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TITLE:

Using *Ustilago maydis* as a Trojan Horse for *in situ* Delivery of Maize Proteins

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SUMMARY:

This work describes the cloning of an *Ustilago maydis* Trojan horse strain for the *in situ* delivery of secreted maize proteins into three different types of maize tissues.

ABSTRACT:

Inspired by Homer´s Trojan horse myth, we engineered the maize pathogen *Ustilago maydis* to deliver secreted proteins into the maize apoplast permitting *in vivo* phenotypic analysis. This method does not rely on maize transformation but exploits microbial genetics and secretory capabilities of pathogens. Herein, it allows inspection of *in vivo* delivered secreted proteins with high spatiotemporal resolution at different kinds of infection sites and tissues. The Trojan horse strategy can be utilized to transiently complement maize loss-of-function phenotypes, to functionally characterize protein domains, to analyze off-target protein effects, or to study onside protein overdosage, making it a powerful tool for protein studies in the maize crop system. This work contains a precise protocol how to generate a Trojan horse strain followed by standardized infection protocols to apply this method to three different maize tissue types.

INTRODUCTION:

The biotrophic pathogen *Ustilago maydis* is the causative agent of the corn smut disease[1]. It infects all aerial parts of maize resulting in large tumors that contain melanized, black spores. On the global level, *U. maydis* is estimated to cause an annual loss of around 2% of corn yield, while tumors are appreciated as a gastronomical delicacy in Mexico. Plant infection is initiated by an appressorium that secretes cell-wall lysing enzymes to penetrate the first layer of maize epidermal cells. From a primary infection site, *U. maydis* grows intracellularly and

intercellularly, invading one to two cell layers every day[1,2]. Successful infection results in plant hypertrophy that turns into visible tumors upon five days post infection[1,3,4]. During all infection stages, fungal hyphae invaginate the plant cytoplasm membrane without any direct contact to the host cytoplasm[1,2]. The tight apoplasmic space between the infecting hyphae and the plant plasma membrane is considered to be the host/pathogen interactive site, called the biotrophic interaction zone. In order to overcome the plant innate immune system, *U. maydis* secretes an array of effector proteins into the biotrophic interaction zone[1]. Some effectors are taken up by plant cells, while others remain in the biotrophic interaction zone[5-8]. One apoplastic effector is UmPit2, which interacts with apoplastic maize proteases to prevent the release of the signaling peptide ZmZIP1 from ZmPROZIP by apoplastic protease activity[9,10].

Over the last decades, *U. maydis* became not only a model for fungal genetics in plant-pathogen interaction, but also a valuable tool in biotechnology due to a well-understood life cycle, easy genetic accessibility and heterologous expression of secreted proteins[11-13]. Signals for both conventional and unconventional protein secretion have been determined allowing the control of posttranslational modifications[14]. Recently, *U. maydis* was employed as a Trojan horse tool to study small, secreted maize proteins *in situ*[15]. The Trojan horse approach was successfully used to analyze the function of the small, secreted protein ZmMAC1 that is involved in another development. ZmMAC1 induces the periclinal division of pluripotent cells and cell fate specification of the newly formed cells[15]. By the same method, the biological function of the maize damage-associated peptide ZmZIP1 was revealed. *U. maydis* secreting the maize ZmZIP1 resulted in impaired tumor formation[10]. Thus, the Trojan horse approach represents a valuable alternative route to protein *in situ* studies with high spatiotemporal resolution that does neither require generation of stable maize transformation lines nor tissue infiltration with heterologously expressed and purified proteins. In particular, the Trojan horse strategy enables the secretion of any heterologous protein into the maize apoplast and direct comparison of infected *versus* non-infected plant cells within the same tissue.

This protocol illustrates the major steps for generating an *U. maydis* Trojan horse strain to study a protein of interest. It further includes precise information on infection procedures of three different maize tissue types (adult leaves, tassels and ears) with *U. maydis*, which is a prerequisite for studying the spatiotemporal infection progression and protein function in these target tissues. No further specifications are given on maize gene amplification and microscopic imaging techniques, since these steps are target-specific and instrument-dependent. Thus, this protocol is addressed to experienced users of standard molecular biology techniques.

PROTOCOL:

1. Construction of an *U. maydis* Trojan Horse

NOTE: See **Figure 1**.

1.1. Amplify a gene of interest from maize cDNA using gene-specific primers and a proofreading DNA polymerase. Clone the primary PCR product and transform the construct into *E. coli*

following the plasmid vendor's instructions. Verify the correct gene of interest sequence by Sanger sequencing prior to use for the next cloning steps.

NOTE: PCR specifications need to be optimized due to primer sequence specificities and optimal DNA polymerase reaction conditions.

1.2. Design primers to amplify the maize gene of interest without the sequence encoding a signal peptide (SP).

1.3. Extend the 5´ end of the reverse primer with the RSIATA motif and an *Nco*I cutting site (**Table 1**).

1.4. Amplify the maize gene of interest with a proofreading DNA polymerase using the PCR construct generated in 1.1 as the PCR template.

1.5. Double-digest the PCR product and the *U. maydis* transformation plasmid, p123-P_{Um}*pit2*-*Sp*$_{Um}$*pit2*-Zm*mac1-mCherry-Ha* [15], with *Xba*I and *Nco*I. Purify the digested PCR product and plasmid.

1.6. Ligate the digested PCR product into the p123-P_{Um}*pit2*-*Sp*$_{Um}$*pit2*-Zm*mac1-mCherry-Ha* template using T4 DNA ligase following the manufacturer´s instructions. Transform the ligation product into *E. coli* and verify the correct gene of interest sequence by Sanger sequencing.

1.7. Linearize the p123-P_{Um}*pit2*-*Sp*$_{Um}$*pit2*-Zm*gene of interest-mCherry-Ha* with the restriction enzyme *Ssp*I and transform DNA into the solopathogenic *U. maydis* strain SG200[16]. Isolate *U. maydis* transformants by carboxin selection and confirm isolated transformants by Southern blot analysis[16].

