Functional Characterisation of Effectors from Biotrophic and Necrotrophic Wheat Pathogens in Heterologous Expression Systems

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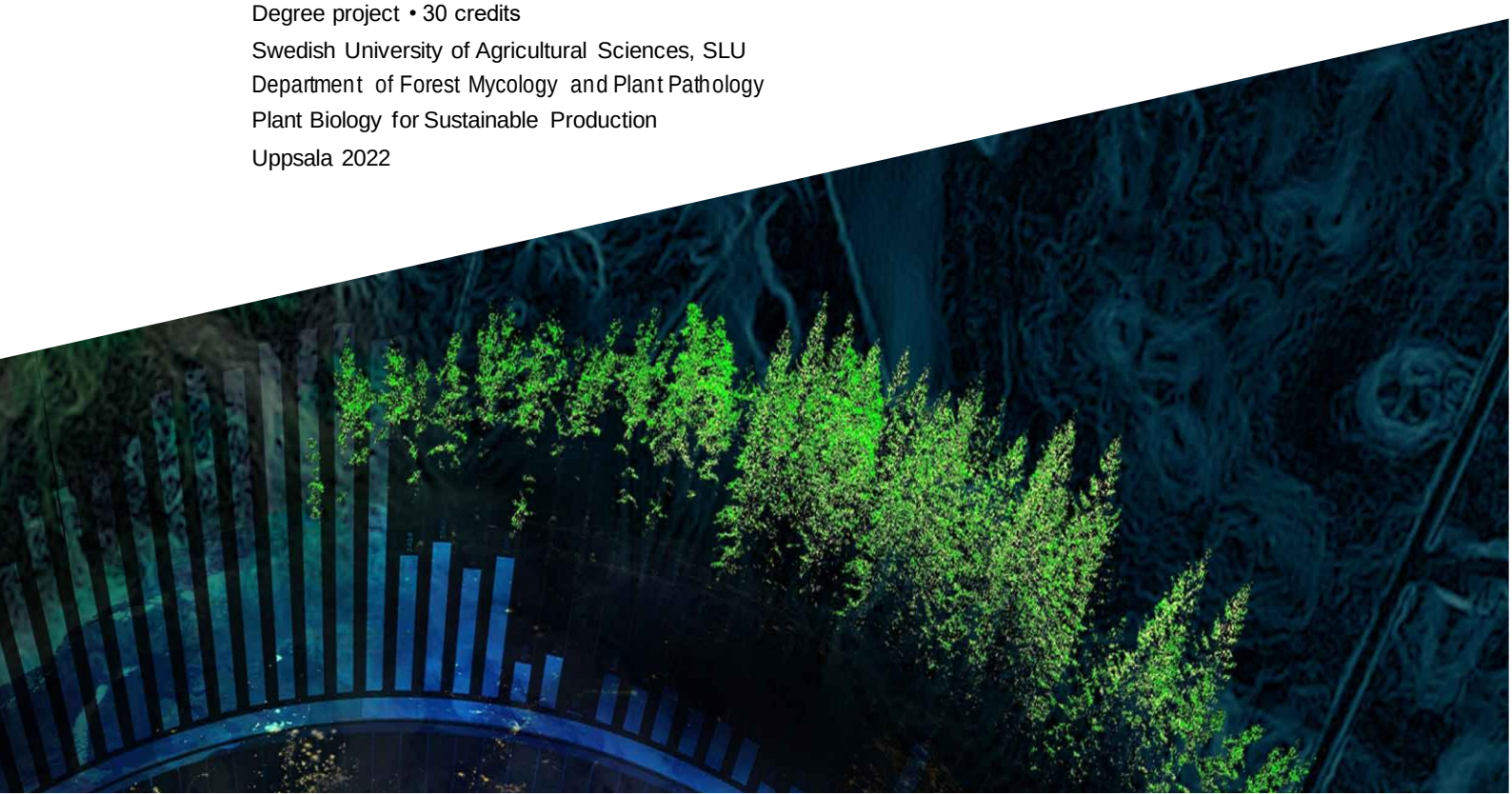

Functional Characterisation of Effectors from Biotrophic and Necrotrophic Wheat Pathogens in Heterologous Expression Systems

Funktionell bestämning av biotrofiska och nekrotrofiska vetepatogeners effektorer i heterologa expressionssystem

Mark Melchior

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Keywords: wheat, *Blumeria graminis*, *Parastagonospora nodorum*, effectors

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Abstract

Effector proteins play an important role in the interactions between fungal pathogens and their host plant. Recognition of effectors can lead to localised cell death in plants. The necrotrophic fungus *Parastagonospora nodorum* utilises this mechanism to induce cell death in wheat and cause Stagonospora nodorum blotch (SNB). The best described protein in this interaction is ToxA, but seven other proteinaceous toxins have been described as well. The gene in wheat that is necessary for the recognition of ToxA is *Tsn1* and is a dominant gene. In this study we tested the susceptibility for SNB in parental lines and commercial wheat varieties. One of the cultivars used in breeding programs was found to be susceptible for disease, but with very weak symptoms. Another part of this study focused on the proteins of the E024 family that are highly expressed by the wheat pathogen *Blumeria graminis* f. sp. *tritici* in field conditions. To study the function of these effectors we used the yeast *Pichia pastoris* and *Agrobacterium tumefaciens* mediated gene transfer for heterologous protein production. The production of these proteins was challenging and therefore their functions could not be tested.

Keywords: wheat, *Blumeria graminis*, *Parastagonospora nodorum*, effectors

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Abbreviations

AOX	Alcohol oxidase
AS	Acetosyringone
HST	Host-selective toxin
LB	Lysogeny broth
MS	Murashige and Skoog
PR	Pathogenesis-related proteins
ROS	Reactive oxygen species
RPM	Revolutions per minute
TAE	Tris-acetate-EDTA
ToxA	Toxin A (host-selective toxic protein)
TGX	Tris-Glycine eXtended shelf life
BMG-CA	Buffered minimal glycerol with Casamino acids
BMM-CA	Buffered minimal methanol with Casamino acids
ETI	Effector triggered immunity
HR	Hypersensitive response
UV	Ultraviolet
BSA	Bovine serum albumin
ER	Endoplasmic reticulum
GPI	Glycosyl phosphatidyl inositol
OD_{600}	Optical density at a wavelength of 600 nm
PAMPs	Pathogen-associated molecular patterns
PTI	PAMP-triggered immunity
SNB	Stagonospora nodorum blotch
TGS	Tris-Glycine-SDS
PCR	Polymerase chain reaction
ML	MD2-related lipid-recognition domain
YMS	Yeast-malt-sucrose medium
YPD	Yeast extract Peptone Dextrose medium

