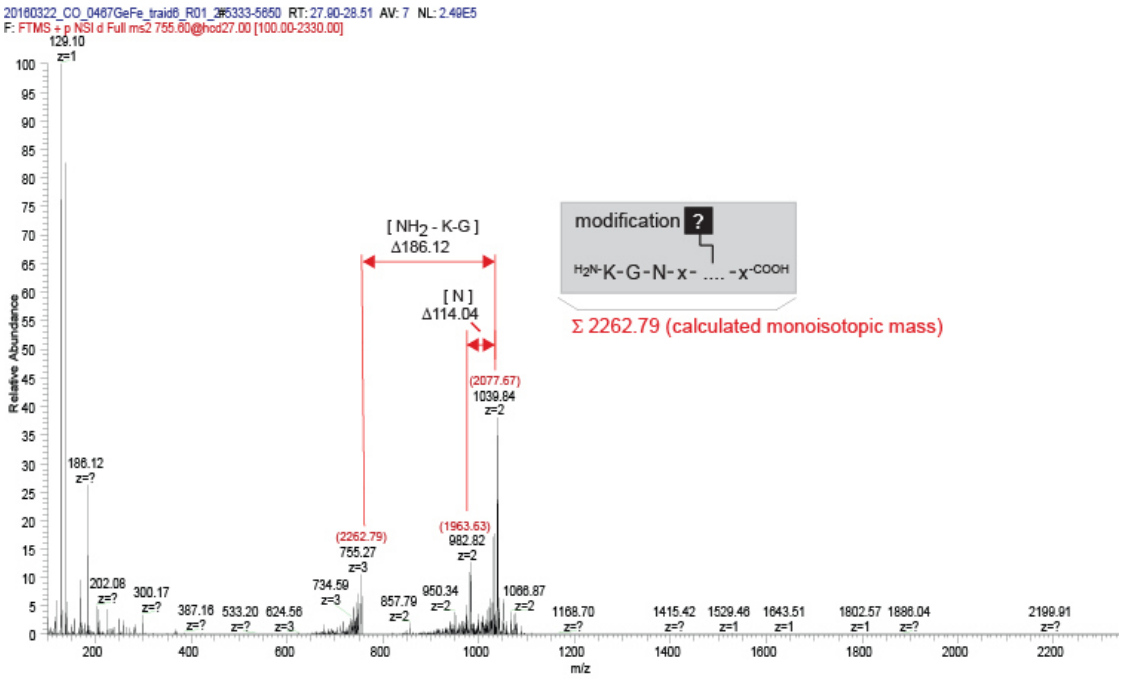

Fig. S4. Fragment spectrum of the Cuscuta factor candidate mass 2262.79.
Spectrum shows incomplete fragmentation of the Cuscuta factor. The inconclusive fragments did only allow for a limited sequence prediction of the N-terminus of the peptide (red arrows and lines). The peptide backbone in minimum consists of five amino acids and the molecular weight of 2262.79 indicates an upper size limit of ~22 amino acids if there are no sugar modifications or some lesser number of amino acids once sugar modifications are accounted for within the estimated molecular weight.
Grey box: Model of the Cuscuta factor 2262.79 Da, N-terminally starting with the amino acids K-G-N, comprising an as yet unknown modification (small black box).

Fig. S5

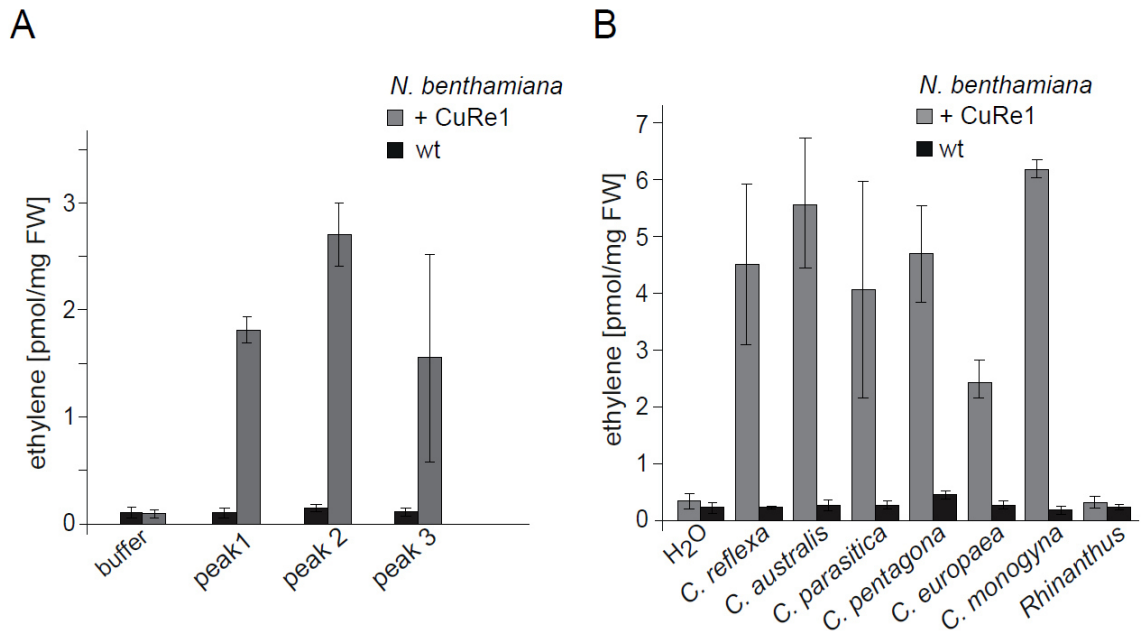

Fig. S5. CuRe1 expressed in *N. benthamiana* leaves is sufficient to confer responsiveness to the forms of Cuscuta factor present in eluate of CEC and in the extracts of different Cuscuta species. Ethylene response in non-transformed and in CuRe1-expressing *N. benthamiana* leaves to (**A**) treatment with Cuscuta factor in peak1, peak2 or peak3 eluting from the CEC column shown in Fig. S 3B (tested at a dilution of 1:5000) and (**B**) treatment with extracts from different *Cuscuta* species and *Rhinanthus alectorolophus*. Bars and error bars represent means of three measurements and standard deviations. Experiments were independently repeated.

Fig. S6

```
SP      MGNIKFLLLVFFLIVVVVNG
LRR-NT  CWEEERNALLELQTNIMSSNGELLV
        DWAGYNAAHFVDCCFWDRVKCSLETG

1       RVIKLDLEADFGTGDGWLFNASLFLPFK
2       SLQVLLLSSQNIIGWTKNEGFS
3       KLRQLPNLKEVDLQYNPIDPKVLLSSLC
4       WISSLEVLKLGVDVDTSFSIPMTYNT
5       NMMSKKCGGLSNLRELWFEGYEINDINILSALG
6       ELRNLEKLILDDNNFNSTIFSSLK
7       IFPSLKHLNLAANEINGNVEMNDII
8       DLSNLEYLDLSDNNIHSFATTKGNK
9       KMTSLRSLLLGSSYSNSSRVIR
10      SLKSFSSLKSLSYKNSNLTSPSIIYALR
11      NLSTVEYLYFKGSSLNDNFLPNIG
12      QMTSLKVLNMPSGGNNGTLPNQGWC
13      ELKYIEELDFLNNNFVGTLPLCLG
14      NLTSLRWLSLAGNNLHGNIASHSIWR
15      RLTSLEYLDIADNQFDVPLSFSQFSDHK
16      KLIYLNVGYNTIITDTEYQNWIPNF
17      QLEFFAIQRC   IALQKLPSFLH
18      YQYDLRILAIEGNQLQGKFPTWLLE
19      NNTRLAAIYGRDNAFSGPLKLPSS
20      VHLHLEAVDVSNNKLNGHIPQNMSL
21      AFPKLLSLNMSHNHLEGPIPSKI
22      SGIYLTILDLSVNFLSGEVPGDLAVV
23      DSPQLFYLRLSNNKLKGKIFSEEF
24      RPHVLSFLYLNDNNFEGALPSNV
25      FLSSLITLDASRNNFSGEIPGCTR
26      DNRRLLQLDLSKNHLQGLIPVEIC
27      NLKIINVLAISENKISGSIPSCVS
28      SLP LKHIHLQKNQLGGELGHVIF
29      NFSSLITLDLRYNNFAGNIPYTIG
30      SLSNLNYLLLSNNKLEGDIPTQIC
31      MLNNLSIVDLSFNKLYGPLPPCLG
ILD     YLTQTKKDAEISWTYFAENYRGSWLNFVIWMRSKRHYHDSHGLLSDLFLMDVETQVQFSTKKNSYTYKGN
32      ILKYMSGIDLSSNRLTGEIPVELG
33      NMSNIHALNLSHNHLNGRIPNTFS
34      NLQEIESLDLSCNRLNGSIPVGLL
35      ELNSLAVFSVAYNNLSGAVPDFKA
        QFGTFNKSSYEGNPFLCGYPLDNKCGMSPKLSNTSNINGDEESSELEDI

TM      QCFYIGFVVSFGAILLGLAAALCLN

CTD     RHWRRAWFRMIEALMFYCYYFVLDNIVTPIKSRWYKNVG*
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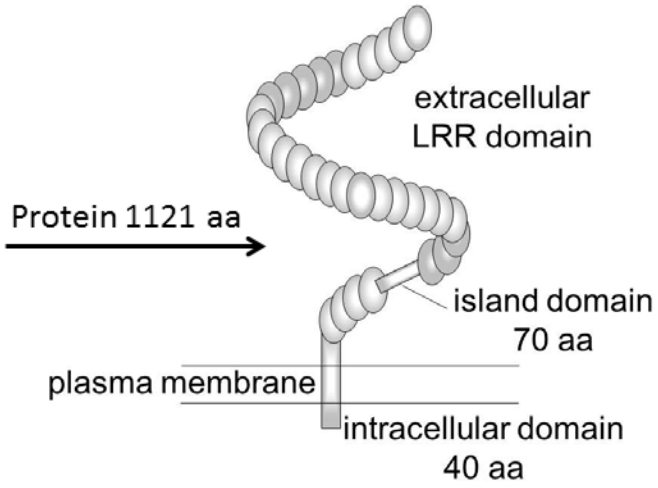

Fig. S6. Left: Amino acid sequence of the CuRe1 protein comprising a signal peptide (SP), a N-terminal domain (LRR-NT), tandemly arrayed LRRs (leucine rich repeats, numbered, partially irregular LRRs #1,2 and 5) interrupted by an island domain (ILD), a single pass transmembrane helix (TM) and a C-terminal cytosolic domain (CTD). Residues characteristic for the plant LRR consensus are highlighted in grey; potential N-glycosylation sites (NxT/S) are highlighted in green and residues characteristic for the N- and C-terminal parts of plant LRR-domains in yellow. Right: Model of the CuRe1 protein with LRRs indicated as ovals.

Fig. S7

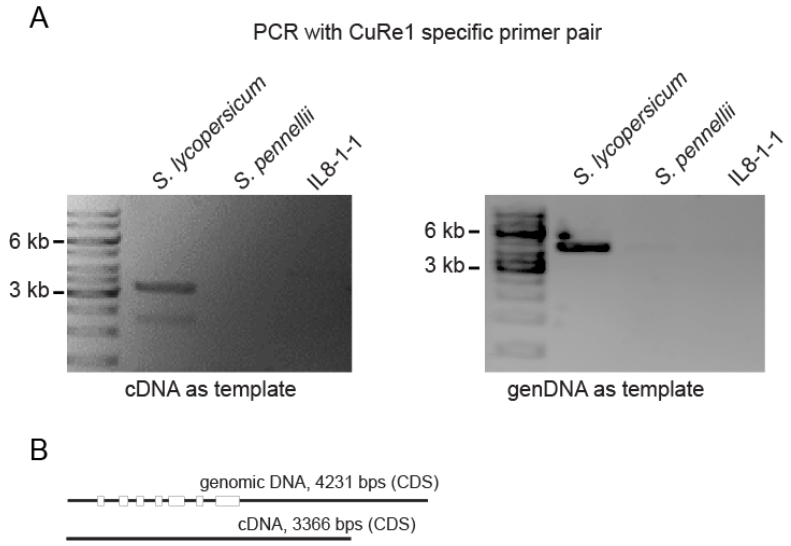

Fig. S7. (**A**) Presence of CuRe1 transcript (left) and gene (right) in *S. lycopersicum, S. pennellii* and IL8-1-1. PCR test with CuRe1 specific primers; separated by Agarose gel electrophoresis (1%). (**B**) Gene model including 7 introns (white boxes) and predicted coding sequence after splicing.

Fig. S8

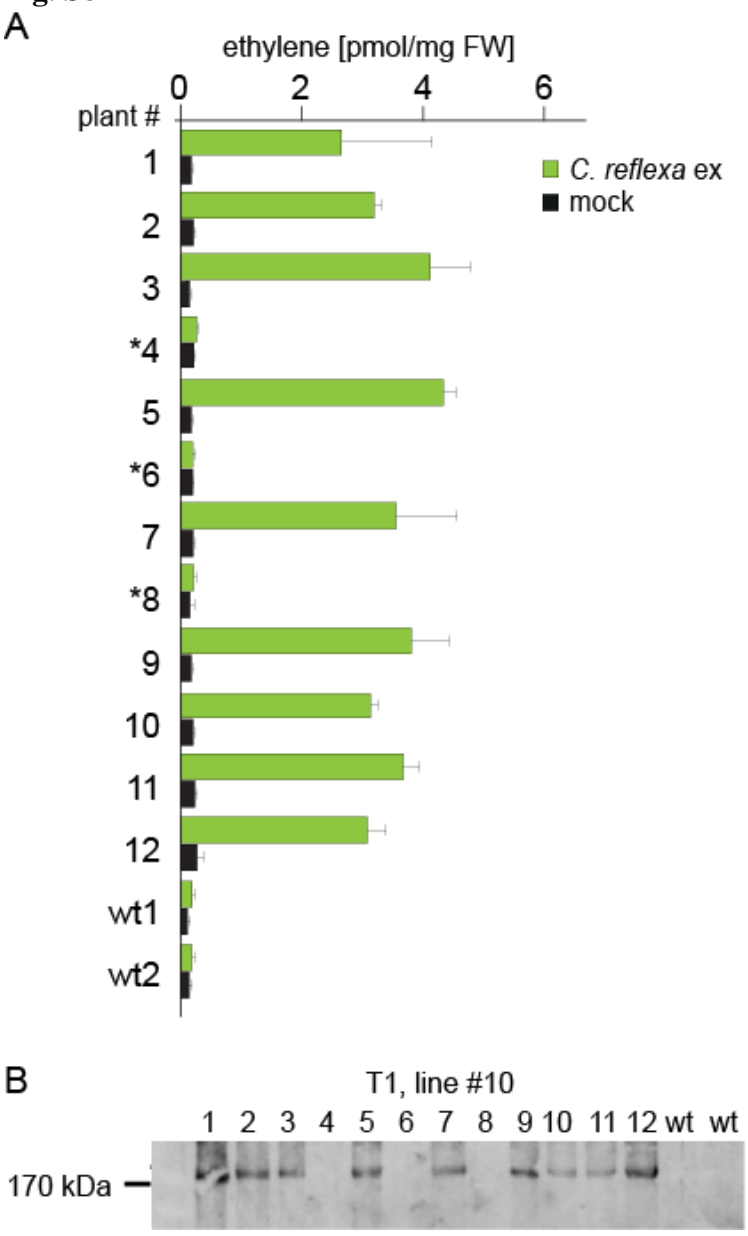

Fig. S8. Stable transformed *S. pennellii* expressing CuRe1 and used for the *C. reflexa* growth assay described in Fig. 3E; segregating T1-line. (**A**) Ethylene response of *S. pennellii* plants. Samples were treated with 10 µl of *C. reflexa* extract; bars represent means of 3 replicates, error bars indicate standard deviations; individuals labeled with * had no CuRe1 protein and were not used for evaluations of the *C. reflexa* growth assay described in Fig. 3E. (**B**) Corresponding western blots were probed with specific antibodies against the GFP-tag present at the CuRe1 C-terminus; Immunoprecipitation against GFP using GFP-trap™ (Chromotek).

