**TITLE:**

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

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**KEYWORDS:**

*Ustilago maydis*, Trojan Horse, Protein Delivery, *Zea mays*, Tassel, Anther, Ear, Cob, Silk, Adult Leaf

**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 disease1. 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 day1,2. Successful infection results in plant hypertrophy that turns into visible tumors upon five days post infection1,3,4. During all infection stages, fungal hyphae invaginate the plant cytoplasm membrane without any direct contact to the host cytoplasm1,2. 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 zone1. Some effectors are taken up by plant cells, while others remain in the biotrophic interaction zone5-8. 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 activity9,10.

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 proteins11-13. Signals for both conventional and unconventional protein secretion have been determined allowing the control of posttranslational modifications14. 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 cells15. 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 formation10. 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. 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. Design primers to amplify the maize gene of interest without the sequence encoding a signal peptide (SP).
  2. Extend the 5´ end of the reverse primer with the RSIATA motif and an *Nco*I cutting site   
     (**Table 1**).
  3. Amplify the maize gene of interest with a proofreading DNA polymerase using the PCR construct generated in 1.1 as the PCR template.
  4. Double-digest the PCR product and the *U. maydis* transformation plasmid, p123-PUm*pit2*-*Sp*Um*pit2*-Zm*mac1-mCherry*-*Ha* 15, with *Xba*I and *Nco*I. Purify the digested PCR product and plasmid.
  5. Ligate the digested PCR product into the p123-PUm*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.
  6. Linearize the p123-PUm*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 SG20016. Isolate *U. maydis* transformants by carboxin selection and confirm isolated transformants by Southern blot analysis16.

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.

1. **Culture Media**
   1. Prepare YEPSlight liquid medium16: 1.0% (w/v) yeast extract, 0.4% (w/v) Bacto-Peptone, and 0.4% (w/v) sucrose. Dissolve all components in ddH2O 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. 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 ddH2O 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. Prepare PD-Charcoal agar21: 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 ddH2O 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. **Plant Infection**
   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.

* 1. Preparation of the *U. maydis* inoculum
     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.
     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 OD600 of 0.2 in 20 mL of YEPSlight medium, and 25 mL of an *U. maydis* culture with an OD600 of 0.8-1.0 are sufficient for infection of 13-16 plants.

* + 1. Scratch *U. maydis* from a PD agar plate using a sterile Pasteur pipette, inoculate in 5 mL of YEPSlight medium and let the culture grow at 28 °C with constant shaking at 200 rpm for 16 h.
    2. 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**).

* + 1. Mix 900 µL of fresh YEPSlight with 100 µL of the overnight culture and measure the OD600 using YEPSlight medium as a blank in the spectrophotometer analysis.
    2. Dilute the overnight culture with fresh YEPSlight medium to an OD600 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 OD600 nm of 0.8–1.0.

NOTE: *U. maydis* cells duplicate every 2 h under these conditions, thus the desired OD600 is reached after 4-5 h of cultivation.

* + 1. Harvest the cells at OD600 of 0.8–1.0 by spinning at 3000 x *g* for 10 min and discard the supernatant.
    2. Wash the cell pellet one time with ddH2O. For this purpose, add one culture volume of ddH2O, spin with 3000 x *g* for 10 min and discard the supernatant.
    3. Resuspend the cell pellet carefully in ddH2O using a 20-mL glass pipette, thereby adjusting the final OD600 to 3.0 (for a Trojan horse assay) or 1.0 (for disease rating).
  1. Verification of the Trojan horse: *in planta* secretion of the maize fusion protein
     1. Infect maize seedlings with a Trojan horse strain18.
     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 ddH2O. To visualize mCherry fusion protein, excite specimen at λ= 561 nm and record emission at λ= 580 – 630 nm.
  2. Infection of adult maize leaves
     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.

* + 1. Transfer the *U. maydis* culture (see 3.2.9) into a 3-mL syringe with a 20G x 1 hypodermic needle.
    2. 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. Mark the meristem on the stalk using a pen.
    4. Inject 1.5 mL of *U. maydis* culture 1 cm above the shoot meristem or inflorescence meristem.
    5. Rate disease symptoms at 6 and 12 days post infection.
  1. Infection of tassels
     1. Grow maize plants until reaching the tassel stage.

NOTE: A detailed timeline on anther and tassel development in maize cv. W23 was previously described17,19,20. 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.

* + 1. Press the stalk carefully to localize the tassel in the stem.
    2. Mark the tip and the base of the tassel on the stem using a pen.
    3. Transfer the *U. maydis* culture (see 3.2.9) into a 3-mL syringe with a 20G x 1 hypodermic needle.
    4. 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.
    5. Rate disease symptoms at 10 days post infection.
  1. 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.

* + 1. Transfer the *U. maydis* culture (see 3.2.9) into a 3-mL syringe with a 20G x 1 hypodermic needle.
    2. Inject the inoculation needle into the space between the husk leaves as deeply as possible without injuring the ear.
    3. Release 1.5 mL of the inoculum around the ear.
    4. Remove the syringe plus needle and carefully massage the cob to distribute the *U. maydis* solution equally.
    5. Rate disease symptoms at 14 days post infection.
  1. Confirm viability of *U. maydis* inoculum
     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-PUm*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 infection22. 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-PUm*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 SG2001 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 secreted7. 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* SP14.