1. Introduction

1.1 Effector proteins

In the interaction between a plant and a pathogen, effectors play an important role. Effectors are small, secreted compounds that can manipulate the host to facilitate the infection of the pathogen. The primary defence that a plant has against fungal infection is the PAMP-triggered immunity (PTI), which is activated upon recognition of pathogen-associated molecular patterns (PAMPs). One of the most well-known examples of this is the recognition of chitin, which is a common molecule found in all fungi (Wan et al. 2008). PTI is not specific to particular species but works against a broad range of pathogens. Fungi have evolved to suppress the PTI by secreting effector proteins. These effectors are secreted cysteine-rich proteins that can inhibit certain plant defence mechanisms (Stergiopoulos & Wit 2009). Effector proteins can in turn activate the second layer of the plant defence system, which is referred to as effector triggered immunity (ETI). ETI is activated when the plant recognises the effector proteins of the pathogen (Patel et al. 2020). As a reaction to ETI, a pathogen can secrete other effectors that suppress the ETI.

In the gene-for-gene model, a pathogen has an avirulence (*Avr*) gene that can be recognised by the corresponding resistance (*R*) gene in the host plant. If the *Avr* corresponds to the *R* in the host, the defence response is activated (Nürnberger & Scheel 2001). A common response is the hypersensitive response (HR) which means that the host kills its own cells locally where the defence has been activated (Keppler et al. 1989). By killing its own cells, biotrophic pathogens that rely on living plant tissue, cannot spread to the rest of the plant. To avoid activating HR, biotrophic fungi secrete effectors that suppress the defence mechanisms of the host. Other functions of biotrophic effectors include protection against plant defence systems (Jaswal et al. 2020), or manipulating the physiology of the host to obtain nutrients (Abera Gebrie 2016). Effectors may also directly or indirectly influence the phyllosphere, which can increase the competitiveness of the pathogen (Chaudhry et al. 2021). These effectors have an ecological effect and are therefore referred to as ecological effectors. Pathogens may evolve to secrete new virulence

components that cannot be detected by *R* genes in the host. The coevolution between host and pathogen is referred to as an evolutionary arms race (Anderson et al. 2010). The HR does not protect the plant against necrotrophic fungi since they feed on dead plant tissue. Due to the nature of necrotrophic fungi, they can have a more aggressive infection in which the plant cells are killed as soon as possible. The function of most necrotrophic effectors is to cause cell death in the host (Vleeshouwers & Oliver 2014). Once the plant cells are dead, the fungus can obtain its nutrients. The interactions of effectors with the host are often studied to get a better understanding of the development of disease. Increased knowledge about the weaknesses in plants will help to breed for more disease resistant crops.

1.2 E024 family powdery mildew

Blumeria graminis f. sp. *tritici* (*B.g. tritici*) is a biotrophic fungus which can only infect wheat. In addition to the gene-for-gene model, with *Avr* and *R* genes interacting with each other, *B.g. tritici* can suppress the recognition of its effector protein. Effector gene expression was examined in an RNA sequencing study (Kälin 2018). The gene expression profiles in lab infected wheat were compared with that from samples from the field. The interesting result was that one particular protein family, E024, was much more expressed in the field than in lab conditions (Kälin 2018).

The E024 effector family consists of eight proteins: Bgt-55138, BgtE-5709, BgtE-5710, Bgt-55135, BgtE-10002, BgtE-20022, Bgt-55136 and BgtE-10005. Of these eight effector proteins, Bgt-55138 was the most highly expressed in field conditions. The proteins Bgt-55138, BgtE-5709, BgtE-5710, BgtE-20022, Bgt-55136 and Bgt-55135 encode for a MD-2-related lipid-recognition (ML) domain. The ML domain is present in other organisms where it is involved with the lipid metabolism and recognition of pathogen components containing lipid (Inohara & Nuñez 2002). MD-2 proteins play a crucial role in protection against bacterial infection in shrimp (Liao et al. 2011). In a study on *Arabidopsis thaliana*, it was shown that MD-2 proteins can have an effect on plant defence systems as well, by regulating the expression of jasmonic acid and salicylic acid (Fridborg et al. 2013). This suggests that the effectors may play an important role in the virulence of the pathogen. However, the exact functions of the proteins in the E024 effector family are not known. One of the differences between infected wheat in lab conditions and field conditions is that in the field there are likely several other pathogens on the wheat. These pathogens are competing with each other to obtain the nutrients from the host. This gives reasons to believe that proteins in E024 increase the

competitiveness of *B.g. tritici*. In a preliminary study with effectors from the E024 family, it was discovered that they might have anti-microbial properties, being able to suppress the growth of *E. coli* (Olsson et al. 2022).

1.3 ToxA – Tsn1 interaction

ToxA was first identified as a host-selective toxin (HST) produced by the fungal pathogen *Prenophora tritici-repentis* (Liu et al. 2006). In later research it was discovered that the necrotrophic fungus *Parastagonospora nodorum* can produce a similar toxin. It is considered that the *ToxA* gene was horizontally transferred from *P. nodorum* to *P. tritici-repentis* (Friesen et al. 2006). ToxA is a protein with a single gene that encodes for its expression (Ciuffetti et al. 1997). This toxin can interact with Tsn1 in an inverse gene-for-gene model and cause cell death. When ToxA is inserted into the apoplast of a sensitive plant without the pathogen, it will cause symptoms similar to those with the presence of the pathogen (Ballance et al. 1989). Without damaging the cell membrane of the host plant, ToxA is internalised in the cell of sensitive plants (Manning & Ciuffetti 2005). Extracellular receptors bind to ToxA as a first step of internalising the protein (Figure 1). Internalisation of the toxin is crucial for necrosis, and the ability to internalise the toxin explains whether a plant is sensitive or not. Inside the cell, ToxA targets two chloroplast-related proteins, ToxABP1 and plastocyanin (Tai et al. 2007; Ciuffetti et al. 2010). Plastocyanin is involved in electron transport during photosynthesis. ToxABP1 is a homolog of Thf1, which is crucial for chloroplast development (Ciuffetti et al. 2010). A vital step towards cell death is the light-dependent accumulation of reactive oxygen species (ROS) in the chloroplasts. The production of ROS causes a decrease in the amount of rubisco, which is an important enzyme for photosynthesis (Manning et al. 2009). A site-specific interaction between ToxA and the pathogenesis related PR-1-5 protein found in wheat, is suggested to mediate the cell death in ToxA sensitive plants (Lu et al. 2014).