Fig. S9

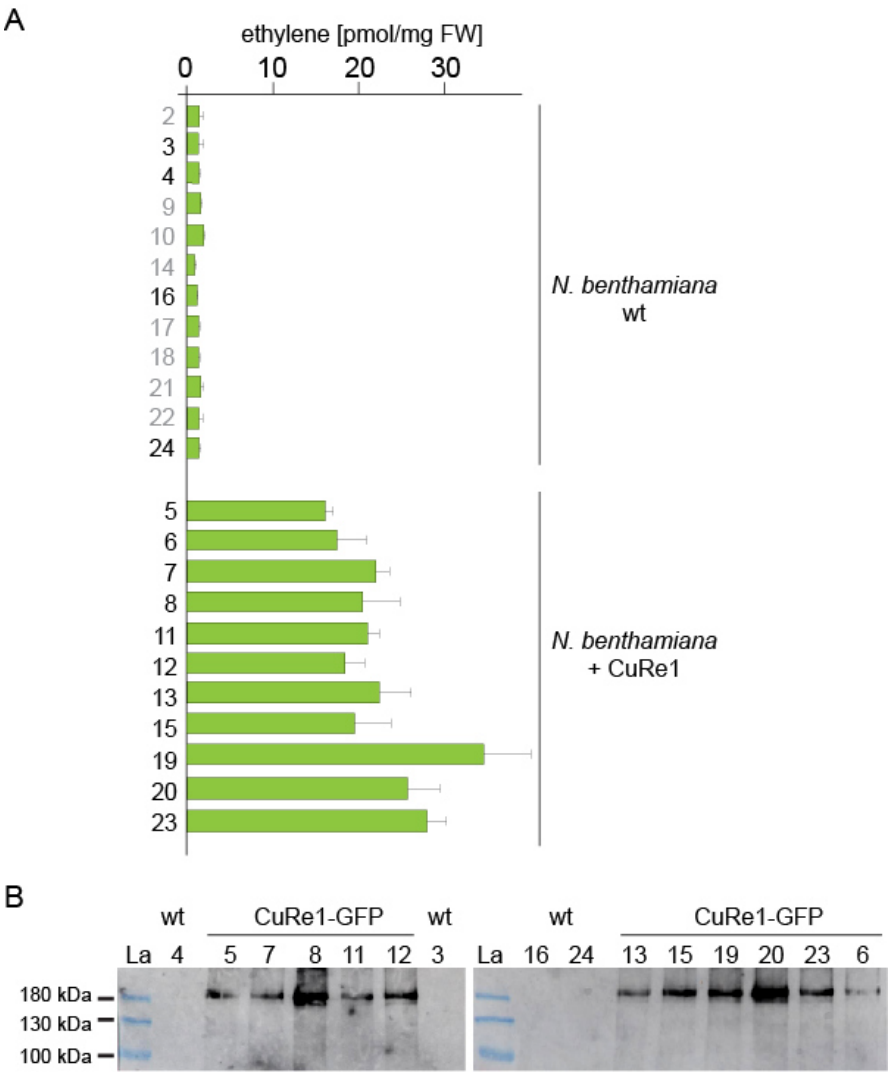

Fig. S9. Data from stable transformed *N. benthamiana* (homozygous T2 line), used for Cuscuta growth assays presented in Fig. 3F. (**A**) Ethylene response of *N. benthamiana* wild type (wt) control plants and plants with CuRe1. Samples were treated with 10 µl of *C. reflexa* extract; bars represent means of 3 replicates; error bars indicate standard deviations. (**B**) Corresponding western blots were probed with specific antibodies against the GFP-tag present at the CuRe1 C-terminus; numbers indicate individual plants. Immunoprecipitation against GFP using GFP-trap™ (Chromotek); La = Ladder (PAGEruler™, prestained, Thermo Fisher).

Fig. S10

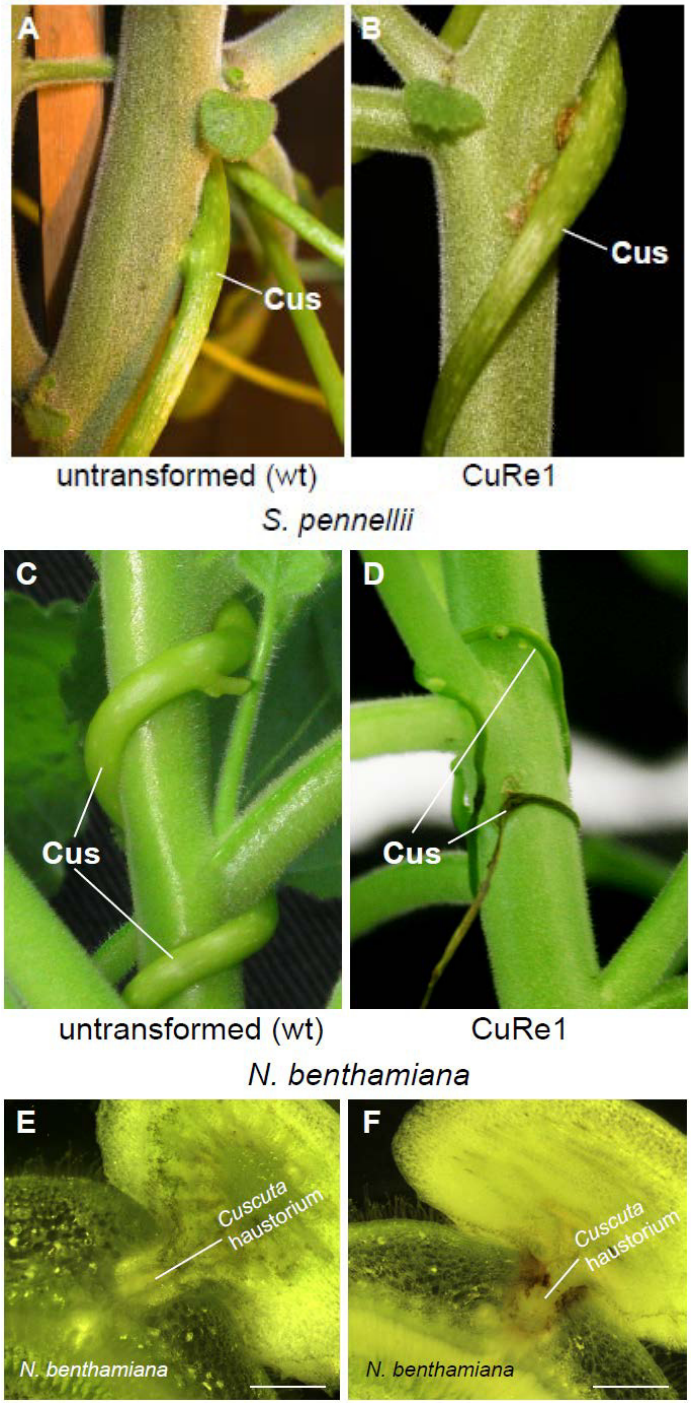

Fig. S10. Photographs of *C. reflexa* (Cus) grown for 14 days on (**A**) untransformed *S. pennellii*, (**B**) transgenic *S. pennellii* expressing CuRe1, (**C**) untransformed *N. benthamiana* or (**D**) transgenic *N. benthamiana* expressing CuRe1. In (**B**) and (**D**), slight HR-symptoms are visible on the host stem at haustoria contact sites and in (**D**) the *C. reflexa* (partially) died off. (**E**) and (**F**) cross sections of penetrating haustoria on (**E**) wt *N. benthamiana* and (**F**) on *N. benthamiana* expressing CuRe1. White bar = 1 mm.

Fig. S11

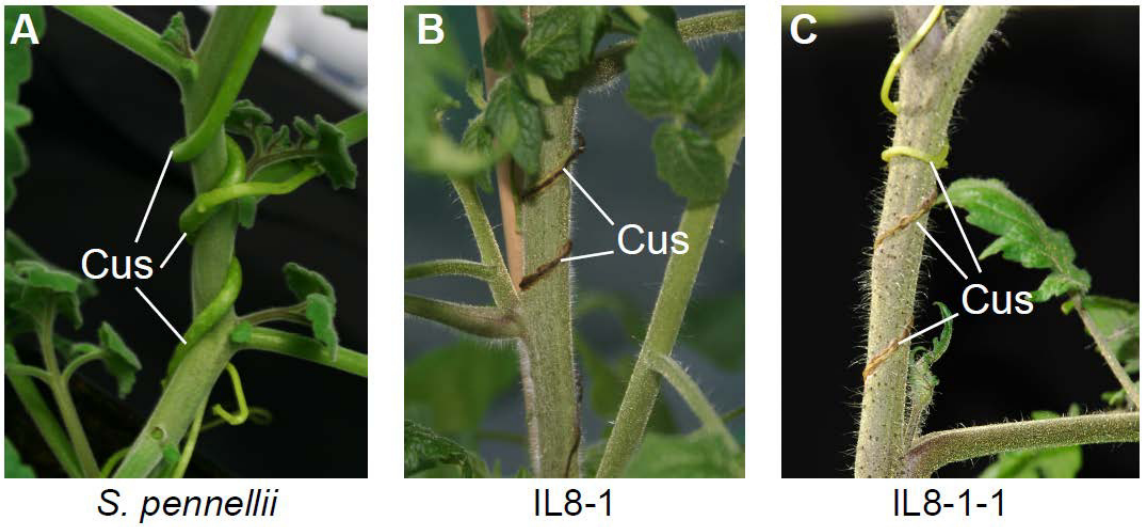

Fig. S11. *Cuscuta reflexa* (Cus) on the susceptible host plant *Solanum pennellii* (**A**) and on the tomato introgression lines IL8-1 and IL8-1-1 (**B** and **C**). IL8-1 and IL8-1-1 are both resistant to *C. reflexa* (Cus) but lack CuRe1 and are insensitive to the Cuscuta factor in the ethylene bioassay (see Fig. 2). Photographs were taken ~14 days after parasite onset.

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Growth Assay for the Stem Parasitic Plants of the Genus *Cuscuta*

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[Abstract] *Cuscuta* spp. are widespread obligate holoparasitic plants with a broad host spectrum. Rootless *Cuscuta* penetrates host stems with so called haustoria to form a direct connection to the host vascular tissue (Dawson *et al*., 1994; Lanini and Kogan, 2005; Kaiser *et al*., 2015). This connection allows a steady uptake of water, assimilates and essential nutrients from the host plant and therefore enables *Cuscuta* growth and proliferation. To quantify the parasites' ability to grow on potential host plants one can use the quantitative growth assay (Hegenauer *et al*., 2016) described herein, which exclusively utilizes fresh weight measurement as readout.
Keywords: *Cuscuta reflexa*, Dodder, Growth assay, Haustoria, Holoparasitic plant

[Background] In research fields of plant-pathogen resistance, either in basic research or in economic plant breeding, it is unavoidable to have an assay to quantify resistance against pathogen infection. To quantify the resistance/susceptibility of different plants against *Cuscuta* infections the simplest way is to measure the gain of biomass of *Cuscuta* growing on a plant of interest. This is a reliable method since *Cuscuta* is a holoparasite and its gain of biomass is completely depending on its ability to successfully infect another plant. Thus, unsuccessful infection of a plant leads to a decrease in biomass and subsequently the death of the parasite *Cuscuta*.

Materials and Reagents

1. Gloves and lab suit (*Cuscuta* sap causes stains on skin and clothes)
2. Mature *Cuscuta* (*e.g.*, *Cuscuta reflexa*; see Note 1 for cultivation)
3. Putative host plants

Equipment

1. Weighing machine/balance (mass range between 0.01-100 g)
2. Wooden planting rods (bamboo; diameter appropriate to the particular host plants stem diameter), available in gardening shops
3. Scissor to cut *Cuscuta* shoots

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Procedure

The whole experiment is performed under optimal conditions for the host plant. The optimal host plant conditions are provided by the seed supplier and can't be generalized.

1. Cut *Cuscuta* shoots (length ~15 cm) including the shoot tip from a mature plant and wind it around upright wooden sticks; be aware of winding direction (counter clockwise from bottom to top; Figure 1A) and integrity of the tip (see also Note 2). Keep the stick with the *Cuscuta* shoot in a vertical position for one day (*e.g.*, by sticking it into Styrofoam, soil or sand). The *Cuscuta* shoot should hold by itself on the planting rod without contact to anything else (Figure 1A).