NOTE: For each protein of interest, at least three independent *U. maydis* transformants should be isolated and analyzed to estimate any phenotype effects of random background mutations.

2. Culture Media

2.1. Prepare $YEPS_{light}$ liquid medium[16]: 1.0% (w/v) yeast extract, 0.4% (w/v) Bacto-Peptone, and 0.4% (w/v) sucrose. Dissolve all components in ddH_2O and autoclave at 121 °C for 15 min; autoclaving at a higher temperature, for a longer period of time or repeatedly would reduce the quality of the medium.

2.2. Prepare potato-dextrose-agar (PD-agar)[21]: 3.9% (w/v) potato dextrose agar, and 1.0% (w/v) 1 M Tris-HCl pH 8.0 (f.c. 0.01 M). Mix all components directly in the bottle for autoclaving and add ddH_2O plus a magnetic stir bar. Autoclave at 121 °C for 15 min; autoclaving at a higher temperature, for a longer period of time or repeatedly would reduce the quality of the medium.

2.3. Prepare PD-Charcoal agar[21]: 3.9% (w/v) potato dextrose agar, 1% (w/v) charcoal, and 1.0% (v/v) 1 M Tris-HCl pH 8.0 (f.c. 0.01 M). Mix all components directly in the bottle for autoclaving and add ddH_2O plus a magnetic stir bar. Autoclave at 121 °C for 15 min; autoclaving at a higher temperature, for a longer period of time or repeatedly would reduce the quality of the medium.

3. Plant Infection

3.1. Perform analysis of maize cell division in response to Trojan horse delivered protein (*e.g.,* microscopy-based cell counting), beforehand. *U. maydis* induced maize cell proliferation and subsequent tumor formation starts around 4-5 days post infection. Quantitative disease assessments are tissue dependent and should be performed from 6 to 14 days post infection.

NOTE: Distinct maize cultivars show different levels of susceptibility to *U. maydis* infection. Maize c*vs.* W23, A188, Gaspe flint, Early Golden Bantam or Va35 show susceptibility towards this pathogen and are thus suitable cultivars for Trojan horse studies.

3.2. Preparation of the *U. maydis* inoculum

3.2.1. Include the progenitor strain SG200 as a negative control in all Trojan horse experiments in order to estimate side effects on the infection by the transgenic strain. Here, use an *U. maydis* strain expressing a non-secreted version of the protein of interest as a negative control. However, for reasons of practicability (*e.g.,* larger screenings), the progenitor strain may be the easier choice of control.

3.2.2. Before starting the experiment, estimate what amounts of infection culture are needed. Keep in mind that infection of each plant requires 1 - 1.5 mL of *U. maydis* suspension dependent on the maize tissue type.

NOTE: Approximately 1 mL of an overnight culture is sufficient for dilution to an OD_{600} of 0.2 in 20 mL of $YEPS_{light}$ medium, and 25 mL of an *U. maydis* culture with an OD_{600} of 0.8-1.0 are sufficient for infection of 13-16 plants.

3.2.3. Scratch *U. maydis* from a PD agar plate using a sterile Pasteur pipette, inoculate in 5 mL of $YEPS_{light}$ medium and let the culture grow at 28 °C with constant shaking at 200 rpm for 16 h.

3.2.4. Prior to infection, examine the *U. maydis* inoculation culture by standard light microscopy for proper growth and bacterial contamination at 400X magnification.

NOTE: In a suitable culture, only the cigar-shaped fungus is visible (**Figure 2**).

3.2.5. Mix 900 µL of fresh $YEPS_{light}$ with 100 µL of the overnight culture and measure the OD_{600} using $YEPS_{light}$ medium as a blank in the spectrophotometer analysis.

3.2.6. Dilute the overnight culture with fresh $YEPS_{light}$ medium to an OD_{600} of 0.2 and let the culture grow at 28 °C with constant shaking at 200 rpm until reaching the mid-log growth phase indicated by an $OD_{600\ nm}$ of 0.8–1.0.

NOTE: *U. maydis* cells duplicate every 2 h under these conditions, thus the desired OD_{600} is reached after 4-5 h of cultivation.

3.2.7. Harvest the cells at OD_{600} of 0.8–1.0 by spinning at 3000 x *g* for 10 min and discard the supernatant.

3.2.8. Wash the cell pellet one time with ddH_2O. For this purpose, add one culture volume of ddH_2O, spin with 3000 x *g* for 10 min and discard the supernatant.

3.2.9. Resuspend the cell pellet carefully in ddH_2O using a 20-mL glass pipette, thereby adjusting the final OD_{600} to 3.0 (for a Trojan horse assay) or 1.0 (for disease rating).

3.3. Verification of the Trojan horse: *in planta* secretion of the maize fusion protein

3.3.1. Infect maize seedlings with a Trojan horse strain[18].

3.3.2. Perform microscopic imaging of infected seedlings at 2 – 3 days post infection using a confocal laser-scanning microscope. To this end, excise a rectangular piece of the leaf 1 cm below the point of injection, place the sample onto a microscope slide and add a drop of ddH_2O. To visualize mCherry fusion protein, excite specimen at λ= 561 nm and record emission at λ= 580 – 630 nm.

3.4. Infection of adult maize leaves

3.4.1. Cultivate maize plants to the stage of adult leaves (when at least leaf 7 grows within the stalk).

NOTE: This stage is reached after four weeks upon sowing under greenhouse conditions of 14 h, 28 °C day/10 h, 22 °C night rhythm using cv. W23. Duration may vary with the maize cultivar and greenhouse conditions.