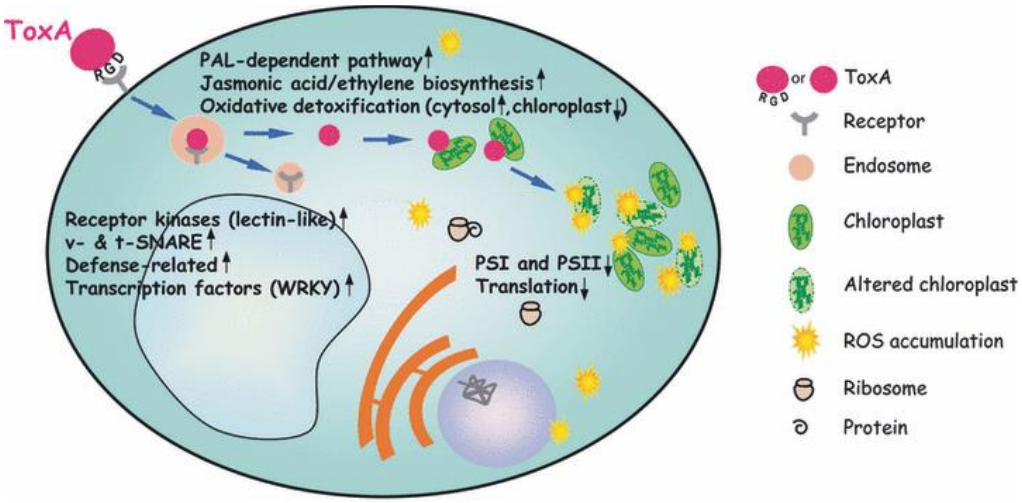

Figure 1. A schematic overview of the internalisation of ToxA and the damage it does to the chloroplasts which leads to necrosis. Adapted from 'Host-selective toxins, Ptr ToxA and Ptr ToxB, as necrotrophic effectors in the *Pyrenophora tritici-repentis*-wheat interaction' by Ciuffetti, L.M. et al. (2010), New Phytologist, 187(4), pp. 911–919. Available at: https://doi.org/10.1111/j.1469-8137.2010.03362.x.

Stagonospora nodorum blotch (SNB) is a disease caused by *P. nodorum* and can reduce the yield of wheat up to 50% (King et al. 1983). There are at least seven other proteinaceous HSTs produced by *P. nodorum* that have been discovered. These proteins are described as SnTox1 (Phan et al. 2016), SnTox2 (Friesen et al. 2007), SnTox3 (Liu et al. 2009), SnTox4 (Abeysekara & Friesen 2009), SnTox5 (Friesen et al. 2012), SnTox6 (Gao et al. 2015) and SnTox7 (Shi et al. 2015). The size of these proteins range between 7 and 30 kDa. Each of the necrotic effectors has a corresponding Snn receptor with which it can interact. The interactions between the HTSs and the receptors are light-dependent, except for SnTox3-Snn3 and SnTox7-Snn7 (Shi et al. 2015).

1.4 Recombinant protein production

A methylotrophic yeast, named *Pichia pastoris*, is often used for heterologous protein production. More than 5000 recombinant proteins have been produced using *P. pastoris* (pichia.com 2022). The popularity of using *P. pastoris* is due to three main reasons: (i) high protein yield; (ii) the ability to modify proteins with processes such as disulphide-bond formation and glycosylation; and (iii) the ease to genetically modify the yeast and insert foreign genes (Cregg et al. 2000). The yeast can use methanol as a carbon source by utilising the metabolic pathway that transforms methanol into chemical energy that can be used by the yeast. The first

step is the oxidation of methanol to hydrogen peroxide and formaldehyde (Cereghino & Cregg 2000). This reaction is catalysed by the alcohol oxidase (AOX) enzyme. AOX is encoded mainly by the gene *AOX1* of which the transcription increases 1000-fold if the yeast is growing in methanol (Cereghino et al. 2002). Glucose and glycerol can also be used as carbon sources by *P. pastoris*, but then the *AOX1* is suppressed. The ability to obtain a high cell density before the recombinant protein is produced can be an advantage if the produced protein is toxic for the yeast. The production of secreted proteins can range from 1 mg/L to more than 10 g/L (Cereghino et al. 2002). Protein production is the highest in fermenter cultures, where important parameters such as carbon feeding and aeration can be controlled to optimise the process (Cereghino et al. 2002).
To produce the desired protein, an expression vector with the gene for the protein has to be inserted in the genome of *P. pastoris*, between the AOX promoter and a terminator sequence (Cereghino & Cregg 2000). Secretion of the proteins is possible if a secretion signal sequence is present in the inserted cassette. However, some recombinant proteins are not properly secreted, with no apparent explanation. Choosing the best secretion signal sequence for a particular protein is a matter of trial and error.

Another method of heterologous protein production involves the use of the bacterium *Agrobacterium tumefaciens.* This bacterium causes crown gall disease by inserting a part of its own T-DNA into the DNA of its host (Chilton et al. 1977). By changing the DNA of the plant, the physiology of the plant changes to benefit the bacterium. This mode of action is often used for various experiments. The T-DNA of *A. tumefaciens* can be altered, so that the bacterium can be used for gene transfer to a plant (Hwang et al. 2017). A gene that is inserted *in planta* will make the plant produce the protein of interest, which makes it possible to test its effects. The bacteria are infiltrated in the leaf, and the transformation happens only in the infiltrated area.

1.5 Objectives

The objectives of this research were to characterise the putative ecological effectors. The thesis consists of two different parts. In the first part there is a focus on heterologous protein expression with *P. pastoris* and *A. tumefaciens*. These proteins are putative ecological effectors of the E024 family, and their effects were to be tested on *P. nodorum* and *Zymoseptoria tritici*. The hypothesis was: The effectors of the E024 family from *B.g. tritici* have an antagonistic effect on *P. nodorum* and *Z. tritici*.

In the second part of the thesis, the virulence of a small *P. nodorum* population was tested on commercial wheat varieties. This can give insights on the susceptibility to SNB in Sweden. It can also give more information on the variety of effector proteins present in the *P. nodorum* populations and the resistance genes in wheat.