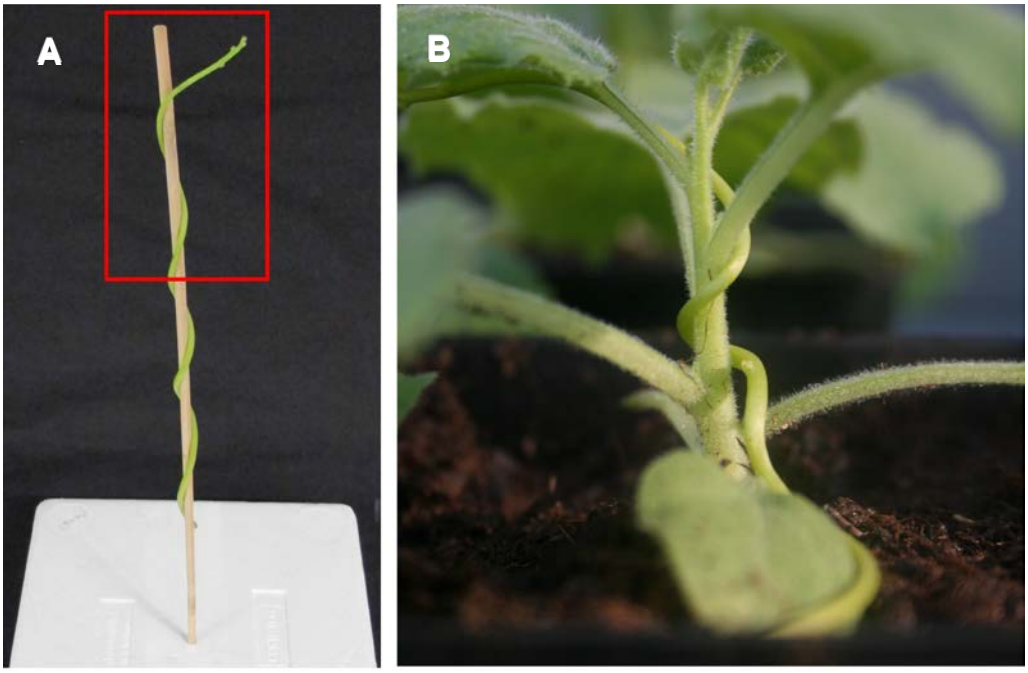

Figure 1. Starting the *Cuscuta* infection. A. *Cuscuta reflexa* shoot enwinding a wooden stick. Winding direction is counter clockwise from bottom to top. Red frame indicates the area where haustoria will most likely form. B. *Cuscuta reflexa* shoot wound around *N. benthamiana* plant after preconditioning around the wooden stick (A).

2. After one day (at the beginning of prehaustoria formation), when the shoot has been carefully uncoiled from the stick, weigh the shoots and transfer them to their host plants considering the winding direction (see also Note 3). The haustoria will form close to the shoot tip (Figure 1, red box) so make sure to wind this part around the host plant stems thoroughly. Remaining of the *Cuscuta* shoot will provide a source of nutrients and water until the haustoria connect with the host's vascular system. Therefore, an equal length (*e.g.*, 15 ± 1 cm) as well as an approximately same weight (*e.g.*, 0.5-0.7 g) of the parasite shoots is relevant for reproducible results.

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3. After transfer (Figure 1B, see also Note 4), let the *Cuscuta* spp. shoots grow for the same time period (14-21 days). After that time of growth, remove individual shoots from the host plant and measure the fresh weight immediately for each individual.

Data analysis

Cuscuta's ability and speed to accumulate biomass depends on the number of haustoria to acquire nutrients, and thus, on the time frame a single shoot needs to establish a successful haustorial connection to the host's vascular tissue. For this reason, the variance of the *Cuscuta* growth can be occasionally high. A big number (> 10) of replicates and multiple repeats are recommended. The outcome of this experiment is the gain of biomass (in g) for every single *Cuscuta* shoot for the distinct timeframe. So resistance against *Cuscuta* (or susceptibility for the infection) can be quantified and compared by its ability to gain biomass on two distinct sets of host plants. A correction factor is not necessary if there is no great variance in the initial weight, never the less if this is the case it could be helpful to show the result as mass change (ΔFw = final shoot weight - initial shoot weight) during the time frame. This depends on the particular experiments experimenters will perform to answer their research questions.
The results can be presented as the mean of the *Cuscuta* shoot biomass 21 dpi (days post infection) of n replicates with standard deviation comparing two types of hostplants.
For the reduction of outlier effects, ranked data analysis and nonparametric tests like Mann-Whitney *U*-test can be used.
See also Note 5.

Notes

1. Cultivation of *Cuscuta*: *Cuscuta* can be cultivated on many plants. In our Lab we use Coleus (*Solenostemon scutellarioides*) because it's a robust plant and its red color gives a good contrast to spot *Cuscuta* shoots. You can easily cultivate *Cuscuta* when you place 6 to 10 host plants in close proximity so that *Cuscuta* can overgrow all of them. It's important to concern that after *Cuscuta* overgrows the host plant it cannot be potted anymore, so make sure to provide enough nutrients for the host plant. *Cuscuta* should be cultivated under long day conditions with 17 h daylight. *Cuscuta* seeds and instructions how to start the culture can be requested in University botanical gardens or other research groups.
2. The pre winding of *Cuscuta* around a wooden planting rod facilitates the later infection of the host plant due to an efficient prehaustoria formation, a clearly visible swelling of the shoot at the shoot-stick contact sites.
3. It is important not to harm the *Cuscuta* shoot during the initial weight measurement and transfer to the host plant.

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4. In the first days of infection the *Cuscuta* shoot may search in the surrounding area of the host plant for other hosts despite infecting the plant it is sitting on. Therefore, the experimenter has to softly redirect the shoot back to its host.
5. Blinded set up of the experiment: Comparison of *e.g.*, *N. benthamiana* wild type plants with resistant transgenic CuRe1-expressing plants was performed as blind experiment in Hegenauer *et al.* (2016). One person randomly assigned numbers to each tested plant and a second person performed the infection with *Cuscuta reflexa* shoots.
6. A successful infection of Coleus by *Cuscuta reflexa* is shown in Figure 2. At the side of haustorial infection *Cuscuta* is thickened (red frame). After successful infection *Cuscuta* starts growing and branching (blue arrow).

Figure 2. Coleus successfully infected by *Cuscuta reflexa*. Red frame: sides of haustorial development. Blue arrow: branching of *Cuscuta.*

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ARTICLE ADDENDUM

OPEN ACCESS

Parasitic *Cuscuta* factor(s) and the detection by tomato initiates plant defense

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ABSTRACT

Dodders (*Cuscuta* spp.) are holoparasitic plants that enwind stems of host plants and penetrate those by haustoria to connect to the vascular bundles. Having a broad host plant spectrum, *Cuscuta* spp infect nearly all dicot plants – only cultivated tomato as one exception is mounting an active defense specifically against *C. reflexa*. In a recent work we identified a pattern recognition receptor of tomato, "Cuscuta Receptor 1" (CuRe1), which is critical to detect a "Cuscuta factor" (CuF) and initiate defense responses such as the production of ethylene or the generation of reactive oxygen species. CuRe1 also contributes to the tomato resistance against *C. reflexa*. Here we point to the fact that CuRe1 is not the only relevant component for full tomato resistance but it requires additional defense mechanisms, or receptors, respectively, to totally fend off the parasite.

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KEYWORDS
Cuscuta; Cuscuta factor; parasitic plant; pattern recognition receptor; plant immunity; plant-plant interaction

Metazoans and plants possess an innate immune system to mount active defense against pathogen attacks. Most plant pathogens are microbes or herbivorous arthropods that the immune systems of plants are able to detect by sensing microbe- or herbivore-associated molecular patterns (MAMPs/HAMPs).[1,2] These molecular patterns, indicative for "non-self," serve as molecular signals that trigger specific plant pattern recognition receptors (PRRs) and initiate plant defense signaling to fend off the pathogen.[3,4] Besides the pathogens mentioned, there exist ~4,500 plant species that live parasitic on other plants and genera such as *Striga, Orobanche* or *Cuscuta* are known to cause tremendous crop loss.

The plant genus *Cuscuta* (dodder) comprises about 200 species distributed in all moderate climate zones. All *Cuscuta* species live as stem holoparasites with a broad host spectrum, preferentially for dicotyledonous plants. The different *Cuscuta* species grow as yellowish, orange or slightly greenish vines that wind around the stems of their host plants.[5] Most dodder species have no or only marginal amounts of chlorophyll and their photosynthesis is insufficient for surviving.[6-9] All *Cuscuta* species possess neither roots nor expanded leaves and penetrate host plants with haustoria that directly connect to the vascular bundles. Right after germination, *Cuscuta* seedlings sense host plant volatiles which support the finding of an appropriate host.[10] In the parasite, initial physical contact induces the formation of haustoria,[11] specific organs which are generally important for parasitic plants to penetrate the host tissue.[12] The penetration phase is accompanied by the expression of cell-wall modifying enzymes leading to structural rearrangements within the cell-walls of the parasite[13] and the loosening of the host tissues.[14,15] After reaching the vascular bundles, the parasitic haustorium connects to the host xylem and phloem. This allows the parasite to withdraw water, nutrients, and carbohydrates to grow and complete its lifecycle.[8,16,17] *Cuscuta* parasites also take up macromolecules such as proteins, viruses or RNAs.[18-22] Recently, RNAs were shown to move between host plant and parasite in a bidirectional manner and to a much higher extent than previously expected.[23]

Not much is known about how host plants can sense parasitic *Cuscuta* spp. and how they initiate cellular programs to fend off plant parasites. In our recent study,[24] we made use of the special case *Cuscuta reflexa* and its resistant host plant *Solanum lycopersicum* (cultivated tomato) to get insights in the early steps occurring in the plant-plant dialog. Tomato displays an active and clearly visible resistance reaction directly at the penetration sites of the parasitic haustoria a few days after the initial contact with the parasite and successfully fends off *C. reflexa*.[25-27]

In this study we show that extracts of *C. reflexa* induce the production of reactive oxygen species (ROS) and the

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biosynthesis of the stress related phytohormone ethylene, plant defense responses usually known to occur during plant–microbe interaction and typically induced by pathogen-associated molecular patterns (PAMPs).[2,28] We could isolate and characterize the trigger of these responses from *C. reflexa*, a 2 kDa peptide with an o-esterified modification, and we further screened an introgression library of *S. lycopersicum x S. pennellii*[29] to map responsiveness to this parasitic factor, since *S. pennellii* is insensitive to parasitic extracts and susceptible for a *C. reflexa* infestation.[13] We identified a gene encoding a plasma membrane-bound receptor, the Leucine-rich repeat receptor like protein (LRR-RLP) "Cuscuta receptor 1′ (CuRe1) which senses the parasitic "Cuscuta factor" (CuF). CuF initiates defense responses in the formerly insensitive host plant *Nicotiana benthamiana* after transient expression of *CuRe1*. Stable transformation of a *CuRe1* construct into *N. benthamiana* lead to a drastically reduced *C. reflexa* growth and to an increased resistance.

Besides CuRe1, there are 3 genes encoding for CuRe1 homologs (CuRe1-likes; Solyc04g0014400; Solyc08g016210; Solyc08g016310) within the tomato genome, sharing 64 – 81 % amino acid sequence identity (Fig. 1A). Receptors with up to 80 % aa-sequence identity to CuRe1 seem exclusively present in Solanaceaus plants. Only receptors with less than 45 % aa-sequence can be found outside the Solanaceae. We cloned all CuRe1-like genes from tomato[24] and expressed them heterologously in *N. benthamiana*. However, in contrast to CuRe1 none of these receptors was able to trigger defense-related responses like ethylene induction when treated with the CuF or crude *C. reflexa* extract (Fig. 1B).

The recognition of the parasitic cell wall associated CuF or related other Cuscuta factors by these receptors could be supposable. Nonetheless, the initiated cellular signaling program must be distinct from the defense related responses induced by CuRe1 as we could not measure the emission of ethylene (Fig. 1) after treatment with CuF.

During a susceptible interaction the parasite has to hook up the host plant's developmental processes to establish a connection to the vascular system. Therefore, the parasite has to (ab-)use existing host mechanisms including the signals and perception systems to succeed in infecting other plants. If the CuRe1-like receptors are critical to recognize and process any molecular cues of *Cuscuta* spp is possible but remains to be demonstrated. The roles of CuRe1-likes for the harbouring host plant e.g. as receptors for endogenous signals involved in developmental processes or as receptors to detect MAMPs is still unclear and up to date no function could be assigned to any receptor of this clade.

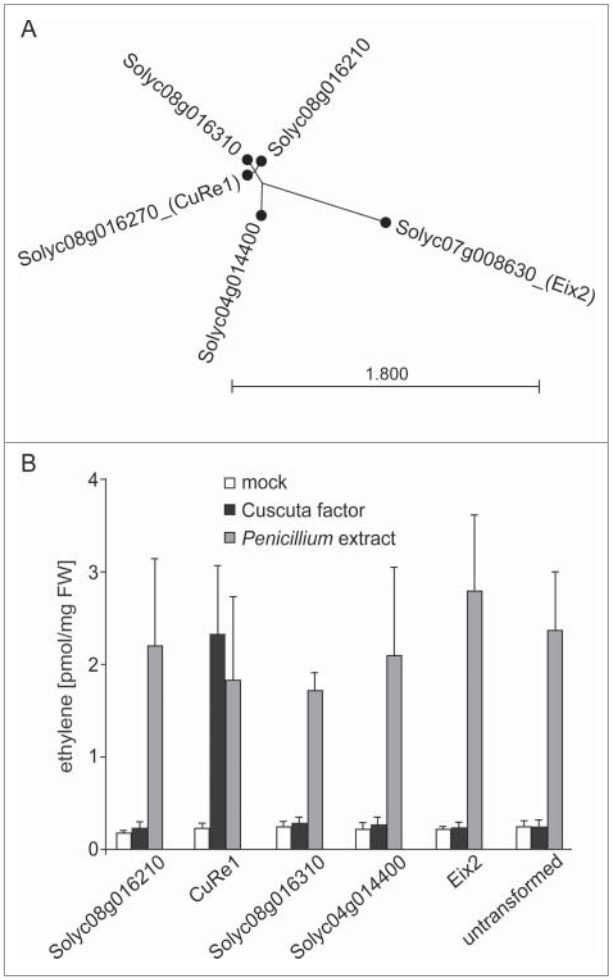

Figure 1. Functionality of CuRe1-like receptors. (A) Tree shows relationship of CuRe1 and CuRe1-like genes; Eix2: receptor for fungal Xylanase[33] served as reference. (B) Ethylene response of *N. benthamiana* leaves expressing receptor CuRe1-like constructs and treated with *C. reflexa* extract or controls (mock = 0.01 mg/ml BSA in water; *Penicillium* extract = positive control); values represent means of n = 3 replicates plus stdv.