3.4.2. Transfer the *U. maydis* culture (see 3.2.9) into a 3-mL syringe with a 20G x 1 hypodermic needle.

3.4.3. Press the stalk carefully to localize the meristem tissue in the stalk. The base of the meristem can be distinguished by a transition from harder stalk to softer tissue.

3.4.4. Mark the meristem on the stalk using a pen.

3.4.5. Inject 1.5 mL of *U. maydis* culture 1 cm above the shoot meristem or inflorescence meristem.

3.4.6. Rate disease symptoms at 6 and 12 days post infection.

3.5. Infection of tassels

3.5.1. Grow maize plants until reaching the tassel stage.

NOTE: A detailed timeline on anther and tassel development in maize cv. W23 was previously described[17,19,20]. Tassels containing pre-meiotic anthers are highly susceptible to *U. maydis* infection; in the maize cv. W23 tassels are the size of 4 - 7 cm.

3.5.2. Press the stalk carefully to localize the tassel in the stem.

3.5.3. Mark the tip and the base of the tassel on the stem using a pen.

3.5.4. Transfer the *U. maydis* culture (see 3.2.9) into a 3-mL syringe with a 20G x 1 hypodermic needle.

3.5.5. Inject 1.5 mL of the inoculum around the tassel. To ensure equal distribution of the inoculum, slowly place 0.5 mL each at the tip, the middle part, and the base of the tassel marked with the pen.

3.5.6. Rate disease symptoms at 10 days post infection.

3.6. Infection of ears

NOTE: The ear tissue development differs in distinct maize cultivars and greenhouse conditions and must be carefully observed prior to inoculation. Ears starting to outgrow silks are highly susceptible to *U. maydis* infection.

3.6.1. Transfer the *U. maydis* culture (see 3.2.9) into a 3-mL syringe with a 20G x 1 hypodermic needle.

3.6.2. Inject the inoculation needle into the space between the husk leaves as deeply as possible without injuring the ear.

3.6.3. Release 1.5 mL of the inoculum around the ear.

3.6.4. Remove the syringe plus needle and carefully massage the cob to distribute the *U. maydis* solution equally.

3.6.5. Rate disease symptoms at 14 days post infection.

3.7. Confirm viability of *U. maydis* inoculum

3.7.1. Drop 10 µL of the inoculum on a PD charcoal agar plate and incubate at room temperature for 2 days.

NOTE: If the respective *U. maydis* culture is able to form filaments, fluffy, white mycelium becomes visible (**Figure 5**).

REPRESENTATIVE RESULTS:

Constructs for *U. maydis* Trojan horse experiments are cloned into the plasmid p123-$P_{Um pit2}$-*Sp*$_{Um pit2}$-*gene of interest-mCherry-Ha*. The maize gene of interest is fused to a *mCherry* fluorescence reporter and an epitope *HA*-tag. The expression of the fusion protein is under control of the *U. maydis* Um*pit2* promoter which is specifically activated during infection[22]. To direct secretion of the protein of interest peptide into the biotrophic interaction zone, the coding region is fused to the signal peptide sequence of *U. maydis* Um*pit2* [22] (**Figure 1**). Upon *U. maydis* transformation, the transgene is inserted into the SG200 *ip*-locus by homologous recombination, and targeted genome insertion can be verified by Southern blot analysis. Secretion of the fusion protein is confirmed by confocal laser scanning microscopic imaging in seedling leaves infected with a Trojan horse strain (**Figure 3**). As an example, seedlings infected with either the *U. maydis* Trojan horse strain SG200Zm*mac1* secreting a ZmMAC1-mCHERRY or a non-Trojan horse control strain expressing noSP-Zm*mac1-mCHERRY* that lack the Um*pit2*-SP, and subsequently does not secret the ZmMAC1-mCHERRY-HA protein, are shown in **Figure 3**[15]. Hyphae secreting ZmMAC1 are surrounded by fluorescent mCherry signal (**Figure 3A**). In contrast, non-secreting hyphae only show fluorescence signal in the fungal cytoplasm (**Figure 3B**).

In particular, tassel infection with *U. maydis* relies on proper tissue inoculation, as described in step 3.5. Improper tassel localization or unequal distribution of the inoculum can result in non-infected tassel (**Figure 4A**) or only partial infection of the tassel (**Figure 4B**). To ensure even distribution, the inoculum needs to be slowly released from the inoculation needle to acquire entire tissue infection (**Figure 4C**). Viability of the inoculum can be verified by placing a droplet on PD-Charcoal agar. Infectious strains form filaments appearing as write fluff on the plate as shown for the solopathogenic Trojan Horse progenitor strain SG200 (**Figure 5A**) while the *U. maydis* strain FB1 requires mating before infectious filament formation (**Figure 5B**).

FIGURE LEGENDS:

Table 1: Sequences of primer additions to add restriction sides and the RSIATA motif coding sequence to the maize gene of interest.

Figure 1: Schematic overview of the *U. maydis* Trojan horse plasmid cloning strategy.

Zm*mac1* (light grey) is released by double digest from p123-$P_{Um pit2}$-*Sp*$_{Um pit2}$-Zm*mac1-mCherry-Ha*. In parallel, the gene of interest (yellow) is amplified by PCR. For cloning purpose, forward and reverse primers are designed which include *Xba*I and *Nco*I cloning sites and a RSIATA linker

(purple). The PCR product is digested with *Xba*I and *Nco*I. After ligation, the Trojan horse plasmid contains the following elements: Driven by the Um*pit2* promoter, an Um*pit2*-SP (blue) is fused N-terminally to a maize gene of interest ORF (yellow). At the C-terminus, a RSIATA linker, a *mCHERRY* reporter gene (red) and an *HA* epitope tag (green) are fused followed by a stop codon. Prior to *U. maydis* transformation, the Trojan horse plasmid is digested with *Ssp*I to allow homologous integration into the *U. maydis ip*-locus (grey).