2. Materials and methods

2.1 Production of E024 proteins

2.1.1 Transforming *Pichia pastoris*

Plasmids carrying the genes for the proteins Bgt-55138, BgtE-5709, BgtE-5710, Bgt-55135, BgtE-10002, BgtE-20022, Bgt-55136 and BgtE-10005 of the E024 protein family, were previously constructed by Olsson, et al. (2022). Briefly, the effector-genes were codon-optimised for their expression in *P. pastoris*, and synthesised by General Biosystems Inc., (Durham, NC, USA). The optimised sequences were subcloned into the plasmid pPIC9H (kind gift from Dr. George Tzelepis, SLU), under the control of the methanol-inducible promotor *AOX1*, using the α-factor secretion signal and the *AOX1* as the terminator sequence.

Glycerol stocks of *Escherichia coli* carrying the different E024 plasmids were grown at 37ºC overnight, in a rotary shaker at 200 RPM. Plasmid extraction was done using the Thermo Scientific GeneJET Plasmid Kit. The instructions of the manufacturers' protocol were followed, except that 55 µL of Elution Buffer was used instead of 50 µL. Plasmids were verified with restriction enzymes *Bam*HI and *Eco*RI (Thermo Scientific) and the digested plasmid DNA were visualised in 1%TAE agarose gels (40 mM Tris base, 20 mM acetic acid, 1 mM EDTA, 1% agarose, 0.002% Nancy 520 stain (Sigma)) under ultraviolet (UV) light in Bio-Rad's Gel Doc 2000. DNA concentration was determined by measuring the absorbance of 1 µL in a nanodrop. Positive clones for the eight effector constructs and the empty vector with the highest concentration were used to transform *P. pastoris*.

P. pastoris strain GS115 (Invitrogen) was transformed according to the PEG 1000 protocol (*Pichia* Expression Kit User Guide, Invitrogen). First, the plasmids were linearized using the restriction enzyme *Bgl*II (Thermo Scientific) and a buffer by

heating at 37°C for 30 minutes. To maximise transformation frequencies, 40 µg salmon sperm DNA was added to each sample as carrier DNA. After the last step in the protocol, the cells were spread on agar plates and placed in an incubator.

The plates were kept in an incubator at 30°C for several days until colonies appeared. From each plate, several colonies were picked with a sterile pipette tip and added to five mL YPD in a 50 mL conical tube. Liquid cultures of *P. pastoris* transformants were grown in a rotary shaker at 200 RPM in a temperature of 30°C. Glycerol stocks were made using filter-sterilised 50% glycerol and were stored in cryotubes at -80°C. Besides the transformants with the *AOX1* inducible promoter, *P. pastoris* was transformed with plasmids carrying the same effectors under the constitutive promoter *GCW14* (Xia et al. 2021). Briefly, the different effector-plasmids were used as templates in a polymerase chain reaction (PCR) with the forward primer "CAA CAA AAA TGA GAT TTC CTT CAA TTT TTA CTG CAG" and the reverse primer "TTC ACC TGG CGG GTG TTT CCC CAT TTG" (IDT-DNA, Belgium), to amplify the backbone of each plasmid (Figure 2). The promoter region for the gene glycosyl phosphatidyl inositol (GPI)-anchored (pGCW14) was amplified from *P. pastoris* with the forward primer "ACA CCC GCC AGG TGA ACC CAC CTA AC" and the reverse primer "AAT CTC ATT TTT GTT GTT GAG TGA AG CG AG" (IDT-DNA, Belgium). All the PCR products were visualised using a 1% TAE-agarose gel. The DNA bands were excised and purified using the GeneJET Gel Extraction Kit (Thermo Scientific). Each effector plasmid was used with GeneArt Seamless Cloning (Invitrogen) to ligate the pGCW14, by following their protocol, except that five µL of each ligation was used to transform *E. coli* Top10 competent cells (Invitrogen). The next day, positive clones were picked and grown as indicated above. Plasmid extraction was done using the Thermo Scientific GeneJET Plasmid Kit, following the instructions in the manufacturers' protocol. Plasmids were verified with restriction enzyme *Pst*I (Thermo Scientific) and the digested plasmid DNA were visualised in 1% TAE agarose gels.

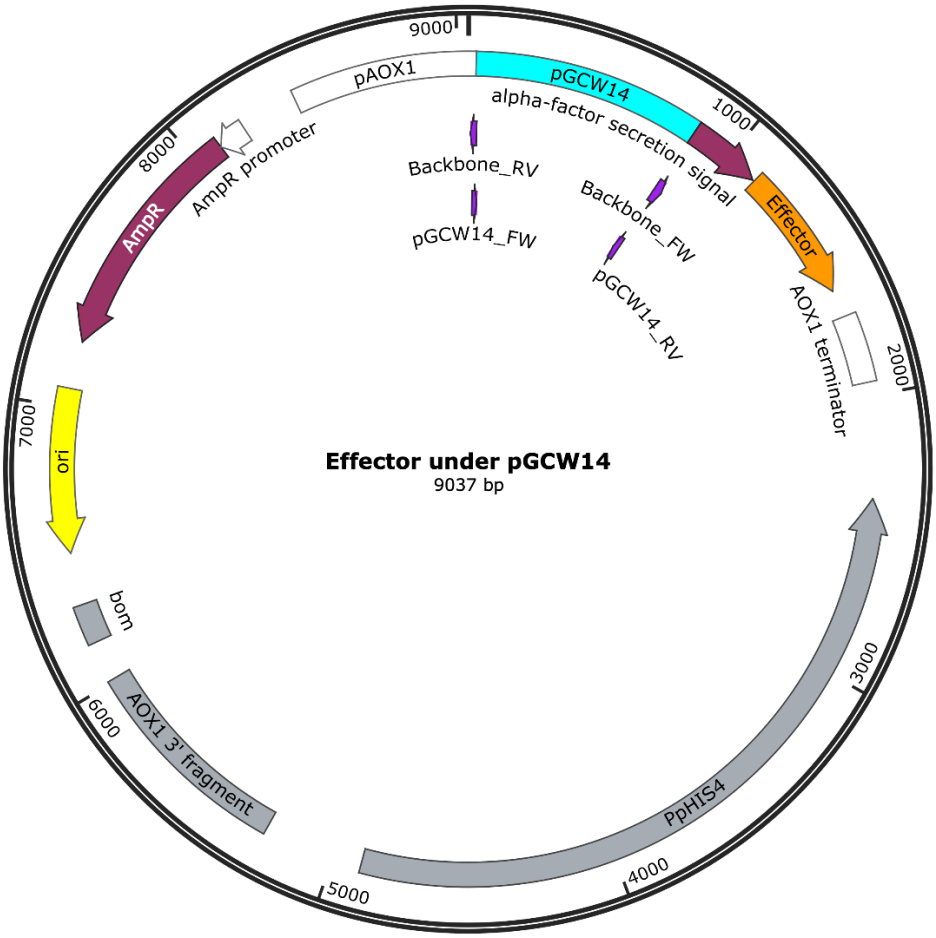

Figure 2. General visualisation of the plasmid backbone using the constitutive promoter *GCW14*.This construct was used for the eight effector-genes and for making the control with the empty vector.