In fact, the specific recognition of the Cuscuta factor by tomato CuRe1[24] and the induction of the plant defense system seems unique and has probably evolved by incident exclusively in tomato. As far as tested, the Cuscuta factor seems present in other *Cuscuta* species as well but seems absent from plant species outside this genus.[24] The full resistance toward parasitic *C. reflexa*, however, seems not to depend on CuRe1 alone but requires additional mechanisms maybe related to those known for Effector triggered immunity (ETI) occurring during plant–microbe interaction (overview in Fig. 2).[24,30,31] An nucleotide binding site leucine-rich repeat (NBS-LRR) protein, as part of a second layer of immunity and as a potential element of ETI, has been found to be relevant for resistance during the plant-plant interaction of cowpea against witch-weed (*Striga* spp.).[32] In case of the *C. reflexa* interaction with tomato

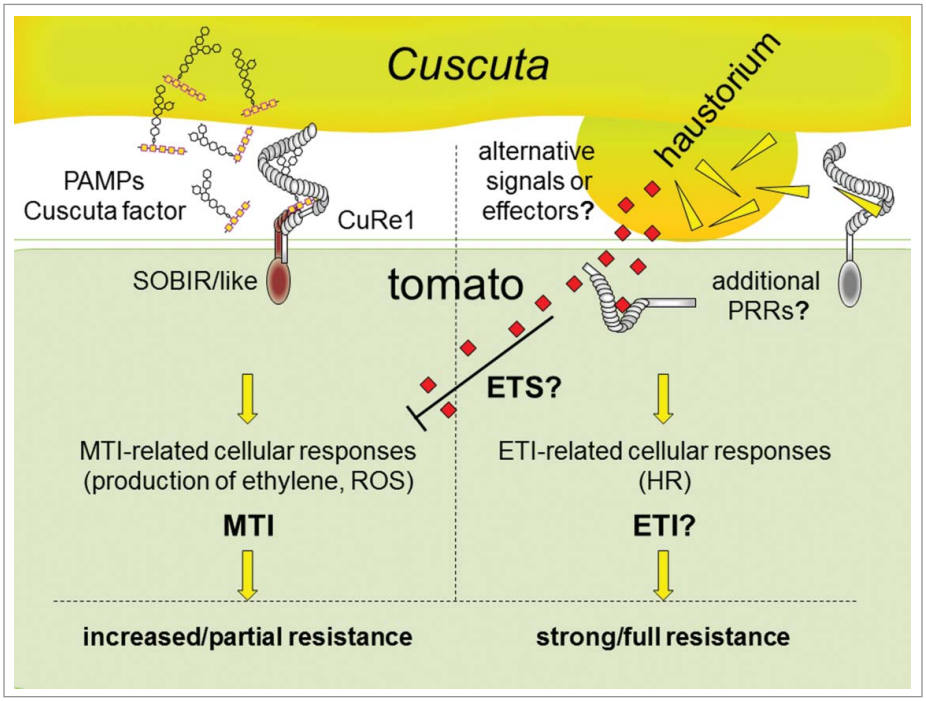

Figure 2. Model for defense and resistance of tomato to *Cuscuta* spp infestation. (Left): The Cuscuta factor is detected as a parasite-associated molecular pattern (PAMP) by the plasma membrane-bound PRR CuRe1 and initiates MTI-type responses in tomato, including the production of ethylene and ROS. MTI, apart from increasing resistance against various microbial pathogens, leads to increased resistance of tomato to *Cuscuta* attacks. (Right): Hypothesized ETS (effector triggered susceptibility), ETI (effector-triggered immunity) or alternative principles in tomato might, synergistically with or independently from MTI, confer full resistance of tomato to *Cuscuta* infestation.

additional components of resistance still have to be identified. If the CuRe1-like or other receptors are involved in such tomato-specific defense—maybe in a long term process—has to be further studied.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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The tomato receptor CuRe1 senses a cell wall protein to identify *Cuscuta* as a pathogen

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Parasitic plants of the genus *Cuscuta* penetrate shoots of host plants with haustoria and build a connection to the host vasculature to exhaust water, solutes and carbohydrates. Such infections usually stay unrecognized by the host and lead to harmful host plant damage. Here, we show a molecular mechanism of how plants can sense parasitic *Cuscuta*. We isolated an 11 kDa protein of the parasite cell wall and identified it as a glycine-rich protein (GRP). This GRP, as well as its minimal peptide epitope Crip21, serve as a pathogen-associated molecular pattern and specifically bind and activate a membrane-bound immune receptor of tomato, the Cuscuta Receptor 1 (CuRe1), leading to defense responses in resistant hosts. These findings provide the initial steps to understand the resistance mechanisms against parasitic plants and further offer great potential for protecting crops by engineering resistance against parasitic plants.

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Characteristic molecular patterns uncover pathogens as external invaders and are critical signatures that are detected by the innate immune system of both, animals and plants. This discrimination between self and non-self allows the host organisms to initiate defense reactions and resist pathogen attacks. As part of their innate immune system, plants evolved cell surface receptors to detect molecular patterns[1–3]. Due to the facts that most plant pathogens are microbes or arthropods and the wider evolutionary distance between plants and those attackers, the presence of "plant pattern recognition receptors" to detect molecular patterns seems a logical consequence of evolution. However, ~4500 higher plant species live as parasites and thus pose an additional threat to plants. Well-known parasitic plants with high agronomical relevance are *Striga* spp., *Orobanche* spp., and *Cuscuta* spp[4–6]. Most host plants are unable to detect an invasion by parasitic plants and the attackers stay unrecognized due to the limited innate immune system of host plants for detecting dangerous parasitic plants. Yet, a few host exceptions are described, which are able to fend off parasitic plants and stay incompatible[7–9]. However, the molecular mechanisms behind these are poorly understood, and molecular patterns of parasitic plants, which could mark a plant parasite as a devastating invader, have not yet been described.

Cuscuta spp. are holoparasitic plants which infect a broad spectrum of hosts by connecting to their vasculature via specific feeding structures, called haustoria (Fig. 1a, b)[10–12]. Cultivated tomato (*Solanum lycopersicum*) is one of few host exceptions that recognizes *Cuscuta reflexa* as an alien invader and actively initiates defense responses[4,13,14] measureable as the emission of the stress-phytohormone ethylene or reactive oxygen species (ROS) in tomato leaves[15] and visible as hypersensitive response (HR) at the infection sites (Fig. 1c)[13–15]. We reported the tomato cell surface receptor "Cuscuta receptor 1" (CuRe1) as a critical component for the detection of *C. reflexa* due to a hypothesized Cuscuta factor or pathogen-associated molecular pattern (PAMP) that can be extracted from the parasitic plant and triggers the defense response in a CuRe1-dependent manner. This Cuscuta factor is a heat stable protein and sensitive to treatments with bases (pH > 11), indicating potential secondary modifications present on the peptide backbone[16]. The Cuscuta factor is found in all organs of *C. reflexa* irrespective of its infectious stage and seems to locate to the parasite's cell wall[15]. Here, we purified this Cuscuta factor from *C. reflexa* extracts and identified it as a Glycine-rich protein (GRP). We further characterized its function as a binding ligand for CuRe1 and the specifically triggered plant defense responses.

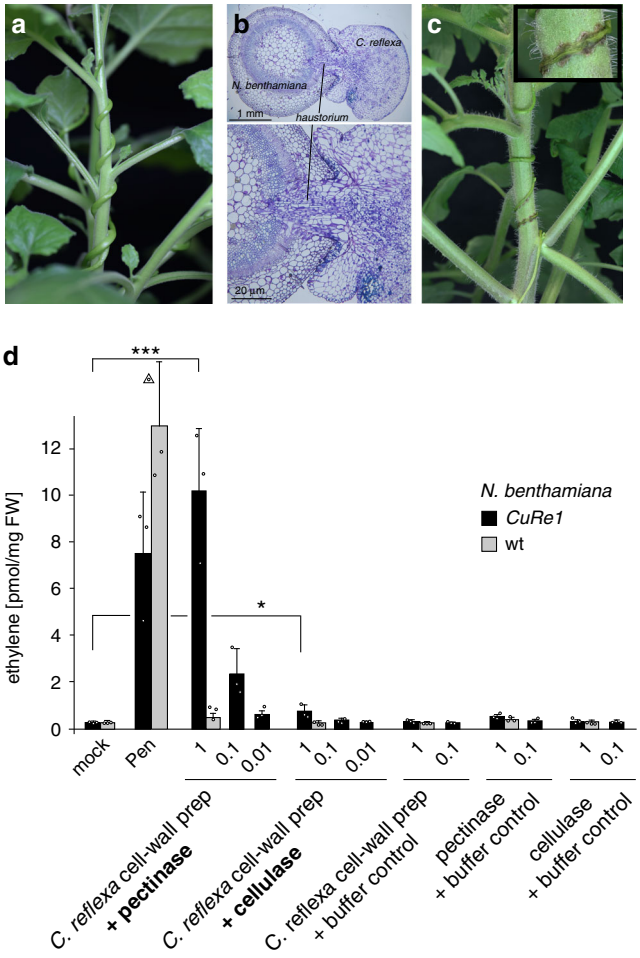

Fig. 1 *Cuscuta reflexa* induces defense in cultivated tomato by a pathogen-associated molecular pattern. a *C. reflexa* can infect nearly all dicot plants as susceptible hosts; picture shows infected *Nicotiana benthamiana*; **b** Parasitic haustoria successfully penetrate the *N. benthamiana* shoot; **c** *C. reflexa* induces visible hypersensitive response (HR) on tomato (*S. lycopersicum*) shoot at the contact sites of the parasite's haustoria. **d** *C. reflexa* cell wall preparations were treated with either cellulase or pectinase; extracts were applied to trigger defense-related ethylene biosynthesis in transgenic *CuRe1*-expressing *N. benthamiana* plants. Numbers on x-axis indicate applied extract volume in µl; bovine serum albumin (BSA; 0.01 mg/ml) buffered in 25 mM MES (pH 5.7) was added as mock control; *Penicillium* extract (0.05 mg/ml) served as positive control[31]. FW, fresh weight. Ethylene measurements show means of three technical replicates; error bars denote SD. Wildtype (wt) *N. benthamiana* plant samples have not been tested with diluted extract preparations (0.1 µl and 0.01 µl) since they did not respond to maximum doses (1 µl) in the ethylene assay. Dots indicate single data points, triangle shows outlier at 16.87; Asterisks show Student's *t* test; ***$p < 0.0028$; *$p < 0.0285$; representative graphs are shown; all experiments were repeated more than three times.

Results and discussion

Purification and identification of a defense-triggering Cuscuta factor. Since we knew that the Cuscuta factor originates from the cell wall, we focused on extracts prepared from the parasite cell wall and tested them for bioactivity in the ethylene bioassay specifically induced via CuRe1 (Fig. 1d). Compared to incubation in buffer/water alone, higher amounts of Cuscuta factor were found to be released from cell wall fractions by treatments with pectinases and, with much lower efficiency, by cellulases (Fig. 1d). Both types of enzymes are known to be present and active in penetrating *Cuscuta* spp. haustoria and can thus lead to an increased release of the Cuscuta factor from the cell walls of intruding haustoria during the infection process[17,18].

To extract sufficient amounts of the Cuscuta factor from collected plant material, we scaled up the previous protocol[15] and used acidic extraction conditions (0.1 M HCl, pH 1). The analysis was also extended to all of the activities that eluted as distinct peaks from the first cation exchange column (Supplementary Fig. 1). When purifying the extracts by cation exchange or reversed phase chromatography, the Cuscuta factor activity detectable by the CuRe1 receptor eluted in several peaks, indicating presence of activity in structurally different forms (Supplementary Fig. 1)[15]. We further purified and enriched the Cuscuta factor(s) from the obtained fractions and performed LC-MS/MS analyses for each sample individually. Several distinct masses correlated with CuRe1-dependent bioactivity and we identified 11 different compounds all of which represented active forms of the Cuscuta factor (Supplementary Fig. 2). MS/MS fragmentation studies of the correlated candidate masses shared similar fragment peaks

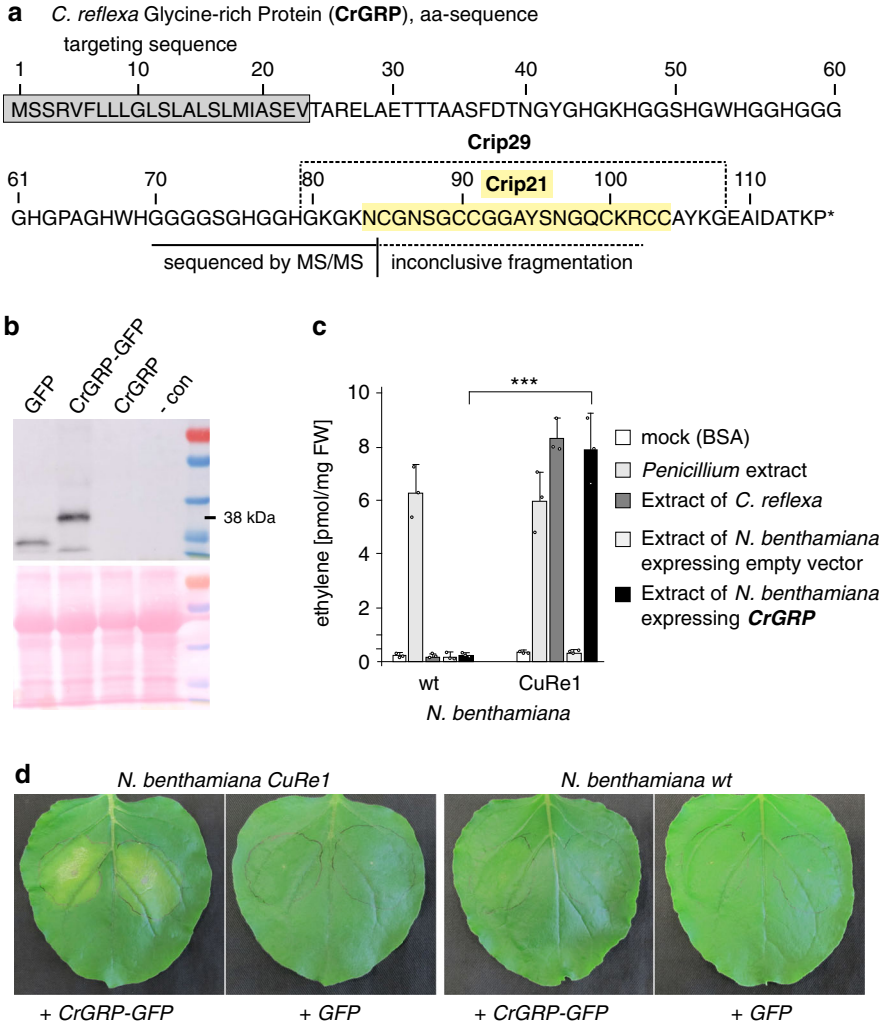

Fig. 2 The *C. reflexa* Glycine-rich protein (CrGRP) triggers CuRe1-dependent defense responses. a Protein sequence of the identified CrGRP; the peptide isolated from *C. reflexa* extracts was sequenced de novo by mass spectrometry and is indicated starting at aa-position 70, dashed line indicates the peptide part which could not be sequenced by MS/MS; Crip21 motif highlighted in yellow. **b** heterologous expression of *CrGRP* gene in *N. benthamiana* leaves; WB shows a c-terminally GFP-tagged CrGRP. **c** Ethylene response in leaves of wt or *CuRe1*-expressing *N. benthamiana*. Plants were treated with extracted CrGRP after heterologous expression shown in b. Bovine serum albumine (BSA; 0.01 mg/ml) buffered in 25 mM MES (pH 5.7) was added as mock control; *Penicillium* extract (0.05 mg/ml) served as positive control[31]. FW, fresh weight; ethylene measurements show means of three technical replicates; dots indicate single data points; error bars denote SD, asterisks denote student's t-test, $p \leq 0.0003$; representative graphs are shown; experiments were repeated more than three times. **d** Expression of CrGRP-GFP in leaves of wildtype (wt) and transgenic *CuRe1*-expressing *N. benthamiana* plants. GFP alone served as negative control; pictures 5 days after expression.