Figure 2: Light-microscopic examination of *U. maydis* inoculation culture. Several cigar-shaped *U. maydis* sporidia are visible, some of which undergo budding (indicated by asterisks). No further cells are present which would indicate a contamination of the culture. Scale bar represents 20 µm.

Figure 3: *In planta* confocal laser scanning microscopic imaging of an *Ustilago* Trojan horse strain. Imaging of the mCherry-fused maize protein ZmMAC1 after maize seedling infection using the Trojan horse strain SG200Zm*mac1* **(A)** or a non-Trojan horse strain SG200Zm*mac1*-noSP lacking the *Umpit2*-SP **(B)**. Secreted ZmMAC1-mCHERRY-HA fusion protein is located on the surface of *U. maydis* hyphae **(A)** [15], indicated by the arrow heads. In SG200Zm*mac1*-noSP only cytoplasmic localization of ZmMAC1 is visible **(B)**, indicated by the asterisk [15]. Scale bars represent 5 µm.

Figure 4: Tassel infection with *U. maydis*. Unsuccessful infection of tassel with *U. maydis* (A), partial tassel infection (B) and complete tassel infection (C) 12 days after infection are shown.

Figure 5: Inoculum viability assay on PD-Charcoal agar. The solopathogenic strain SG200[1] was used for Trojan horse generation. SG200 is self-stimulating and forms infectious filaments on a PD-Charcoal agar plate **(A)**. The haploid *U. maydis* strain FB1 requires mating with a compatible strain prior to filamentous grow **(B)**[23]. Scale bars represent 2 mm.

DISCUSSION:

Modern crop research demands protocols for molecular analysis on genetic and protein levels. Genetic accessibility *via* transformation is not available or inefficient and time-consuming for most crop species such as maize. Moreover, reliable genetic tools such as promoter reporter systems are scarce, which makes it difficult to study *in situ* protein function with high spatiotemporal resolution at distinct tissue sites. Apoplastic proteins can be studied by infiltration of heterologously expressed and purified proteins into tissues. However, despite advances in heterologous protein expression, targeted infiltration into crop tissues remains difficult and often inefficient for protein functional analysis. The Trojan horse strategy is an alternative approach that does not require transformation or protein infiltration. By employing the secretion apparatus of the maize pathogen *U. maydis*, delivery of theoretically any protein of interest into the plant apoplast of infected tissue can be achieved. Regarding the size of a protein of interest, the limits of this technique have yet to be explored. In former assays, the 290 amino acids *U. maydis* effector Cmu1 fused to mCherry was successfully secreted[7]. However, the applicability of the Trojan horse method to bigger proteins remains to be tested.

If additions of posttranslational modifications to the protein of interest are undesirable, unconventional secretion may be used as an alternative route to classical secretion *via* SP[14].

U. maydis is employed as a standard tool in protein biotechnology because of reliable protein folding, posttranslational modification, and secretion efficiencies[11-13]. Nevertheless, protein secretion for each new Trojan horse strain needs to be carefully analyzed as described in the Step 3. It is recommended to perform an initial testing of every newly generated Trojan horse strain by infecting seedlings that are easy to infect and to inspect by microscopic imaging.

SG200 is a solopathogenic strain that does not require mating prior to the Trojan horse experiments and is thus easier to handle. Although the infection efficiency of compatible strains like FB1 and FB2 is higher[24], the efficiency of SG200 is sufficient for Trojan horse experiments. Some *U. maydis* effector proteins are taken up by host cells, while others remain in the apoplast. The differentiation between both groups seems to be a controlled and specific process; however, the underlying mechanisms remain elusive[8] and cannot be taken into account when designing an experiment. Therefore, proteins of interest that have to be integrated into the cell wall or that have to act intracellularly are no suitable candidates for the Trojan horse approach.

Since *U. maydis* is omnipotent in infecting diverse aerial maize tissues, the Trojan horse method can be applied for multiple proteins, in distinct tissues and at different plant developmental stages, such as seedling leaves, adult leaves, tassels, and ears. To name just a few useful applications, the Trojan horse allows testing local overdosage, offside protein effects, or functional characterization of distinct protein domains.

Maintaining an uncontaminated *U. maydis* culture is crucial for all described experiments since co-infection with a large amount of bacteria triggers the plant's immune response and alters its reaction towards the protein of interest, thus rendering any results inconclusive. Trojan horse studies in adult leaves, tassels and ears have to be performed with maximally 1.5 mL infection culture as higher volumes may result in tissue damage. Tassel and ear infections can be trained using food color-stained water instead of *Ustilago* inoculum.

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DISCLOSURES:
The authors have nothing to disclose.