*U. maydis* is employed as a standard tool in protein biotechnology because of reliable protein folding, posttranslational modification, and secretion efficiencies11-13. 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 higher24, 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 elusive8 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.

**REFERENCES:**

1 Kämper, J. *et al.* Insights from the genome of the biotrophic fungal plant pathogen *Ustilago maydis*. *Nature* **444**, 97-101 (2006).

2 Doehlemann, G. *et al.* Establishment of compatibility in the *Ustilago maydis*/maize pathosystem. *Journal of Plant Physiology* **165**, 29-40 (2008).

3 Matei, A. *et al.* How to make a tumour: cell type specific dissection of *Ustilago maydis*-induced tumour development in maize leaves. *New Phytologist* (2018).

4 Doehlemann, G. *et al.* Reprogramming a maize plant: transcriptional and metabolic changes induced by the fungal biotroph *Ustilago maydis*. *The Plant Journal* **56**, 181-195 (2008).

5 Doehlemann, G. *et al.* Pep1, a secreted effector protein of *Ustilago maydis*, is required for successful invasion of plant cells. PLOS Pathogens **5,** e1000290 (2009).

6 Redkar, A. *et al.* A secreted effector protein of *Ustilago maydis* guides maize leaf cells to form tumors. *The Plant Cell* **27**, 1332-1351 (2015).

7 Djamei, A. *et al.* Metabolic priming by a secreted fungal effector. *Nature* **478**, 395-398 (2011).

8 Tanaka, S. *et al.* A secreted *Ustilago maydis* effector promotes virulence by targeting anthocyanin biosynthesis in maize. *eLife* **3**, e01355 (2014).

9 Mueller, A. N., Ziemann, S., Treitschke, S., Assmann, D., Doehlemann, G. Compatibility in the *Ustilago maydis*-maize interaction requires inhibition of host cysteine proteases by the fungal effector Pit2. *PLOS Pathogens* **9**, e1003177 (2013).

10 Ziemann, S. *et al.* An apoplastic peptide activates salicylic acid signalling in maize. *Nature* *Plants* 4, 172-180 (2018).

11 Juárez-Montiel, M. *et al.* The corn smut (‘Huitlacoche’) as a new platform for oral vaccines. *PLoS One* **10**, e0133535 (2015).

12 Sarkari, P., Feldbrügge, M., Schipper, K. in *Gene Expression Systems in Fungi: Advancements and Applications*(eds Monika Schmoll & Christoph Dattenböck) 183-200 (Springer International Publishing, 2016).

13 Monreal-Escalante, E. *et al.* The corn smut-made cholera oral vaccine is thermostable and induces long-lasting immunity in mouse. *Journal of Biotechnology* **234**, 1-6 (2016).

14 Stock, J. *et al.* Applying unconventional secretion of the endochitinase Cts1 to export heterologous proteins in *Ustilago maydis*. *Journal of Biotechnology* **161**, 80-91 (2012).

15 van der Linde, K. *et al.* Pathogen Trojan horse delivers bioactive host protein to alter maize (*Zea mays*) anther cell behavior *in situ*. *The Plant Cell* **30**, 528-542 (2018).

16 Bösch, K. *et al.* Genetic manipulation of the plant pathogen *Ustilago maydis* to study fungal biology and plant microbe interactions. *Journal of Visualized Experiments*, e54522 (2016).

17

18 Chavan, S. ,Smith, S. M. A rapid and efficient method for assessing pathogenicity of *Ustilago maydis* on maize and teosinte lines. *Journal of Visualized Experiments*, e50712 (2014).

19 Kelliher, T., Walbot, V. Emergence and patterning of the five cell types of the *Zea mays* anther locule. *Developmental* *Biology* **350**, 32-49 (2011).

20 Egger, R. L., Walbot, V. Quantifying *Zea mays* tassel development and correlation with anther developmental stages as a guide for experimental studies. Maydica **60**, M34 (2015).

21 Holliday, R. in *Bacteria, Bacteriophages, and Fungi: Volume 1* (ed Robert C. King) 575-595 (Springer US, 1974).

22 Doehlemann, G., Reissmann, S., Aßmann, D., Fleckenstein, M., Kahmann, R. Two linked genes encoding a secreted effector and a membrane protein are essential for *Ustilago maydis*-induced tumour formation. *Molecular* *Microbiology* **81**, 751-766 (2011).

23 Banuett, F.,Herskowitz, I. Different *a* alleles of *Ustilago maydis* are necessary for maintenance of filamentous growth but not for meiosis. *Proceedings of the National Academy of Sciences* **86**, 5878-5882 (1989).

24 Bortfeld, M., Auffarth, K., Kahmann, R., Basse, C. W. The *Ustilago maydis a2* mating-type locus genes *lga2* and *rga2* compromise pathogenicity in the absence of the mitochondrial p32 family protein Mrb1. *The Plant Cell* **16**, 2233-2248 (2004).