(Supplementary Fig. 2) and had the fragment mass of 2077 Da in common. Another characteristic feature of all corresponding MS-spectra was an inconclusive fragmentation pattern with only a few clear but characteristic fragment masses (Supplementary Fig. 2) that have been previously observed[15]. Since the commonly present fragment of 2077 Da is a y-fragment and the spectra of the heavier candidate masses contained the lighter candidate masses as their y-fragments, we assumed a common origin of all fragments from the same protein. The mass differences of the N-terminal fragmentations could be correlated to those of single amino acid residues, which allowed us to deduce the N-terminal part of the peptide sequence (Supplementary Fig. 2). The information obtained from overlays of seven individual MS/MS fragmentation analyses in four individual LC-MS/MS runs revealed the sequence of the first 15 N-terminal amino acids of the peptide (Fig. 2a and Supplementary Fig. 2). A p-blast search against a translated transcriptome database from *C. reflexa*[19] resulted in a perfect hit on a glycine-rich protein (GRP) of *C. reflexa* (Fig. 2a). CrGRP consists of 116 amino acids with an n-terminal targeting sequence that predicts an extracellular localization (Fig. 2a). According to the current classification of GRPs[20], the CrGRP belongs to the class II which comprises a distinguishing c-terminal cysteine-rich region (Fig. 2a). We cloned the corresponding *CrGRP* gene from *C. reflexa* genomic DNA and transiently expressed it in *N. benthamiana* leaves for ~72 h with c-terminal GFP or tagRFP fusion tags. We confirmed the predicted localization of GRP within the cell wall with confocal microscopy of *N. benthamiana* leaves transiently expressing a tagRFP-tagged version of the CrGRP (Supplementary Fig. 3). Western blot analyses showed that the protein migrated at the calculated size and does not appear to be secondarily modified in *N. benthamiana* (Fig. 2b). Extracts of these leaves, expressing *CrGRP*, induced ethylene production in a CuRe1-dependent manner like the original *C. reflexa* extract (Fig. 2c). Moreover, when expressing *CrGRP* in *N. benthamiana* leaves for 5–7 days, clear hypersensitive cell death can be observed only when CuRe1 is present but not in control plants (Fig. 2d) lacking the receptor. These findings demonstrate that

CrGRP is the trigger to initiate the CuRe1-dependent defense program.

The minimal peptide epitope of CrGRP. Due to unspecific degradation of the full-length CrGRP during our initial extraction protocol, the extracted forms of the Cuscuta factor were rather small peptides in a range between 2000 and 4000 Da[15] (Supplementary Table 1). We therefore assumed a minimal peptide motif within the CrGRP full-length protein (~11.5 kDa), which must be sufficient to trigger the defense program. We thus tested a synthetic peptide representing $CrGRP_{82-106}$ for activity. The peptide was highly active and triggered ethylene production at concentrations ≥0.1 nM (Fig. 3a, b), which is in the range we previously estimated for the Cuscuta factor purified from plant extracts[15]. To further narrow down the active motif we tested shortened versions of the peptide, resulting in a 21 aa peptide, termed as crip21 for cysteine-rich peptide 21, that still retained the full activity found with $CrGRP_{82-106}$ or full-length CrGRP (Figs. 2a and 3a, b). Peptides further shortened from the N- or C-terminus showed only reduced activity or no activity, respectively (Fig. 3a and Supplementary Fig. 4). A 15 aa peptide representing the N-terminal sequence present on the purified peptide was inactive (Supplementary Fig. 4), further demonstrating that activity resides in the C-terminal part with the Crip21 motif of CrGRP (Fig. 2a).

Treatment of *C. reflexa* extracts or purified Cuscuta factor with NH_4OH at pH ≥ 11 leads to a total loss of bioactivity and indicated potential secondary modifications on the CrGRP[15,16]. However, synthesized peptides trigger responses in a picomolar range (Fig. 3b and Supplementary Fig. 4) suggesting that no such secondary modifications are required for the bioactivity of Crip21. Much like the purified Cuscuta factor[15], synthesized Crip21 peptide, comprising no secondary modifications, lost all of its activity when treated with NH_4OH (Supplementary Fig. 5a). We analyzed the NH_4OH-treated peptide by MS/MS and observed multiple reaction products of Crip21 among which the most prominent ones were 82 or 99 Da smaller (Supplementary Fig. 5b), clearly showing that the treatment of Crip21 with NH_4OH is modifying the peptide itself.

We infiltrated Crip21 into the leaves of resistant *S. lycopersicum*, susceptible *Solanum pennellii* and the introgression line IL8-1-1 lacking *CuRe1*[15], to check whether Crip21 also induces visible HR in tomato. After 7 days, only the cultivated tomato or an introgression line (IL) with functional CuRe1 showed Crip21-dependent cell death while *S. pennellii* and the IL lacking CuRe1 did not (Fig. 3c).

Binding of CrGRP and Crip to the receptor CuRe1. To test for a direct interaction of the peptide epitope Crip with the receptor CuRe1, we n-terminally labelled a 29-aa-long Crip peptide with biotin (bio-Crip29). The bio-Crip29 peptide is an N-terminally (+4 aa) and C-terminally (+4 aa) prolonged Crip21 (Fig. 2a) and was as active as Crip21 (Fig. 3b). The Crip21 minimal epitope has been prolonged to introduce a higher number of Lysine residues to increase the chance for a successful chemical crosslinking of NH_2 groups on the ligand with NH_2 groups on the receptor. We then examined the interaction of CuRe1 proteins with bio-Crip29 in affinity-crosslinking experiments *in planta*. *N. benthamiana* leaves expressing the myc-tagged receptor CuRe1 were first incubated with the bio-Crip29 derivative, either alone or together with an excess of non-modified Crip as competitor, and the leaves were subsequently treated with a chemical cross-linker. When analyzed for the presence of biotin, immunoprecipitates of CuRe1 showed clear labelling which was absent in samples treated with an excess of non-modified Crip as competitor (Fig. 3d). In turn, binding of bio-Crip29 was not out-competed when using structurally unrelated peptides such as flg22 (Fig. 3d). These findings demonstrate the CrGRP derived peptide epitope Crip as the specific ligand for the CuRe1 receptor. To corroborate direct protein–protein interaction of the full-length CrGRP with CuRe1 as it may occur under physiological conditions, both proteins were co-expressed with different c-terminal tags and the interaction of both could be demonstrated in co-immunoprecipitation assays (Fig. 3e).

To identify aa-residues of Crip21 which are critical for CuRe1-activation, the 21 aa-residues were individually substituted by alanine or serine (substitutions for the cysteines), respectively (Supplementary Table 1). Replacement of the cysteine residues at positions 7, 17, 20, and 21 by Serine led to a reduced functionality or in case of C7 to a complete loss of function. In contrast, the other aa residues seemed less important and had no measurable effects on activity (Supplementary Table 1).

GRP and Crip21 homologs in other plants. When p-blasting the CrGRP or Crip21 aa-sequences against a database of the translated *C. campestris* genome[21] and transcriptome[22], we found a GRP homolog (Supplementary Fig. 6) which also contains a peptide motif (CcCrip21) with a sequence similarity of ~70% to the *C. reflexa* Crip21. Especially the glycine residues and the six cysteines are highly conserved (Supplementary Fig. 6). A comparable GRP sequence has been also found in the sequence database of *C. australis*[23] with the CaCrip21 peptide showing exactly the same 21 aa sequence long peptide as CcCrip21 (Supplementary Fig. 6a). The *C. campestris* CcCrip21, or *C. australis* CaCrip21, respectively, showed full activity at similar concentrations in CuRe1-dependent ethylene induction (Supplementary Fig. 6b). This corroborates previous findings in which we could show that the defense-triggering Cuscuta factor is also present in other *Cuscuta* species[15]. In general, GRPs are widely distributed all over the plant kingdom. Even in cultivated tomato (*S. lycopersicum*) we found a homolog with an aa-sequence similarity of 57% to CrGRP and we thus assumed the corresponding peptide motif to Crip21, SlCrip21 could serve as an endogenous trigger for tomato CuRe1. We therefore tested the synthesized peptide SlCrip21 in our bioassays where SlCrip21 exhibited only residual activity when applied at concentrations ≥1000 nM (Supplementary Figs. 6c and 7).

By substitution of single aa-residues within Crip21 using SlCrip21 as a template (Supplementary Fig. 7), and testing those peptides for bioactivity via CuRe1, we discovered that replacement of the Alanine at position 11 in Crip21 by a Tyrosine residue (as is the case in SlCrip21) abolished its CuRe1-dependent activity (Supplementary Fig. 7), which is possibly important to avoid autoimmune responses in tomato. However, substituting the tyrosine (Y11) of SlCrip21 by Alanine did not restore activity, indicating that additional changes in the peptide sequence of SlCrip contribute to avoiding self-recognition in tomato (Supplementary Fig. 7). The biological function of the full-length protein SlGRP is unclear and SlGRP may probably play other roles in tomato not related to cellular defense responses and independent of tomato CuRe1. In general, assigned functions of plant GRPs are multifaceted and range from the stabilization of cell walls to hypothesized regulating functions during abiotic and biotic stress reactions[24,25], which makes it difficult to speculate about the role of the respective GRP in tomato or *Cuscuta*. Future work will have to reveal what the in vivo function of CrGRP for *C. reflexa* could be. By BLAST searching for Crip21 peptide homologs, we got hits for this peptide motif related to GRPs of many plant species. Peptides giving the best hits and showing the highest sequence identity to Crip21 were synthesized and tested for their capability to trigger ethylene in samples of *CuRe1*-expressing *N. benthamiana* as well as in cultivated tomato (*S. lycopersicum*;

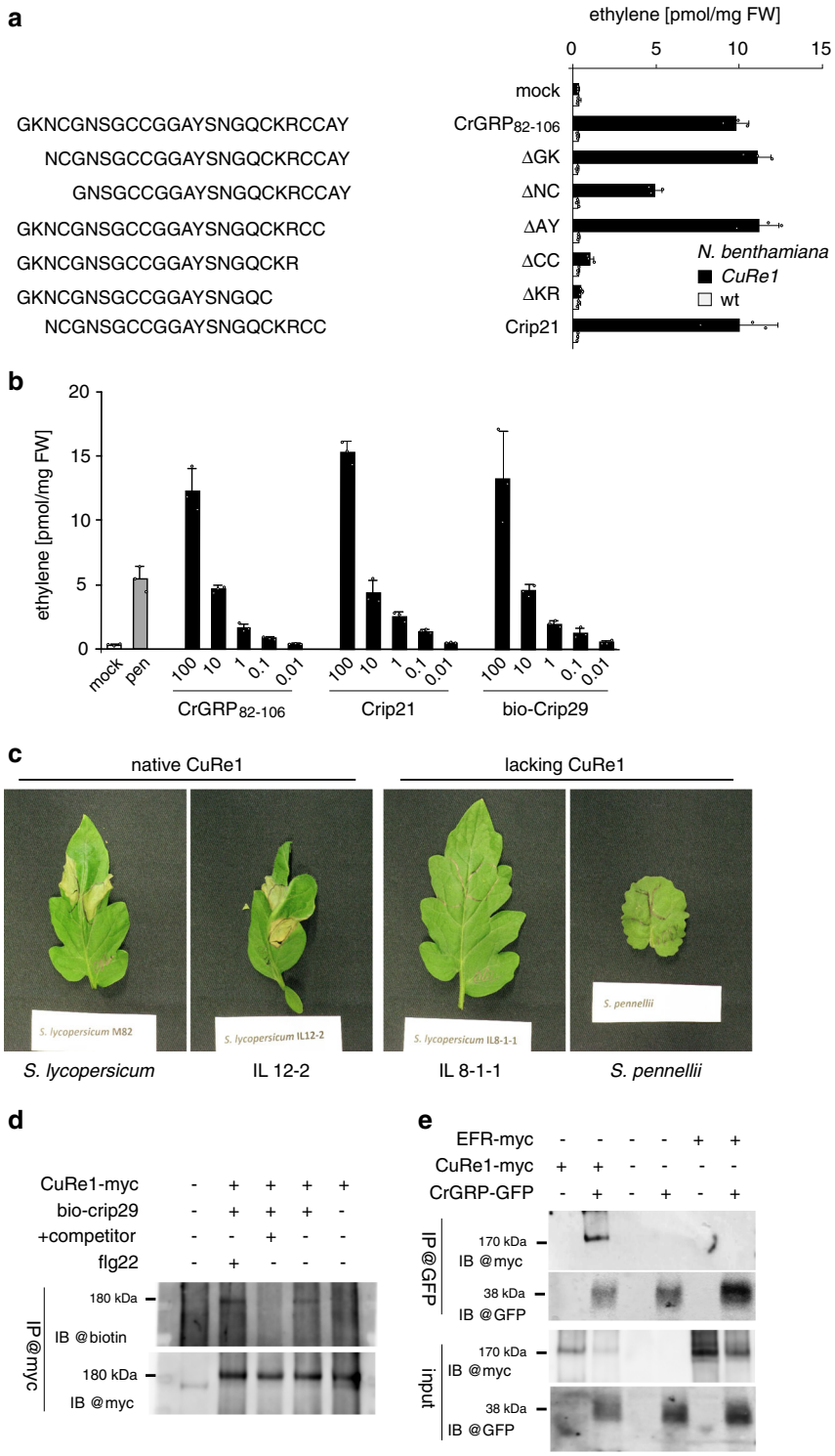

Fig. 3 The peptide epitope crip21 of CrGRP triggers the tomato receptor CuRe1. a Synthesized peptides deriving from CrGRP induce ethylene production in transgenic CuRe1-expressing *N. benthamiana*. Peptides were applied at concentrations of 1 µM each. **b** Dose-dependent induction of ethylene by the CrGRP derived, synthesized peptides in *CuRe1*-expressing *N. benthamiana*; numbers on x-axis indicate peptide concentrations in nM; bio-crip29 is the N-terminally biotinylated peptide used in binding studies; for a and b: Bovine serum albumin (BSA; 0.01 mg/ml) buffered in 25 mM MES (pH 5.7) was added as mock control; *Penicillium* extract (0.05 mg/ml) served as positive control[31]. FW, fresh weight; ethylene measurements show means of three technical replicates; dots indicate single data points; error bars denote SD, representative graphs are shown, experiments were repeated independently more than three times. **c** crip21 peptide induces HR-type of cell death in a CuRe1-dependent manner. Leaves of *S. lycopersicum*, an introgression line (IL 12-2) with functional CuRe1and leaves of IL 8-1-1 and *S. pennellii* (right; both lacking CuRe1) were infiltrated with 100 nM crip21 and photographs were taken 7 days later; painted lines indicate infiltrated leaf area. The effects shown are representative for ten infiltrated leaves per tomato IL or species, respectively; experiments have been independently repeated three times. **d** Affinity-crosslinking of crip29-biotin with CuRe1 in planta. Solubilized proteins were immune-precipitated and analyzed for myc-tagged (bottom) and biotinylated proteins (top); $CrGRP_{82-106}$ served as competitor; **e** Co-immunoprecipitation experiments demonstrate interaction of CuRe1 with CrGRP full length protein after co-expression for ~48 h *in planta*.