2.1.2 PCR validation

To verify the transformants of *P. pastoris* by PCR, the yeast cell walls had to be broken to access the DNA. Each colony was picked from the glycerol stock and resuspended in 10 µL of water. Lyticase (Sigma) was added, and the mix was incubated for 10 minutes at 30°C. After incubation it was snap frozen in liquid nitrogen. In a SimpliAmp Thermal Cycler (Thermo Scientific) the mix was heated at 95°C for five minutes. In the next step, the cell lysate was added to a solution containing the forward and reverse primers, deoxynucleotide triphosphates (dNTPs), and sterile water. We also included either DreamTaq Green PCR Master Mix or Phusion High-Fidelity PCR Master Mix (Thermo Scientific). Sometimes Bovine Serum Albumin (BSA) (0.002 µg/µL) was added to the master mix as well. It was then placed in a SimpliAmp Thermal Cycler (Thermo Scientific) with parameters depending on the polymerase that was used. The PCR products were

visualised in a 1% TAE agarose gel using UV light in Bio-Rad's Gel Doc 2000, and pictures were taken.

2.1.3 Protein production with transformed *P. pastoris*

To test the protein production of the transformed *P. pastoris* cultures, an adjusted protocol for protein expression from *Pichia* Expression Kit (Invitrogen) was followed. The total duration of growing *P. pastoris* from glycerol stock to obtaining pictures from the protein gel was five days (Figure 3). From the glycerol stock an aliquot was added to two mL YPD (1% yeast extract, 2% peptone, 2% sucrose) in a sterile 50 mL conical tube (Sarstedt). The yeast was grown for one day at 30°C in a rotary shaker at 200 RPM. After one day the cell density was determined by the optical density (OD) of the liquid. The OD_{600} of a 10-times dilution of each colony was measured using a SpectraMax Plus 384 Microplate Reader (Nordic Biolabs). In new 50 mL conical tubes, an appropriate amount of the culture in YPD was diluted in BMG-CA (100 mM potassium phosphate pH 6.0, 1.34% YNB, 4 × 10-5% biotin, 1% glycerol, 0.5% Casamino acids) to reach an OD_{600} of 0.1 in a final volume of two mL. It was placed back at 30°C at 200 RPM.

After one day of growing in BMG-CA, the OD_{600} was measured again. The transformed yeast cultures were then transferred to new tubes with BMM-CA (100 mM potassium phosphate pH 6.0, 1.34% YNB, 4 × 10-5% biotin, 0.5% methanol, 0.5% Casamino acids) at a starting OD_{600} of 0.1. These tubes were placed at 30°C at 200 RPM. After 18 hours in BMM-CA, the cultures were kept on ice and the OD_{600} was measured again. The yeast in the growth media were transferred to sterile 2 mL reaction tubes (Greiner Bio-One) and centrifuged at 21,000×g for 10 minutes at a temperature of 4°C using Fresco 21 Microcentrifuge (Thermo Scientific). The supernatant was mixed with 2X Laemmli buffer (31.5 mM Tris-HCl, pH 6.8, 10% glycerol, 1% SDS, 0.005% Bromophenol Blue) and heated for five minutes at 95°C. After heating, 10 µL of each sample was loaded into the wells of an 8–16% Mini-PROTEAN Tris-Glycine with eXtended shelf life (TGX) Precast Protein Gel (BioRad), with 15-wells per gel. The first well was loaded with five µL PageRuler Plus Prestained Protein Ladder, 10 to 250 kDa (Thermo Scientific). The gel tank was filled with the appropriate amount of 1X TGS running buffer (25 mM Tris, 192 mM glycine, 0.1% SDS) for running two gels simultaneously. Gel electrophoresis was performed with Bio-Rad's Mini-PROTEAN Tetra cell at 200V for 35 minutes. After electrophoresis, the gels were removed from the cassette and stained overnight in QuickBlue Protein Stain (LubioScience) while gently rocking at room temperature. The next day, the stain was removed, and the gels were gently

washed with deionised water. Images of the gels were made in Bio-Rad's Gel Doc EZ using the Coomassie Blue protocol.

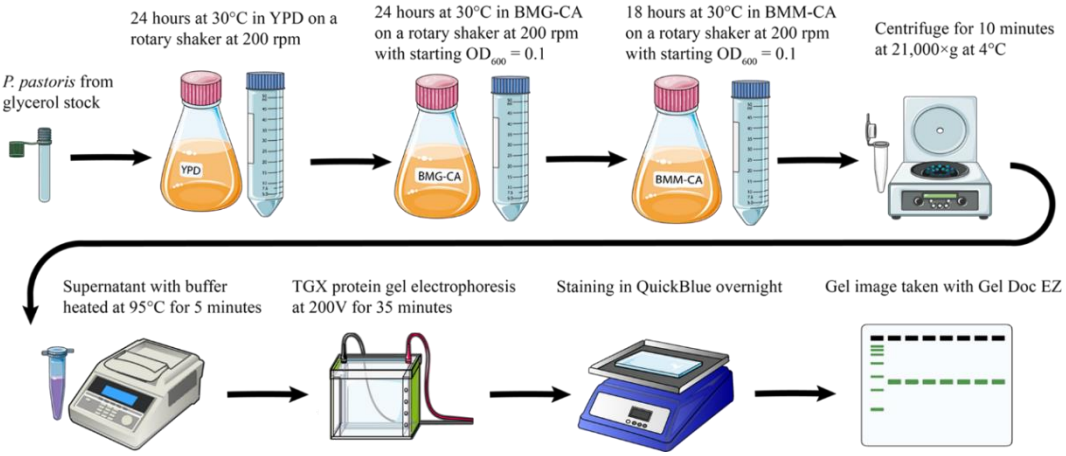

Figure 3. Schematic overview of heterologous protein production in *P. pastoris* and validating the proteins on a gel. The total duration of growing *P. pastoris* from glycerol stock to taking the gel image was five days. tube-screwcap-open-green icon, bath flask icon, falcon-50ml-empty icon, microtube-open-translucent icon, centrifuge icon, microtube-closed-blue icon, electrophoresis-chamber icon, counting-chamber-3d-1 icon by Servier https://smart.servier.com/ is licensed under CC-BY 3.0 Unported https://creativecommons.org/licenses/by/3.0/. shaker icon, electrophoresis-gel icon, thermalcycler-pcr icon by DBCLS https://togotv.dbcls.jp/en/pics.html is licensed under CC-BY 4.0 Unported https://creativecommons.org/licenses/by/4.0/.