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Figure 1

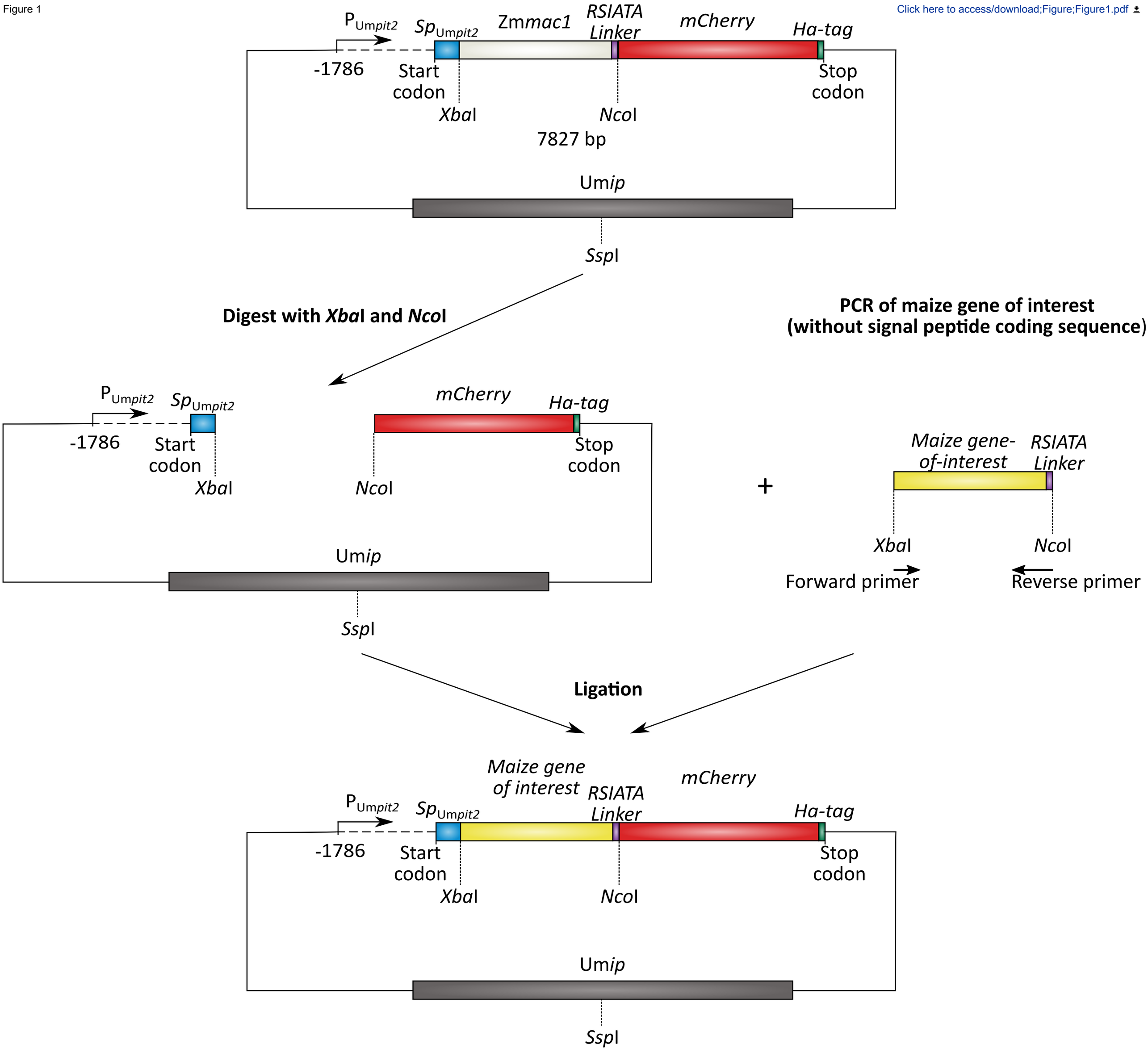

Figure 2

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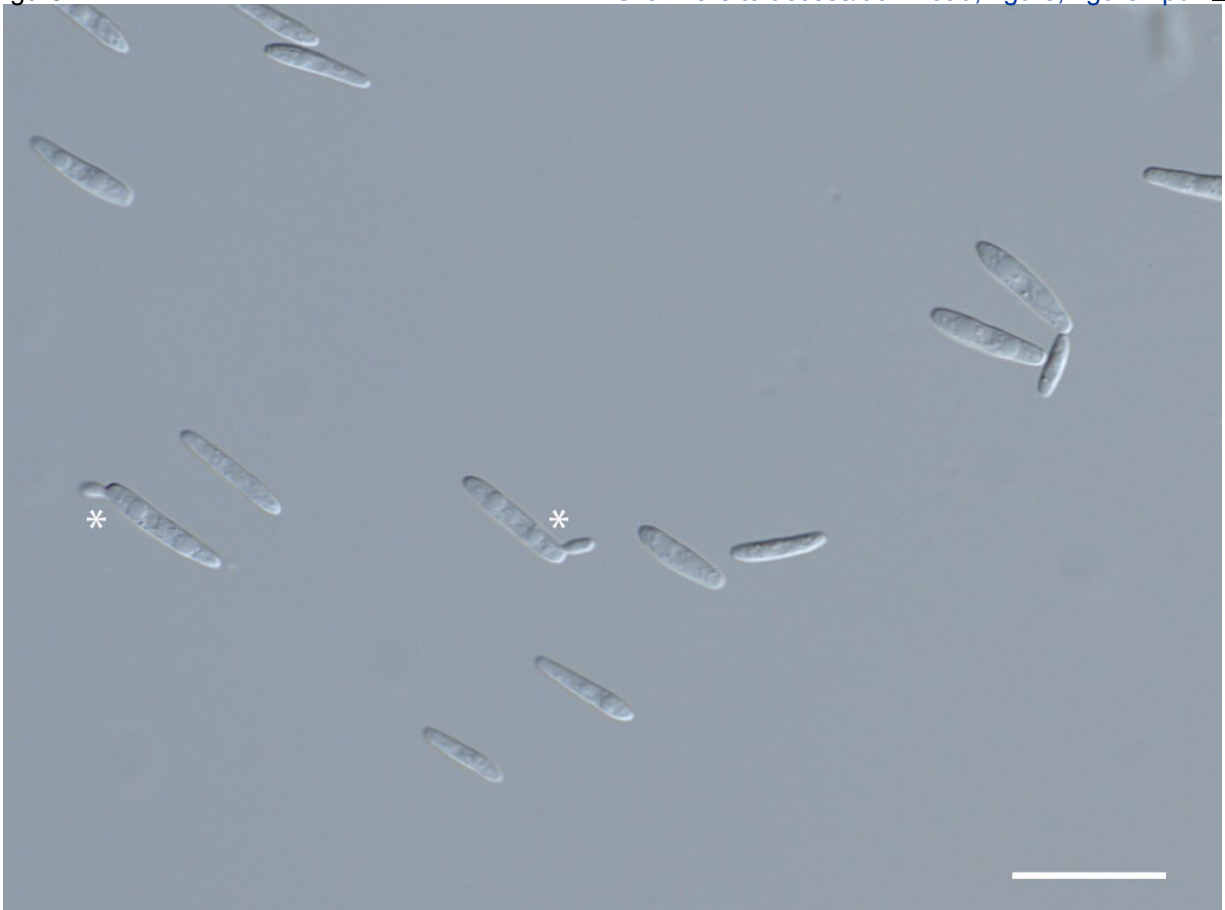
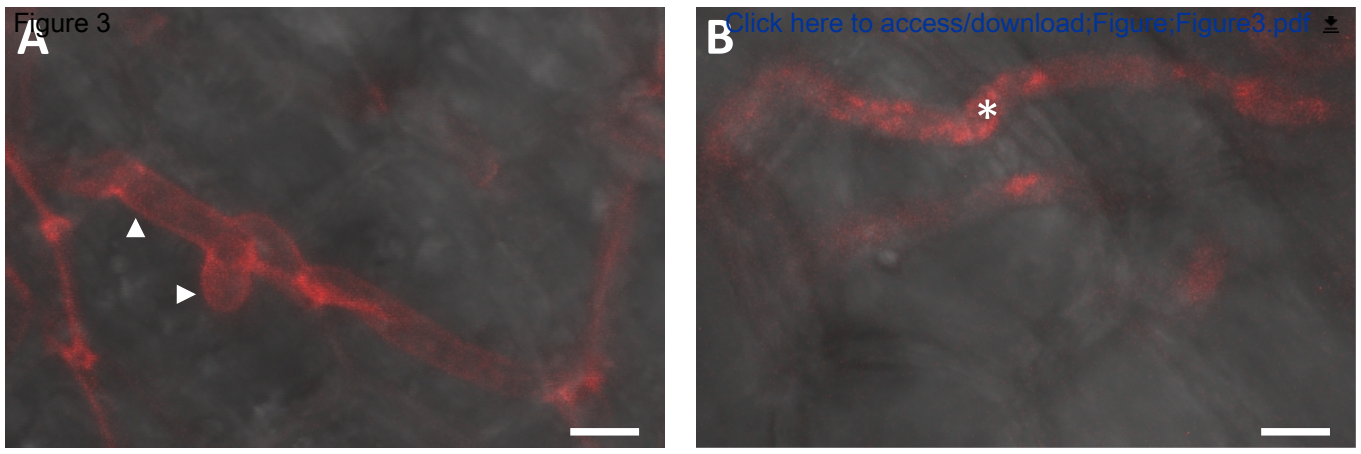