Supplementary Fig. 6c). Crip21 peptides from blue picotee (*Ipomoea nil*), Indian lotus (*Nelumbo nucifera*), and lettuce (*Lactuca sativa*) were able to induce defense related ethylene production in a CuRe1-dependent manner (Supplementary Fig. 6c). Importantly though, the SlCrip21 peptide from tomato showed only residual activity at 1000 nM, suggesting that CuRe1 could have evolved in *Solanum* as a perception system for a molecular pattern of non-self that is characteristic for any attacking invader such as dodder described here.

In summary, we showed that CrGRP comprises the molecular pattern with the characteristic Cys-residues that mark the plant parasite *C. reflexa* as an alien attacker to host plants with the cell surface receptor CuRe1. These findings about the molecular dialogue between host plants and attacking parasitic plants will help to understand resistance during plant–plant interactions and open new possibilities to improve resistance against *Cuscuta* spp. as well as to design resistance against parasitic plants in general.

Methods

***Cuscuta* spp. extract preparation, purification and identification of CrGRP**. *C. reflexa* extract was prepared as described[15], with modifications outlined below. *Cuscuta* ssp. shoots were harvested, frozen in liquid nitrogen and freeze-dried for storage. Extraction was performed with 100 mM HCl (pH ~1.0) at 60 °C for 16 h. Extract was adjusted to pH 5.5 with 25 mM MES, filtered (0.22 µm MCEM filters, Merck Millipore or for higher volumes through silica gel 60, Macherey-Nagel), loaded on a cation-exchange column (SP Trisacryl® M, Sigma; 25 mM MES pH 5.5) and eluted with 600 mM KCl. The obtained elution was supplied with $(NH_4)_2SO_4$ to a final salt concentration of 3 M; bulk protein was precipitated at 4 °C and removed while the bioactive protein/peptides stayed in solution. The solution was then desalted by loading on a C18 reversed-phase column (Chromabond, bench-top, 20 mM ammoniumacetate/acetic acid, pH 4.5) and eluted with 40% acetonitrile. This pre-purified Cuscuta extract was sequentially separated (Fig. S1) on a strong cation exchange (SCX) column (GE healthcare; Fig. S1A). Active fractions were equilibrated with 25 mM MES (KOH, pH 5.5) and a first run on a C18 reversed phase column (ZORBAX Rx- C18, Agilent) with 20 mM ammonium acetate/acetic acid (pH 4.5) and elution with a gradient of acetonitrile (0–20%) was performed (Fig. S1B). Active fractions were again pooled and a second run on the same column with 0.1% acetic acid (pH 4.5) and elution with a gradient of acetonitrile (0–20%). Fractions with highest activity from this second run were further separated on a reversed phase column (Waters, ACQUITY C18 HSST3) equilibrated with 0.1% formic acid and eluted with a gradient of methanol (0–30%, 60 min). The CuRe1 responsive eluate of this final step was analyzed under similar LC conditions by LC-MS (Waters Acquitiy UPLC - Synapt G2 LC/MS system, electrospray ionization). We correlated distinct masses in these fractions to the CuRe1-dependent responses in transgenic *N. benthamiana* leaf samples. In all, 1 µl of the obtained fractions were tested for their capability to induce ethylene production in CuRe1 transgenic *N. benthamiana* plants. The identified masses were further analyzed by MS/MS fragmentation studies using an Easy nano-LC (Thermo Scientific) coupled to an LTQ Orbitrap Elite mass specrometer (Thermo Scientific) as previously described[26] and evaluated with mMass[27]. The aa sequences were calculated manually from the fragment spectra obtained.

The Cuscuta factor (CuF) could be inactivated by incubating it either with 12.5% NH_4OH or 70% ethylamine for 1 h at 45 °C.

***C. reflexa* cell wall purification and Pectinase treatment**. Lyophilized *C. reflexa* was ground to fine powder (liquid nitrogen) and subsequently washed with 70% Ethanol, Chloroform/Methanol (1:1 v/v), 200 mM $CaCl_2$ (5 mM Sodium Acetate pH 4.6), 10 mM EGTA (5 mM Sodium Acetate pH 4.6), and 3 M LiCl (5 mM Sodium Acetate pH 4.6). The washed cell wall was dried with Acetone. In all, ~100 mg purified cell wall was incubated with Pectinase (from *Aspergillus niger*, Sigma-Aldrich) or Cellulase (from *Trichodoma reesei* ATCC 26921, Sigma-Aldrich) according the supplier's guidelines. Extracts, similarly prepared from tomato or tobacco served as controls.

Plant response assays. All obtained extracts or collected fractions after each purification step, as well as the isolated CrGRP or synthesized Crip peptides were tested for bioactivity in the ethylene assay as previously described[15]. For this we cut leaf samples of analyzed plants in 3 × 3 mm squares and float them on a water surface. After incubation over night at RT, three leaf pieces are carefully collected into glass tubes (6 ml) with 500 µl water. Samples were treated as indicated as well as amounts of used extracts or concentrations of peptides. After treatment tubes were sealed with a rubber plug and incubated at RT on a horizontal shaker (85–100 rpm) for 3 h. All samples were analyzed with a Gaschromatograph (Shimadzu, GC-2014, glass column 3 mm × 1.6 m with Al_2O_3) by manually injecting 1 ml of the gaseous phase.

DNA extraction and cloning of *CrGRP*. *C. reflexa* plants were grown under long day conditions (16 h day/8 h night) at 22 °C, in a greenhouse. Genomic DNA was extracted from frozen tissue using the Plant DNA Preparation Kit (Jena Biosciences, Germany), and PCR was performed with gene specific primers for the candidate gene (C_ref_ r2_000247) CrGRP): FW: ATGAGTTCAAGGGTCTTT CTTCTCC, REV: AGGCTTCGTCGCATCAATGGC; The PCR products were cloned to the pCR8/GW/TOPO TA-cloning vector (Invitrogen™, Thermo Fisher). Reverse primers without stop codon allowed for C-terminal fusion to a GFP tag after recombining via LR-reaction (LR-clonase® II Plus enzyme mix, Invitrogen™) into respective vectors (pB7FWG2.0, pK7FWG2.0, both with C-terminal GFP tag; plant systems biology, university of Gent). For cloning of a *CrGRP* cDNA construct, total RNA was extracted from tomato plants (RNeasy Plant Mini Kit, Quiagen), and cDNA was synthesized by reverse transcription (First-Strand cDNA Synthesis Kit, GE Healthcare Life Sciences); PCR was performed with primers above. For subcellular localization, CrGRP has been cloned via LR-reaction into a modified version of pGWB660, including a tagRFP[28].

***CrGRP* expression and protein isolation**. The 35S::CrGRP:GFP construct (in vector pB7FWG2.0; plant systems biology, university of Gent) was transiently transformed into *N. benthamiana* leaves using *Agrobacterium tumefaciens* (strain GV3101). *A. tumefaciens* cultures ($OD_{600} = 0.1$ in 10 mM $MgCl_2$, 150 µM Acetosyringone) were infiltrated into leaves of 4 weeks old *N. benthamiana* plants, according to the described protocol[29]. About 48 h post infiltration, leaves were harvested, ground under liquid nitrogen to fine powder, supplemented with buffer (~3x volume), and centrifuged (45 min, 100,000 rcf, 4 °C). The supernatant was then collected for further testing. An extract of *N. benthamiana* leaves expressing GFP alone (pB7WGF2.0) was prepared similarly and served as mock control for treatments.

For monitoring hypersensitive responses (HR) in leaves, *35S::CrGRP:GFP* or *35S::GFP* constructs were expressed in either transgenic, *CuRe1*-expressing or wt *N. benthamiana* plants. Leaves were infiltrated as described above using the defined volume of 200 µl; infiltration area was carefully labeled with a black marker. Pictures were taken 7 days post infiltration. For detecting HR-symptoms in tomato plants, two leaves of three plants (six leaves total; per introgression line or wildtype) were infiltrated with 100 µl of a 100 nM peptide solution; infiltrated leaf area was labeled with a pen immediately after infiltration and photographs were taken 7 days post peptide infiltration.

Confocal microscopy. Images of transiently transformed *N. benthamiana* were taken 5 days after *A. tumefaciens* infiltration with a Zeiss confocal laser scanning microscope (LSM880, Carl Zeiss Microscopy GmbH, Carl-Zeiss-Promenade 10, 07745 Jena, Germany) and the attached C-Apochromat ×10/0.45 W M27 objective. The tag-RFP fluorescence was excited with 561 nm and emission was detected at 563–607 nm. Autofluorescence of plant cell walls (lignin) was excited at 405 nm and emission was detected at 410–466 nm. Pinhole, detector gain and digital gain settings were adjusted to provide an optimal balance between fluorescence intensity and background signal. Data were processed with the ZEN 2.3 software.

Binding assays and immunoprecipitation assays. Direct interaction of CrGRP with CuRe1 was tested by co-immunoprecipitation. For immunoprecipitation, leaves of *N. benthamiana* were transiently transformed with *35S::CuRe1:myc* or *35S::CrGRP:GFP* alone each, or co-expressed in combination for ~48 h. Leaf material was harvested, frozen in liquid nitrogen and ground to fine powder. Samples of 300 mg were solubilized and used for immunoprecipitation as reported[29] using α-GFP trap Sepharose beads (ChromoTek, IZB Martinsried, Germany). Samples were separated by SDS-PAGE (8% Acrylamide gels) and transferred to nitrocellulose membrane. Western blots were probed using the α-GFP (Acris (now OriGENE) Polyclonal Antibody to GFP; Cat. No.: R1091P; dilution 1:5000 in 5% BSA; goat; UniProt: P42212) or α-myc (Sigma Polyclonal anti-c-Myc antibody; Cat. No.: C3956; dilution: 1:5000 in 5% BSA; from rabbit; UniProt: P01106) antibodies, diluted according to the instructions of the suppliers, and developed with secondary antibodies conjugated to alkaline phosphatase as described[29,30] (Sigma; Anti-Goat IgG (whole molecule) - Alkaline Phosphatase antibody produced in rabbit; Cat. No.: A4187, dilution: 1:50,000 in 5% BSA; OR: Sigma; Anti-Rabbit IgG (whole molecule) - Alkaline Phosphatase antibody produced in goat, Cat. No.: A3687, dilution: 1:50,000 in 5% BSA).

In vivo cross-linking of biotin-Crip29 (Crip29 aa-sequence: GKGKNCGNSGC CGGAYSNGQCKRCCAYKG) to CuRe1 was performed as described[30]; leaves of *N. benthamiana* expressing 35S::CuRe1:myc, or control plants (*N. benthamiana* expressing *35S::RLP23:myc*) were infiltrated with biotinylated bio-Crip29 (10 nM in ddH2O) with or without unlabeled Crip21 (or unlabeled Crip82-106) (10 µM) as competitor or with flg22 peptide as competition control. Five minutes after peptide treatment 2 mM EGS (ethylene glycol bis(succinimidyl succinate) in 25 mM HEPES buffer (pH 7.5) was infiltrated into the same leaves for cross-linking of peptides to the receptor proteins. Twenty minutes after cross-linking, leaf samples were harvested and frozen in liquid nitrogen; immunoprecipitations were performed against the myc tag present at the c-terminus of CuRe1 using myc-trap agarose beads (ChromoTek, IZB Martinsried, Germany) as described above. All peptides, including biotinylated bio-Crip29, were synthesized by GenScript® and ordered with a purity of >95%. Biotinylated Crip29 was detected on blots by

Streptavidine-conjugated Alkaline Phosphatase (Strep-AP, Roche diagnostics; Streptavidin-AP conjugate, Cat. No.: 11089161001, dilution: 1:1000 in 5% Albumin Fraction V, biotin-free).

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

Source data are provided with this paper. Any other supporting data are available from the corresponding author upon request.

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Author contributions

V.H. isolated and identified the *C. reflexa* GRP as defense trigger. V.H., M.K., B.K., and B.L. pepared *Cuscuta* extracts and purified the GRP. M.K. did microscope work and photography. J.A.B., A.R., N.S., and K.K. helped with bioinformatics, gave access to unpublished *Cuscuta* sequencing data and helped with the identification of the GRP gene/RNA. V.H. and P.S. helped with primer design, GRP cloning, expression, and minimal peptide motif identification. I.D.B., J.S., F.L.H.M., B.M., V.H., G.F., and M.S. did mass spec analyses and helped with MS-data interpretation. I.A. and R.B. performed binding studies; B.L. tested peptides for activity in bio-assays. V.H., K.K., T.N., G.F., M.S., and M.A. designed and discussed the experiments. All authors discussed the data referring to their respected experience and helped with interpretations and data analyses. V.H., G.F., K.K., N.S., M.S., and M.A. wrote the manuscript.

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Competing interests

The authors declare no competing interests.