2.2 *In planta* production of E024 proteins

Seeds of *Nicotiana benthamiana* (kind gift from Dr. Beat Keller, University of Zürich) were surface sterilised and placed on ½MS agar (2.165% MS salts (Duchefa Biochemie), 10% sucrose, pH 5.7, 0.8% agar) in an incubator. After two weeks, the seedlings were planted in soil and placed in the phytotron. Climate conditions in the phytotron were: temperature 20°C, relative humidity 70%, light 300 µMOL, 16 hours light, and eight hours dark. Soil used for *N. bentamiana* was prepared by mixing 1 L of regular soil with 500 mL P-soil, 500 mL perlite and 500 mL liquid fertiliser (see table S1 for nutrient content). The plants were regularly watered with deionised water.

Agrobacterium tumefaciens strain GV3101 (kind gift from Dr. Beat Keller, University of Zürich) was previously transformed by Olsson, et al. (2022), with constructs for the eight E024 effector proteins in the pIPKb004 overexpression vector, a pIPKb004 (negative control), and a construct encoding the auto-active immune receptor $Pm3A^{HR}$ (positive control). Aliquots of glycerol stocks from these

agrobacteria were added to 50 mL conical tubes containing five mL Lysogeny broth with antibiotics (LB_{GSR}) medium (2.5% tryptone, 2.5% NaCl, 1.25% yeast extract, gentamicin 0.005%, spectinomycin 0.01% and rifampicin 0.005%). The transformants were grown overnight at 25°C with shaking at 180 RPM. The next day, 100 µL of the LB_{GSR} with agrobacteria were transferred to a new 50 mL conical tubes with five mL LB_{GSR} and placed back on the shaker at 25°C to grow overnight. On the day of infiltrations, the agrobacteria were taken out of the 25°C room. The tubes were centrifuged at 3000×g for five minutes. Carefully, the supernatant was discarded and five mL of LB medium without antibiotics was added to the cell pellet, and it was homogenised by pipetting up and down. This was then placed at 25°C at 180 RPM for 30 minutes. After the 30 minutes, the OD_{600} of the agrobacteria was measured in a 10 times dilution in a 1 mL cuvette. The liquid with agrobacteria was diluted in AS medium (10mM $MgCl_2$, 10 mM MES-KOH (pH 5.6) and 20 µM acetosyringone (Sigma)) to a final OD_{600} of 1.2. The agrobacteria in AS medium were placed on a shaker in 25°C at 180 rpm for four hours. The infiltrations in *N. benthamiana* were performed with 1 mL needleless syringes. The edges of the infiltrated area were marked with a non-toxic black marker. After infiltrations with agrobacteria, the plants were placed back in the phytotron and regularly watered.

Photographs of the leaves were taken seven days after the agroinfiltrations. The infiltrated leaves were placed on a black background and a Nikon D3100 camera was attached to a mount. Pictures were taken in a dark room with the flash from the camera as the only source of light.

2.3 Wheat infiltrations

2.3.1 Plant growth conditions

For the first wheat infiltrations, three reference cultivars were grown: 'Bobwhite', 'Fielder' and 'Chinese Spring'. Each cultivar was grown in 24 pots with four seeds per pot, which makes a total of 72 pots and 288 seeds. The soil for wheat was prepared by mixing the following: 1 L regular soil, 1 L U-soil and 500 mL liquid fertiliser (see table S1 for nutrient content). The pots with the sowed seeds were placed in the phytotron with climate conditions: temperature 20°C, relative humidity 70%, light 300 µMOL, 16 hours light, and eight hours dark. During the first days after sowing, the pots were covered with a plastic cover to keep the soil moist. The cover was removed after the seeds had germinated. The plants were

regularly watered with deionised water to prevent the wheat from drought. After 16 days the leaves were infiltrated with *P. nodorum* culture filtrate.

Parental lines of four different RIL populations were also tested (Dr. Francesca Desiderio, CREA, Italy). Eight different cultivars were used, with ten seeds used per cultivar. They were grown with one seed per pot. The soil that was used was prepared in the same way as described before. Leaf infiltrations of the parental lines were performed 17 days after sowing. Commercial lines of bread wheat from the Swedish Agricultural Cooperative (Lantmännen Lantbruk, Sweden) were used as well. These plants were first used for detached leaf assays in another project, and the remaining attached leaves were used for infiltrations with *P. nodorum* culture filtrates when the plants were three to four weeks old. The total number of commercial wheat cultivars was 30, and together with the eight parental lines they were renamed to SW001 to SW038 to keep the names anonymous.

2.3.2 Preparing culture filtrates

A small population of 22 isolates of *P. nodorum* was cultivated on agar plates (Prof. Bruce McDonald, ETH, Zürich, Switzerland). To keep the used material anonymous, the names of the isolates were changed to Sn001 up to Sn022. Liquid cultures were prepared by transferring a small piece of mycelium from the agar plates containing *P. nodorum* to YMS_{KAN} (0.4% yeast extract, 0.4% malt extract, 0.4% sucrose, 0.005% Kanamycin). The fungi were grown in volumes of 10 mL in 50 mL conical tubes. The tubes were placed on a shaker at 200 RPM at a temperature of 20°C. After 17 days the tubes with liquid culture were centrifuged at 3000×g for five minutes and the supernatant was transferred to a new tube. The supernatant was centrifuged for a second time and transferred to a new tube, without any of the mycelium. The culture filtrate was used shortly after harvesting. Any remaining culture filtrate was stored at -20°C. For the infiltrations of parental lines and commercial cultivars, only isolates Sn001, Sn002, Sn005 and Sn021 were prepared.