Figure 3



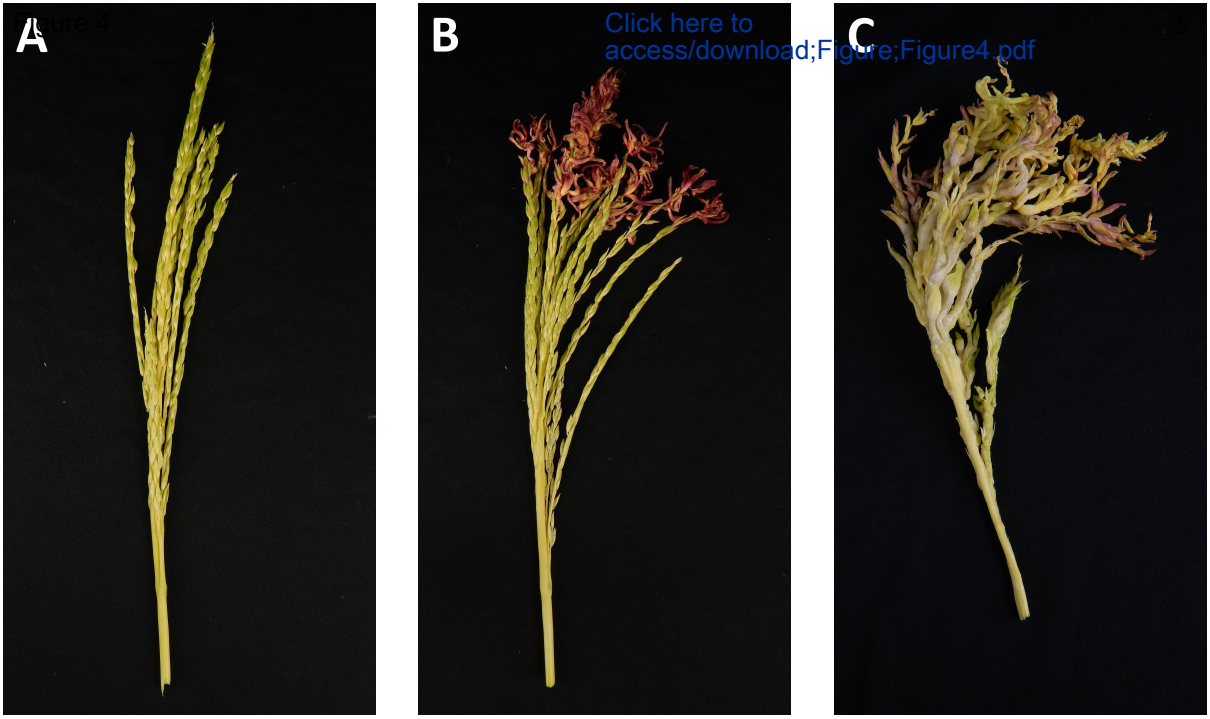
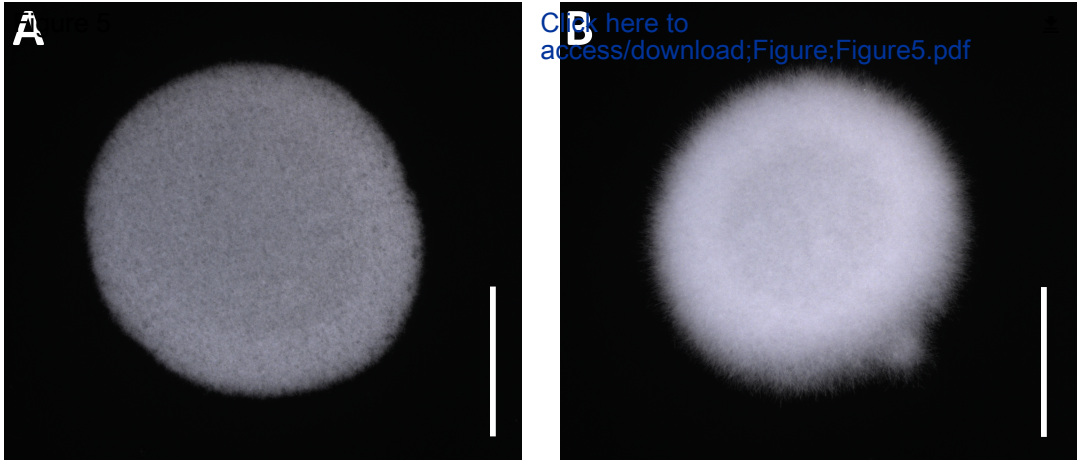