Additional information

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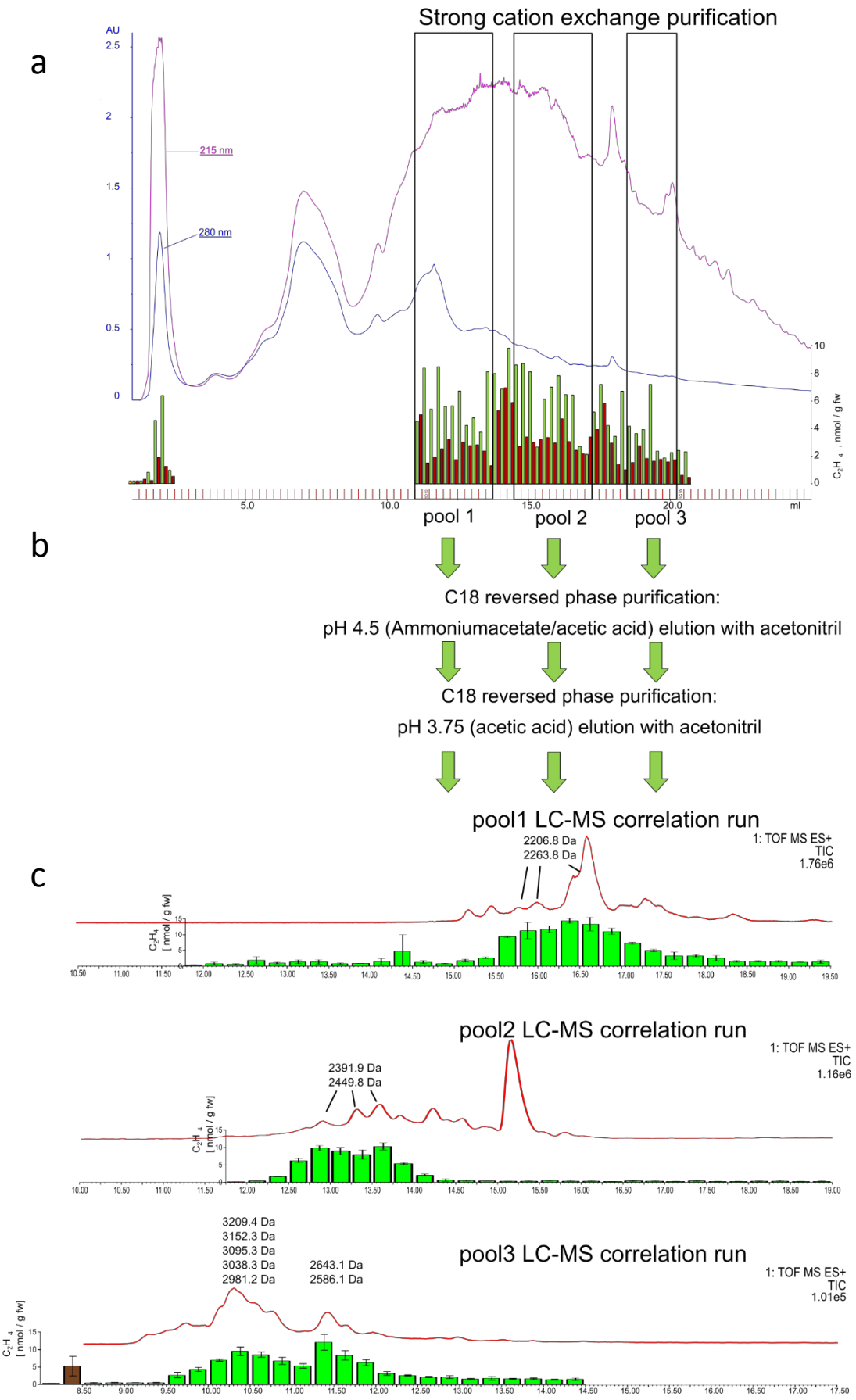
Supplementary Data


a
Strong cation exchange purification
AU
215 nm
280 nm
ml
pool 1
pool 2
pool 3
b
C18 reversed phase purification:
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C18 reversed phase purification:
pH 3.75 (acetic acid) elution with acetonitril
c
pool1 LC-MS correlation run
2206.8 Da
2263.8 Da
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TIC
1.76e6
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TIC
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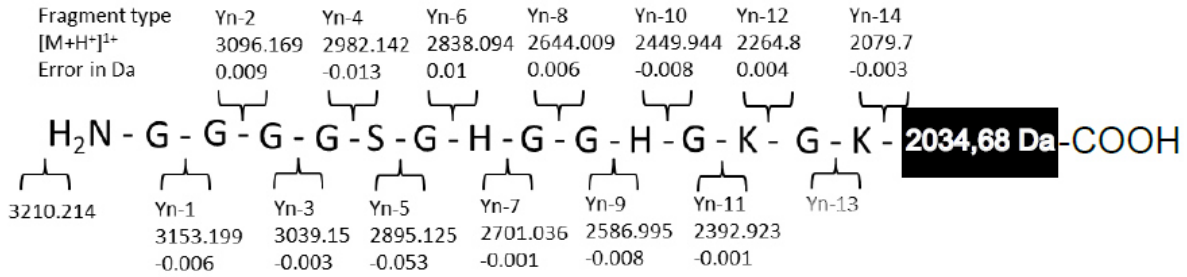
2

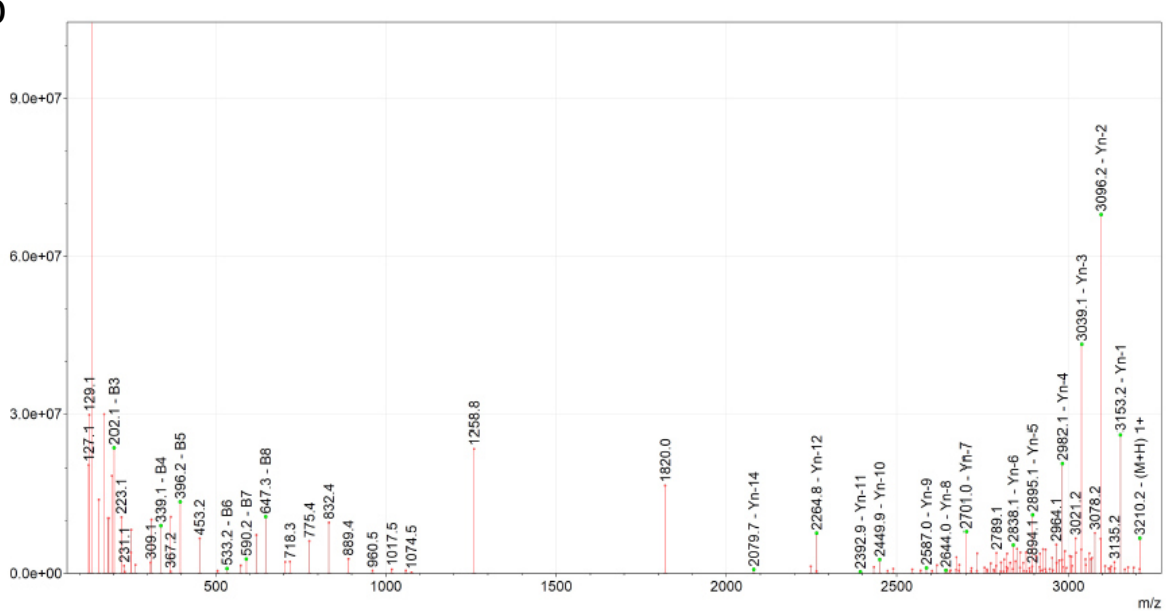
3

Supplementary Fig. 1. Purification of *C. reflexa* extract and identification of CuRe1 inducing molecules. (a) Separation of pre-purified *C. reflexa* extract by strong cation exchange chromatography (SCX). Eluting fractions (gradient 0-700 mM KCl; fractions in ml) were tested for induction of ethylene production in *CuRe1*-expressing *N. benthamiana*. Fractions with activity were combined to three pools as indicated. (b) Overview illustrating the subsequent purification steps on reversed phase C18 columns under two different pH regimes performed with the three pools separately. (c) Final analysis of the activities by LC-MS. Purified activities originating from pools 1, 2 and 3 from (a) were analysed with LC-MS to obtain a total ion chomatograms. In parallel runs, fractions were tested for activity in the ethylene bioassay. Bioactive peptides with masses ranging from 2206.8 Da to 3209.4 Da are indicated.

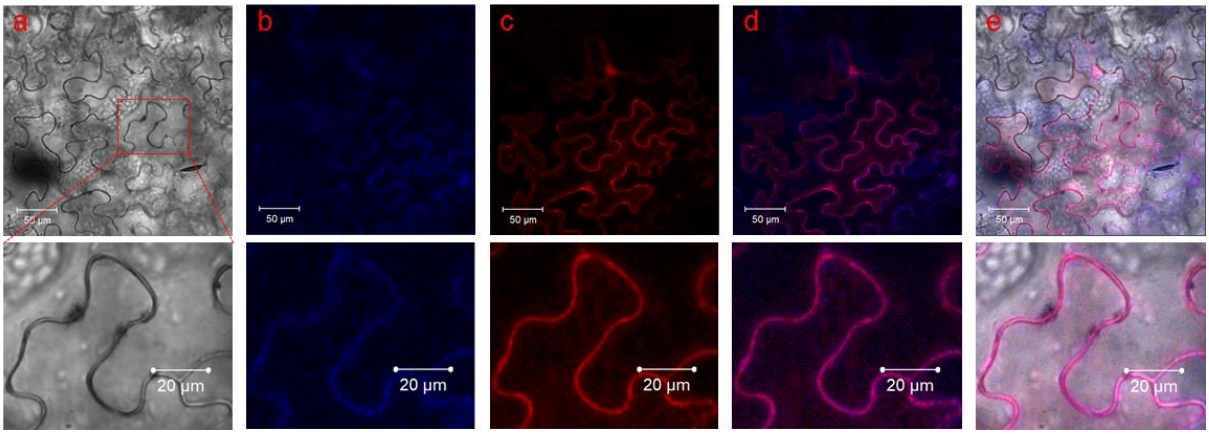
a



b

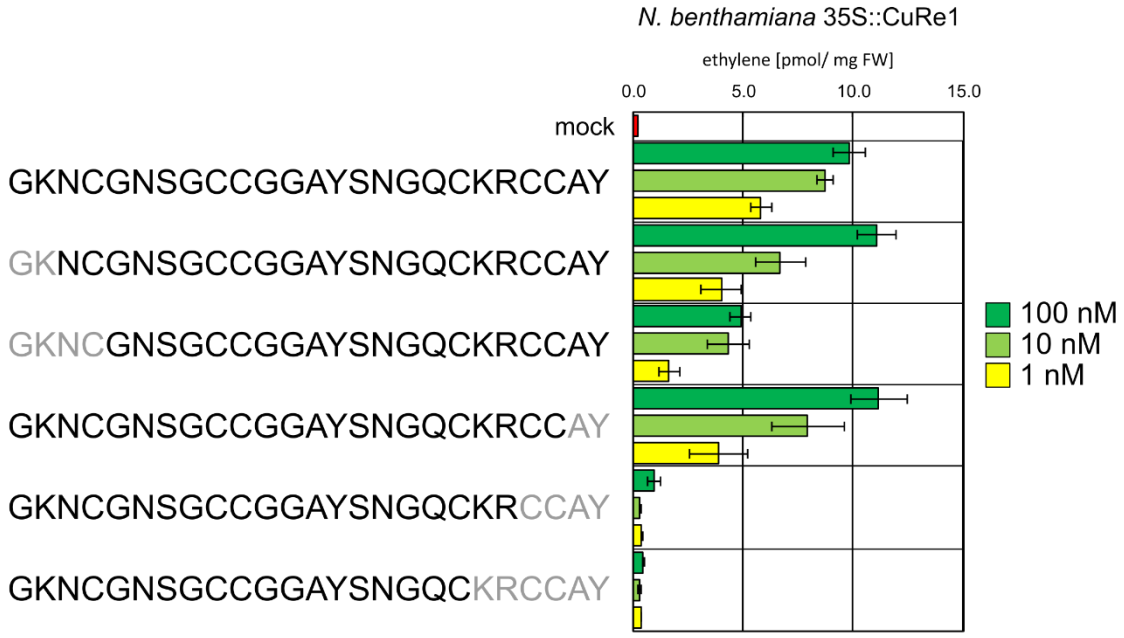


Supplementary Fig. 2. *de novo* sequenced peptide of CrGRP and corresponding MS-spectrum. (a) N-terminal peptide sequence of the Candidate mass 3209.2 Da calculated from the observed Y-fragments in the deconvoluted MS/MS spectrum; black box shows the peptide part which could not be sequenced due to inconclusive fragmentation. The mass repetitively occurred in the analyzed samples and was the responsible bioactive part (e.g. in ethylene measurements), later identified as Crip21 within the CrGRP sequence. (b). Y-fragments are labeled and indicated with green dots.



Supplementary Fig. 3. Confocal microscopy and subcellular localization of CrGRP-RFP in *N. benthamiana* leaf epidermal cells; a) bright field; b) blue: autofluorescence of the epidermal cell walls (lignin); excitation at 405 nm and emission was detected at 410-466 nm; c) Fluorescence of the RFP-tag present at the CrGRP c-terminus; excitation at 561 nm and emission was detected at 563-607 nm; d) overlay of b) and c); e) overlay of a), b) and c); lower panel: focused detail as indicated by red box in a); purple color in d) and e) arises from overlay of RFP and lignin fluorescence.