2.3.3 Infiltrating wheat

Culture filtrate of *P. nodorum* was used to infiltrate wheat. As a control, YMS_{KAN} was used. Using a 1 mL needleless syringe, the culture filtrate was injected into the leaf of wheat. The borders of the infiltrated area were marked with a black non-toxic marker. The infiltrated plants were kept in the phytotron and watered

regularly. Seven days after infiltrations the plants were taken out of the phytotron, and the infiltrated leaves were cut. These leaves were taped to A4 paper and scanned using a flatbed scanner. After first screening all 22 isolates of *P. nodorum* with the infiltrations in 'Bobwhite', 'Fielder' and 'Chinese Spring', four isolates were chosen for assays on the parental lines and commercial varieties.

3. Results and discussion

3.1 Protein production in *P. pastoris*

The aim of the protein production in yeast was to test the functions of the proteins of the E024 family. The first goal was to find cultures of *P. pastoris* that produce the proteins in a small-scale production method. After screening, the best-performing yeast cultures would be used for heterologous protein production on a larger scale. The produced proteins would be used for bioassays to test whether they have antifungal properties against *P. nodorum* and *Z. tritici*.

3.1.1 Using the AOX promoter

Cultivation of *P. pastoris* was first tried in 96-Well plates but was unsuccessful. A large number of cultures did not grow. This may be because it was grown in 25°C, instead of the 30°C that are described by Kaushik et al. (2020). Another problem with this micro-scale cultivation is aeration. Due to limitations in the lab, it was not possible to test whether this was the case. The cultivation in 50 mL conical tubes did work for most cultures. A few cultures had a low growing rate, and some did not grow at all. The cultures with a lower growth rate also showed a different result on the protein gels (Figure 4). For the majority of *P. pastoris* cultures the growth rates were the same and they looked similar on the protein gels as well. For the first protein gels that were run, there was no visible difference between the yeast with constructs for the E024 effector proteins and the ones with the empty vector (Figure 4).

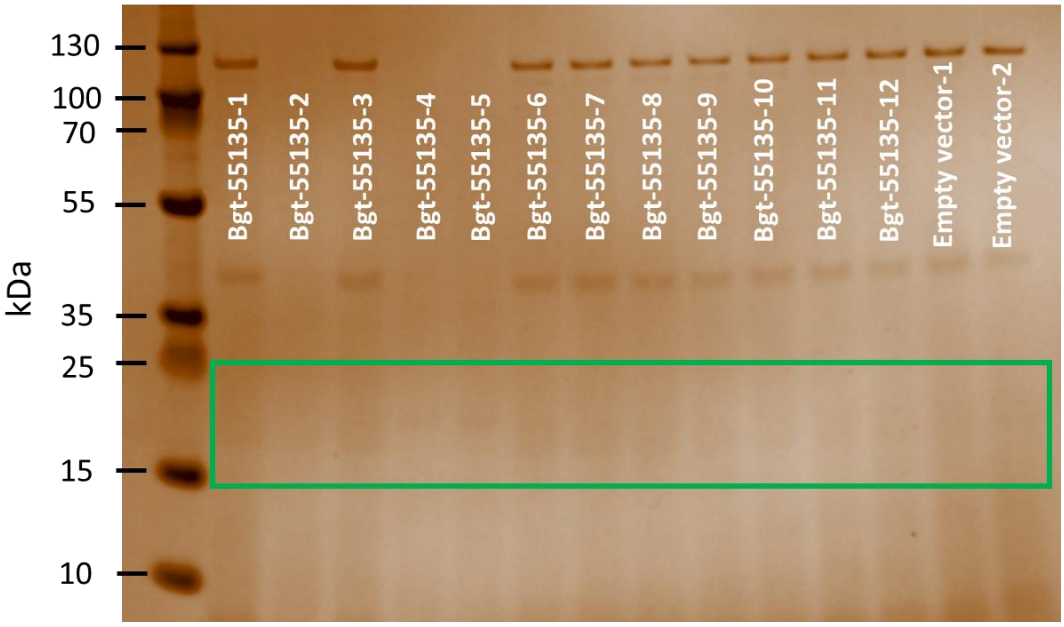

Figure 4. Protein gel with *P. pastoris* cultures previously made by Olsson, et al. (2022), with the *AOX1* promoter. There were 12 cultures that all carry the construct for the same effector, and two colonies with an empty cassette as controls. The protein bands were expected within the area of the green square. Bgt-55135-2, Bgt-55135-4 and Bgt-55135-5 were alive but had a lower growth rate than the other cultures.

There was no yeast culture that resulted in a clear band on the gel at the expected size (~19 kDA). One explanation for this could be that the constructs for heterologous protein production were not properly inserted in the yeast. Another reason might be that the protein was produced but not secreted. This can be caused by a fault in the secretion signal sequence. Not all secretion signal sequences work well for each recombinant protein (Cereghino & Cregg 2000). A study by Barrero et al. (2018), showed two possible ways for protein secretion to be unsuccessful. The protein may fold in the cytosol and be unable to go across the endoplasmic reticulum (ER) membrane. If that happens, the protein gets stuck and will not enter the secretory pathway. The other possible way is for the protein to aggregate in the ER lumen and get trapped. Protein gels that were loaded with the cell lysate showed that there were many proteins inside the cell. There were clear bands in the expected size of the effector proteins, but they were also visible in the lysate of the yeast with the empty vector. This suggests that *P. pastoris* has native proteins in the same size as the effector proteins. Another reason for the lack of bands on the expected size could be that the heterologous protein production was too low to be visible on the gel. Protein glycosylation could be another cause of not finding proteins of the expected size. Glycosylation of proteins is a post-translational change which adds a carbohydrate to the protein. These changes to proteins have been found to occur

in *P. pastoris* as well (Cereghino et al. 2002). If protein glycosylation occurred to the effector proteins it means that the size and function could change. There were a few cultures that seemed to have very faint bands in the expected size on one gel.

Western blot, a method to qualitatively detect proteins, could not be used because the recombinant proteins did not have a histidine tag. The proteins did not have a histidine tag because the goal was to characterise the functions of the proteins and it was shown that effectors from *B. graminis* are particularly sensitive to epitope tagging, loose their function, are not secreted anymore, or are simply not possible to tag (Bourras et al. 2019). However, this does not necessarily mean that it is impossible to tag the effectors of the E024 family. Due to the time limitation of this project, it was initially decided to not take the risk of effectors losing their function because of a tag. However, when the protein gels did not show any bands in the expected size, new transformants were made for effector Bgt-55136 with a histidine tag. These new transformants had growth issues for unknown reasons and there was no time to use them for protein expression or performing a Western blot. To gain confidence in the results and exclude possible user errors, we have also produced new transformants of *P. pastoris* carrying an empty cassette with the *AOX1* promoter. The new empty vector did show a different result compared to the constructs with the effector proteins (Figure 5). However, it has to be noted that this new empty vector was compared to the *AOX1* transformants made by another MSc student. Therefore, it cannot be guaranteed that similar methods were used. Perhaps the comparison between the previously constructed and new *AOX1* transformants is not completely justified.