Figure 5



Click here to access/download;Figure;Figure5.pdf

Table1: Sequences of primer additions to add restriction sides and the RSIATA motif coding sequence to the maize gene of interest.

Name	Primer addition	Sequence (5´→3´)
Forward	*Xba* I-maize gene	GCTCTAGA…
Reverse	*Nco* I-RSIATA-maize gene	CATGCCATGGAGGCGGTGGCGATCGAGCG….

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
2 mL syringe	B. Braun	4606027V	
23G x 1 1/4 hypodermic needle	B. Braun	4657640	
Bacto Peptone	BD	211677	
cDNA from maize			from maize tissue expressing the gene of interrest
Charcoal	Sigma-Aldrich	05105	
Confocal laser scanning microscope			use locally available equipment
Cuvette (10 x 4 x 45 mm)	Sarstedt	67742	
Incubator-shaker set to 28 °C, 200 rpm			use locally available equipment
Light microscope with 400-fold magnification			use locally available equipment
Nco I	NEB	R0193	
p123-$P_{Um pit2}$ -*Sp* $_{Um pit2}$ -Zm*mac1-mCherry -Ha*			please contact the corresponding author
Pasteur pipet (glass, long tip)	VWR	14673-043	
pCR-Blunt-II-TOPO	Thermo Fisher Scientific	K280002	can be exchanged for other basic cloning vectors like pENTR or pJET
Potato Dextrose Agar	VWR	90000-745	
Sharpie pen			use locally available equipment
Spectrophotometer			use locally available equipment
Ssp I	NEB	R0132	
Sucrose	Sigma-Aldrich	S0389	
T4 DNA ligase	NEB	M0202	
TRIS	Sigma-Aldrich	TRIS-RO	
Xba I	NEB	R0145	
Yeast extract	BD	212750	

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Author(s):	Isabell-C. Fiedler, Arne Weiberg, Karina van der Linde

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Regensburg, 09.09.2018

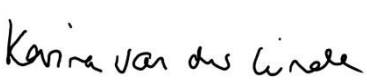
Dear Dr. Steindel,

We are pleased to resubmit the manuscript “Guidelines for using *Ustilago maydis* as a Trojan horse for in situ delivery of maize proteins” and would like to thank the editors and reviewers for their time and helpful comments, which allowed us to improve the manuscript quality.

We revised our manuscript according to the editorial instructions (e.g., adding “Notes” and splitting some of the steps) and added further information on issues which seem to have been unclear before and which were kindly highlighted by you and the reviewers. This involves adding more detail to the protocol (e.g., regarding the preparation of *U. maydis* inoculum or recommended time frames for the respective assays) as well as including more information in the discussion as was requested by reviewers 2 and 3 (e.g. regarding the size limit of candidate proteins, possible uptake of the protein by the plant cell, or use of crossed, compatible *U. maydis* strains).

Please find a detailed response to all editor and reviewer comments on the next pages. Major changes in figures, supplemental figures, and the manuscript are highlighted in red.

Best regards,



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1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Author response:
We performed a final proofreading of the manuscript and removed all remaining issues.

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Author response:
We did not reuse any figures from previous publications for this manuscript.

3. Please use SI abbreviations for all units: L, mL, µL, h, min, s, etc.

Author response:
We made corrections throughout the manuscript.

4. Please include a space between all numbers and their corresponding units: 15 mL, 37 °C, 60 s; etc.

Author response:
We changed the manuscript accordingly.

5. Please use centrifugal force (x g) for centrifuge speeds.

Author response:
We changed this throughout the manuscript.

6. Please revise the protocol text to avoid the use of any personal pronouns (e.g., “we”, “you”, our” etc.).

Author response:

We changed the manuscript accordingly.

7. Please revise the protocol to contain only action items that direct the reader to do something (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol.

Author response:

Regarding the phrase “should be”, we altered the manuscript.

8. Any text that cannot be written in the imperative tense may be added as a “Note.” Please include all safety procedures and use of hoods, etc. However, notes should be used sparingly and actions should be described in the imperative tense wherever possible.

Author response:

We added notes at multiple steps throughout the manuscript.

9. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please simplify the Protocol so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step. Use sub-steps as necessary. Please move the discussion about the protocol to the Discussion.

Author response:

We altered the manuscript accordingly in steps 1.1, 1.4, and 1.5.

10. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. Some examples:2.3.2: Please add more details here about how to investigate the culture. What is observed?What type of water is used, deionized/distilled? What volume of water is used to wash? Please specify throughout.2.3.7: What volume of water is used to resuspend the cells?

Author response:

Regarding step 2.3.2 (now step 2.3.4), please see the newly added Figure 2 and line 145.