a



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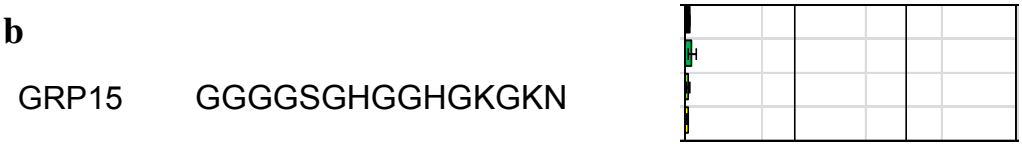
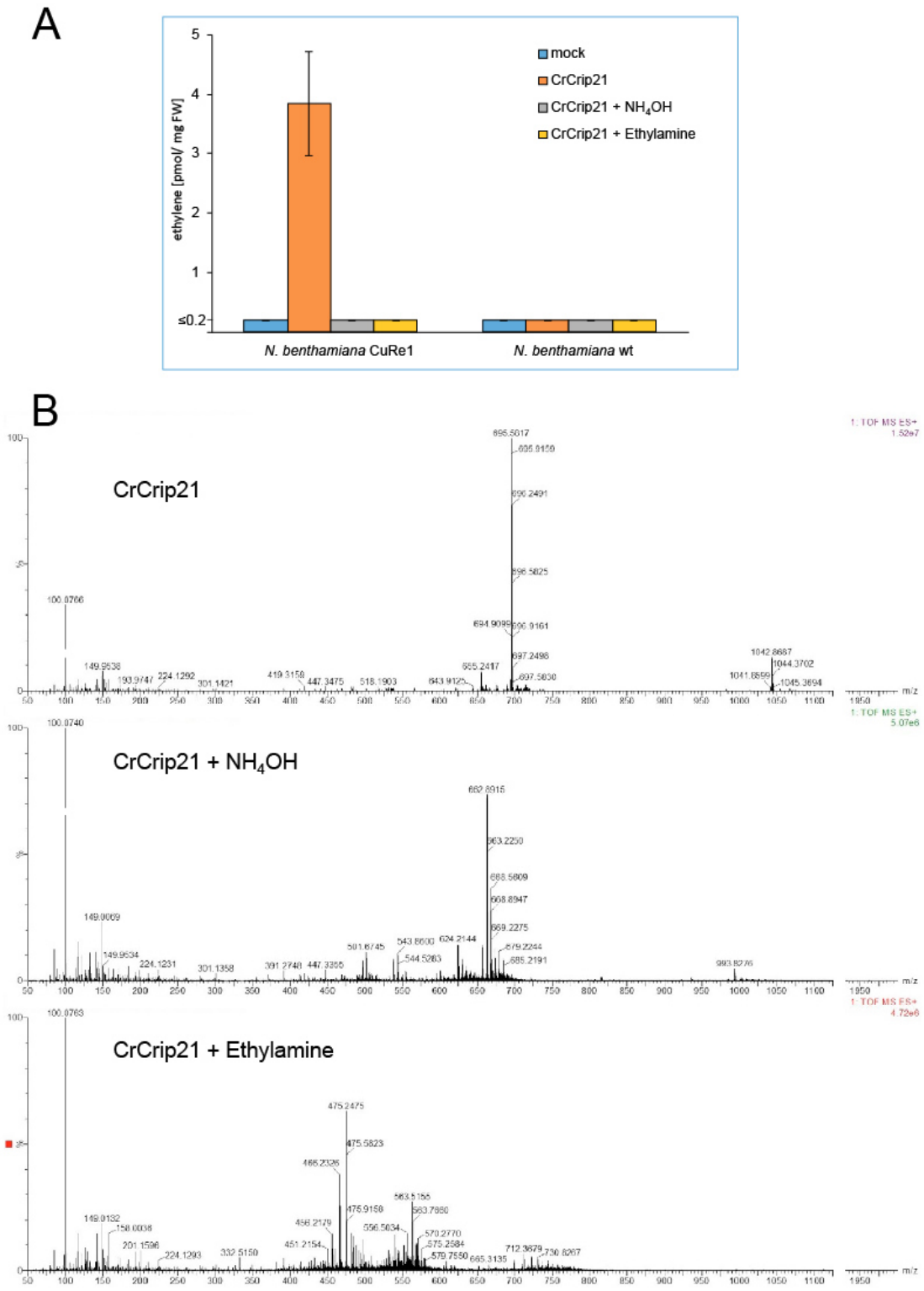


Supplementary Fig. 4. Synthesized peptides derived from CrGRP induce ethylene production in *CuRe1*-expressing *N. benthamiana*. a) The $CrGRP_{82\text{-}106}$ was n- and c-terminally truncated for finding the minimal motif triggering CuRe1; peptides were applied at the concentrations indicated. b) the peptide GRP15 which served as a fingerprint for the identification of CrGRP was completely inactive and added to the CuRe1 expressing samples at concentrations 1000, 100, 10 and 1 nM (from top). Bovine serum albumin (BSA; 0.01 mg/ml) buffered in 25 mM MES (pH 5.7) was added as mock control; FW, fresh weight; ethylene measurements show means of three technical replicates; error bars denote SD. Experiments have been repeated more than three times.



Supplementary Fig. 5. Inactivation of Crip21 by treatments with NH_4OH or Ethylamine; (a) Crip21 peptide treated with 12.5% NH_4OH or 70% Ethylamine, respectively, loose activity to induce ethylene biosynthesis in *CuRe1*-expressing *N. benthamiana* leaves. Peptide samples were applied at concentrations of 100 nM. Bovine serum albumin (BSA; 0.01 mg/ml) buffered in 25 mM MES (pH 5.7) was added as mock control. FW, fresh weight; ethylene measurements show means of three technical replicates; error bars denote SD. (b) incubation with NH_4OH or Ethylamine leads to disappearance of the peptide mass signal and appearance of multiple as yet unidentified reaction products with different masses.

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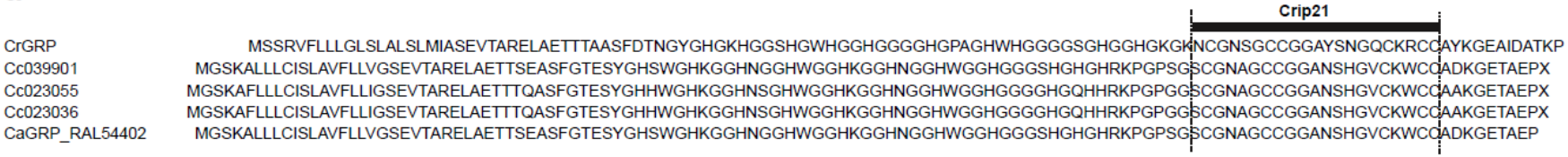
Peptide name	sequence	minimum effective concentration [nM]
		10000 1000 100 10 1 0.1
Crip 21	NCGNSGCCGGAYSNGQCKRCC	
Crip 21-C2S	N**S**GNSGCCGGAYSNGQCKRCC	
Crip 21-C7S	NCGNSG**S**CGGAYSNGQCKRCC	
Crip 21-C8S	NCGNSGC**S**GGAYSNGQCKRCC	
Crip 21-C17S	NCGNSGCCGGAYSNGQ**S**KRCC	
Crip 21-C20S	NCGNSGCCGGAYSNGQCKR**S**C	
Crip 21-C21S	NCGNSGCCGGAYSNGQCKRC**S**	
Crip 21-CC1SS	NCGNSG**SS**GGAYSNGQCKRCC	
Crip 21-CC2SS	NCGNSGCCGGAYSNGQCKR**SS**	
Crip 21_A3	NC**A**NSGCCGGAYSNGQCKRCC	
Crip 21_A4	NCG**A**SGCCGGAYSNGQCKRCC	
Crip 21_A5	NCGN**A**GCCGGAYSNGQCKRCC	
Crip 21_A6	NCGNS**A**CCGGAYSNGQCKRCC	
Crip 21_A9	NCGNSGCC**A**GAYSNGQCKRCC	
Crip 21_A10	NCGNSGCCG**A**AYSNGQCKRCC	
Crip 21_A12	NCGNSGCCGGA**A**SNGQCKRCC	
Crip 21_A13	NCGNSGCCGGAY**A**NGQCKRCC	
Crip 21_A14	NCGNSGCCGGAYS**A**GQCKRCC	
Crip 21_A15	NCGNSGCCGGAYSN**A**QCKRCC	
Crip 21_A16	NCGNSGCCGGAYSNG**A**CKRCC	
Crip 21_A18	NCGNSGCCGGAYSNGQC**A**RCC	
Crip 21_A19	NCGNSGCCGGAYSNGQCK**A**CC	

Supplementary Table 1. Activity of Crip21 peptides with substituted aa-residues; replacements of aa-residues are indicated; peptides were tested dose-dependent in ethylene bio assays using *CuRe1*-expressing *N. benthamiana* leaves (see supplemental data set s1); bars (right column) show the minimum effective concentrations; fully active peptides were similarly active as Crip21 at minimum effective concentrations of ≥0.1 nM; the peptides Crip21-C7S and CC1SS were both inactive at the tested maximum concentration (10,000 nM; bars in light grey). Asterisks indicate the Cysteine residues within Crip21 which are critical for full function in triggering CuRe1.

For raw data of ethylene bio-assay see also dataset s1 (.xlsx)

a



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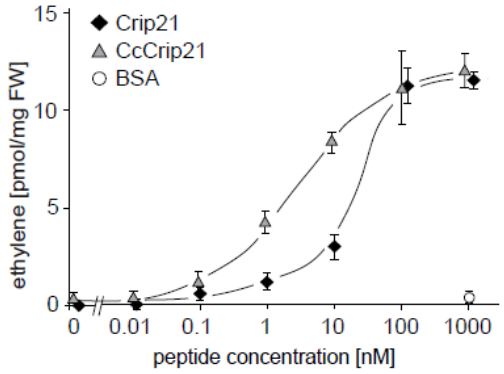
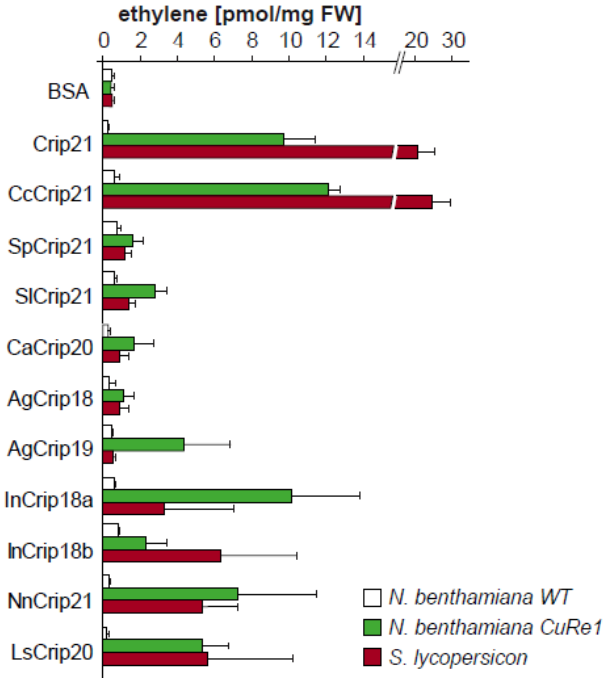
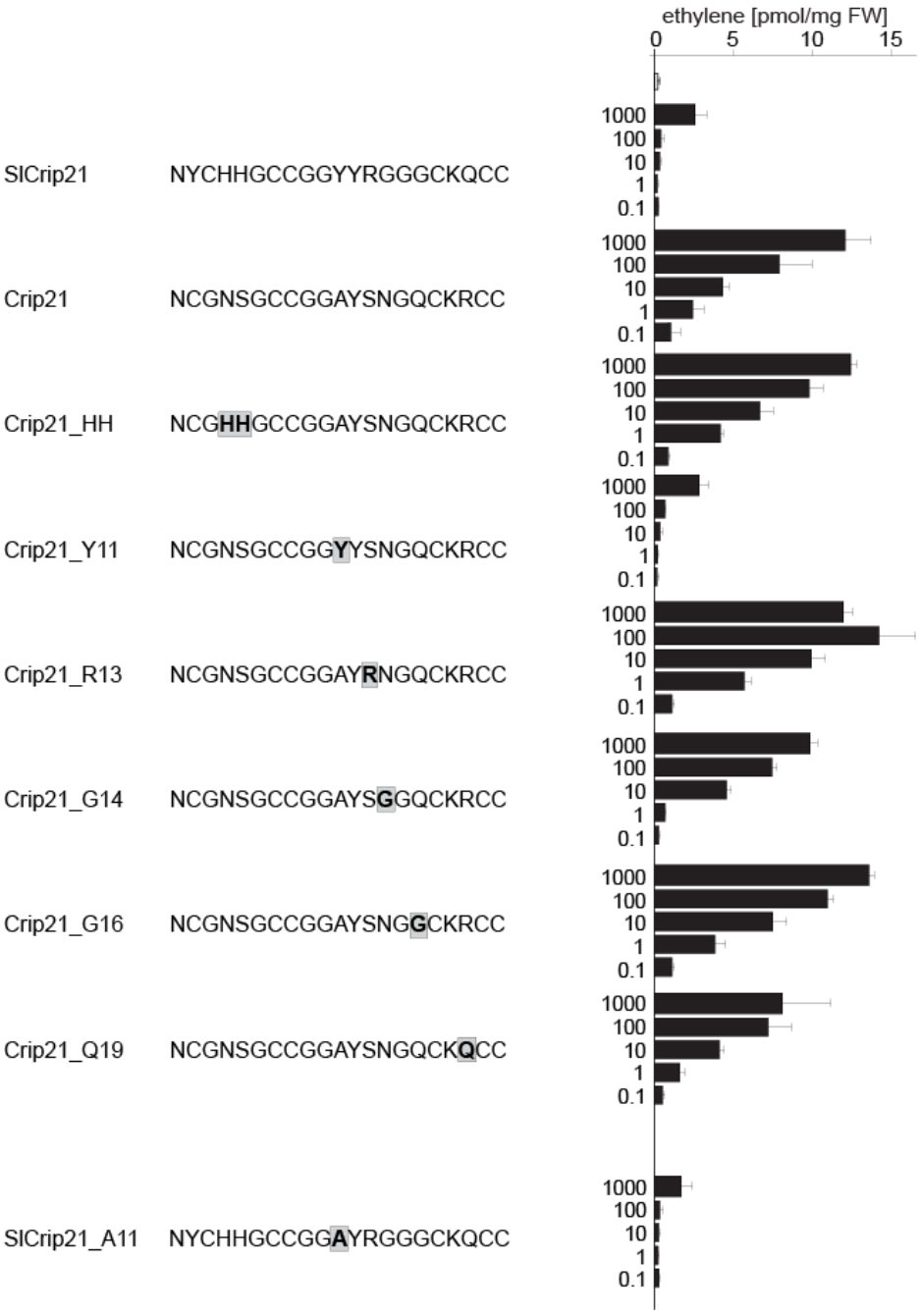


c

source organism	peptide sequence
Bovine serum albumine (control)	
Cuscuta reflexa	NCGNSGCCGGAYSNGQCKRCC
Cuscuta campestris	SCGNAGCCGGANSHGVCKWCC
Solanum pennellii	NYCRHGCCSGYYRGGGCKQCC
Solanum lycopersicum	NYCHHGCCGGYYRGGGCKCC
Cicer arietinum	YCGNGCCGGYYSGSCRRCC
Arabidopsis glauca	WCRHGCCYAGSNGCIRCC
Arabidopsis glauca	GCKYGCCGSYANGQCSACC
Ipomoea nil	GCHHGCCASYHGGCNRCC
Ipomoea nil	GCQYGCCGHGNYGCQRCC
Nelumbo nucifera	HCRYGCCGGGGYHGGGCRRCC
Lactuca sativa	YCRYGCCGGGRYYNGGCRCC



Supplementary Fig. 6. Activity of Crip peptides representing GRP sequences of other *Cuscuta* species and other plant species. (a) GRP protein sequences of *Cuscuta reflexa* (first lane), *Cuscuta campestris* (Cc0…) and *Cuscuta australis* (CaGRP; lowest line); Crip21 epitope highlighted; (b) Dose-dependent measurement of ethylene in CuRe1-expressing *N. benthamiana* leaves; the Crip21 peptides of *C. reflexa* and *C. campestris* (Crip21 sequence identical to *C. australis*) were applied in doses as indicated; (c) Crip peptides of other plant species (found by BLAST search) were tested for bioactivity in the ethylene bioassay; peptides applied at 5 µM each. (b) and (c)



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80 **Supplementary Fig. 7** Activity of Crip21 peptides with substituted aa-residues according to the
81 SlCrip21 sequence; replacements of aa-residues are indicated; peptides were tested dose-dependent
82 (0.1 to 1000 nM) in ethylene bio assays using *CuRe1*-expressing *N. benthamiana* leaves; The Crip21
83 with a Tyrosine residue at position 11 (Crip21_Y11) was only triggering a defense response at the
84 maximum concentration of 1000 nM and thus seems critical to abolish the peptide activity of
85 SlCrip21. SlCrip21_A11 shows, that the single replacement of tyrosine by alanine in SlCrip21 at
86 position 11, is not sufficient to restore the function as a defense trigger.

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