Regarding the type of water, we replaced H_2O by ddH_2O.

Regarding step 2.3.7/2.3.8: please see new steps 2.3.8 and 2.3.9.

Moreover, further details were added to the protocol for steps 2.3.1, 2.3.3 (formerly part of 2.3.1), 2.3.4 (formerly 2.3.2), 2.3.8 (formerly 2.3.6), 2.3.9 (formerly 2.3.7), 2.4.2, 2.5.3 and 2.8.1. Steps 2.3.2, 2.5.6, 2.6.6 and 2.7.6 were newly added (see also response to reviewer #2, point 5).

11. Please reference Table 1 in the manuscript.

Author response:

Please see line 95.

12. Discussion: Please discuss critical steps within the protocol and any limitations of the technique.

Author response:

For discussion of critical steps within the protocol, please see lines 358-363 and 368-373.

Possible limitations are addressed in lines 339-345 and 356-368.

13. For in-text references, the corresponding reference numbers should appear as superscripts after the appropriate statement(s) in the text (before punctuation but after closed parenthesis). The references should be numbered in order of appearance.

Author response:

We changed references throughout the manuscript.

14. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage (YEAR).] For more than 6 authors, list only the first author then et al.

Author response:

We changed the references to the above-mentioned style.

15. References: Please do not abbreviate journal titles. Please include volume and issue numbers for all references.

Author response:
We include full journal titles, volumes, and issue numbers in all references.

Reviewer #1:

1. I wonder if the method can be used for the introduction of genes from an origin distinct to plants.

Author response:
As mentioned in line 71 and line 345-350 of the manuscript, theoretically any protein of interest can be
secreted into the apoplast, thus including non-plant proteins.

Reviewer #2:

Major concerns:

1. Determining protein function(s) in situ is critical to advancing our understanding of the roles they play. But this method seems tailored to the analysis of small, secreted maize proteins that exert their function from the apoplast on a cellular process that must be measured with a microscope. Can the Trojan horse strategy be used to study the function of other types of proteins like an enzyme, transcription factor, signaling molecule, receptor or structural protein? If so, please give examples where this as worked.

Author response:
We now added some information on this issue within the discussion (lines 340-350); see also Editorial comments #11 and Reviewer #1.

2. The abstract states the strategy can be used to transiently complement a loss-of-function phenotype, characterize the function of protein domains, and study off-target or over-accumulation

effects. This was done quite elegantly for the ZmMAC1 protein but have the authors shown this will work for any other protein, even another small secreted peptide (e.g., any of the CLE-related peptides)?

Author response:
As mentioned in line 65-67, the method was also successfully applied to the investigation of the maize peptide ZmZIP1.

3. Do the secreted proteins remain in the apoplast where they exert their effects at the plasma membrane? Or are they taken up by the adjacent cells where they might also function in the cytoplasm or nucleus? This might be mentioned as it would be important for choosing what type of protein to use with this strategy.

Author response:
We added information about this to the discussion (lines 344–366).

Minor concerns:

1. Is there a size limit or recommended maximum on the size of the protein that can be expressed and secreted (especially since it is fused to mCherry already)?

Author response:
We now added information on this issue in lines 345-350.

2. Smut tumors appear within 7, 10 or 12 days depending on tissue and maize cultivar used. I assume tumor formation might affect the analysis of the protein being studied and so is there a recommended time frame for when protein effects should be assessed (e.g., between 1 - 3 days after inoculation but not past 5 days)?

Author response:
We added Note 2.1, which gives instructions on the proper timing for different experiments.

3. In line 113, three independent U. maydis transformants should be used to assess protein function. Does this mean, each independent transformant should be used in replicated inoculations? Or can the transformants bulked prior to inoculation? Or should this be avoided?

Author response:
A more detailed explanation towards this issue is now added to this step (lines 110-112). We thank the reviewer for highlighting any misleading phrasings.

4. For the negative control, you suggest using the untransformed SG200 strain but also you suggest using a "no signal peptide" version of the studied protein. Are both needed? It seems, the "not secreted" version of the studied protein would be the best negative control. If there is another compelling reason to also use the untransformed SG200 strain, please state why.

Author response:
We now added further information on this issue within the protocol (see 2.3.1).

5. The two tables are split on multiple pages making them difficult to read.

Author response:
We thank the reviewer for its thorough revision. Both tables have been changed to fit on one page.

6. Some of the grammar and word choices are not correct. Copy editing would help. For example:Line 32 "offside"….. "onside". Perhaps "off-target" and "target (on-target?)" are better.Line 85 The Trojan horse strategy enables "the secretion of" any heterologous…..There are others too.

Author response:
Regarding general grammar/word choices, please view our response to the Editorial comments (#1). We applied the reviewer's suggestion for "off-target" instead of "offside" (line 32).

Reviewer #3:

1. Therefore, the authors need to describe in the introduction the following papers:The Corn Smut ('Huitlacoche') as a New Platform for Oral Vaccines. PLoS ONE 10(7): e0133535. https://doi.org/10.1371/journal.pone.0133535; and The corn smut-made cholera oral vaccine is thermostable and induces long-lasting immunity in mouse. Journal of Biotechnology (2016), Pages 1-6. https://doi.org/10.1016/j.jbiotec.2016.04.047; in this papers the production of an oral vaccine using the Ustilago maydis expression system has been successfully achieved.

Author response:

We thank the reviewer for the suggestion and added the respective literature to our citations in the introduction as well as in the discussion (see lines 56 and 348).

2. Another important point is that the authors propose to use the strain SG200, this strain being only pathogenic would have the advantage of not requiring the crossing of 2 compatible strains; however, in my experience the infection efficiency is low with the SG200 strain, compared to using compatible strains such as FB1 and FB2, so the authors could mention the possibility of using compatible strains in their system.

Author response:

We now addressed this issue in the discussion (see line 358-366).