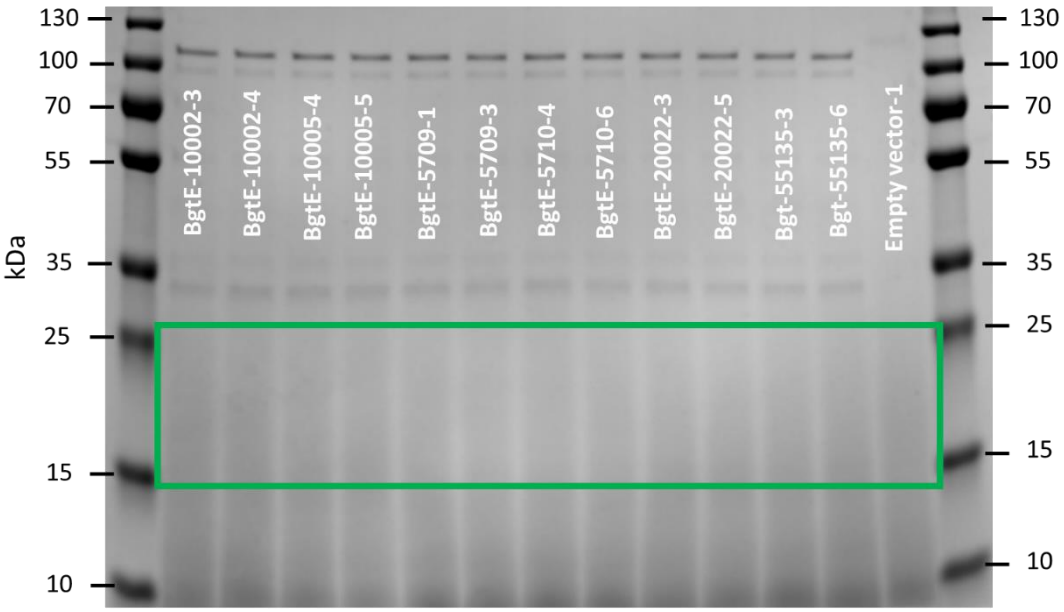

Figure 5. Protein gel with *P. pastoris* cultures previously made by Olsson, et al. (2022), with the *AOX1* promoter, and a new empty vector with the *AOX1* promoter. The protein bands were expected within the area of the green square.

3.1.2 PCR validation of new transformants

The PCR validated 32 new *P. pastoris* transformants. These 32 transformants include six effector proteins BgtE-55709, BgtE-5710, Bgt-55135, BgtE-20022, Bgt-55136 and BgtE-10005 with the constitutive promoter, and one empty vector with the AOX1 promoter. Not all cultures could be validated to carry the vector (Figure 6). Some PCR products showed no bands at all on the DNA gel. Because of this, different parameters for the thermal cycler were tested. After first using DreamTaq (Thermo Scientific), better results were obtained with Phusion polymerase (Thermo Scientific). With the addition of BSA to the mastermix, the yield of PCR amplification increased. BSA is known to prevent PCR inhibitors from interacting with the DNA polymerase, but can also enhance the PCR amplification in a reaction free of PCR inhibitors (Farell & Alexandre 2012).

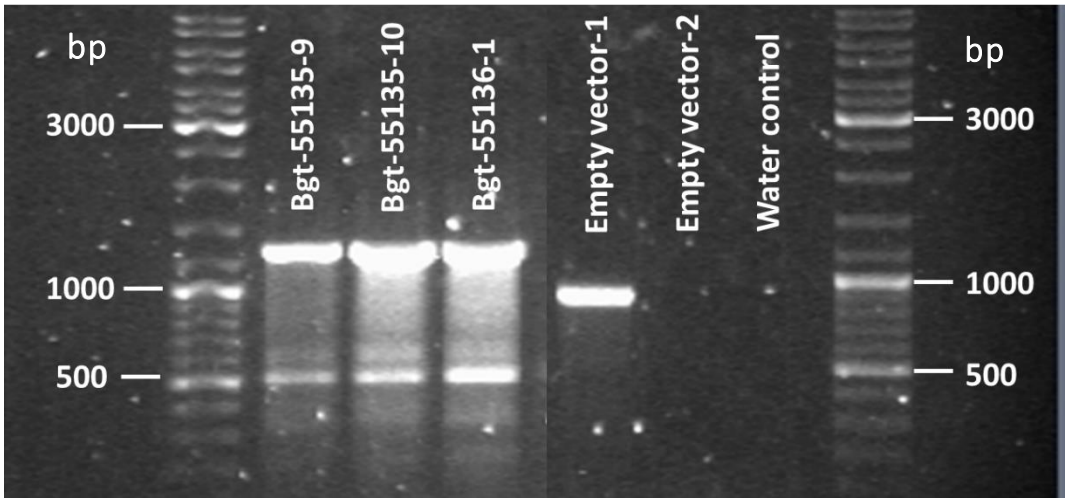

Figure 6. A DNA gel with five *P. pastoris* transformants with the *GCW14* promoter tested by PCR. The cultures 35-9, 35-10, 36-1 and empty vector 1 are shown to carry the inserted vector. The size of the product in the empty vector 1 is lower than the others because it does not carry the gene for a protein, as it is the empty vector.

3.1.3 Using a constitutive promoter

Since the yeast transformants with the *AOX1* promoter did not give the expected result, new transformants were made with the constitutive *GCW14* promoter. These yeast transformants did not need to be induced in methanol to produce the protein. On the protein gels with the constitutive promoter faint bands were visible, but they were higher than the 35 kDa ladder mark, and thus larger than the expected size of ~19 kDA (Figure 7). In contrast to the results with the *AOX1* promoter, there were no sharp bands of any size visible on the gels. The protein expression of induced *P.*

pastoris with the *AOX1* promoter is higher than that of the *GCW14* promoter (Liang et al. 2013). This could explain the reason why there are no sharp bands visible on the gel, because the protocol that was used was the same as for the inducible promoter, except that the cultures were grown for 18 hours in BMG-CA instead of BMM-CA. The successful production of recombinant proteins is dependent on many different factors, so by changing the growth conditions there could be a change in the results on the gels. A longer growing period may result in more protein production, but because of time restraints this was not tested.

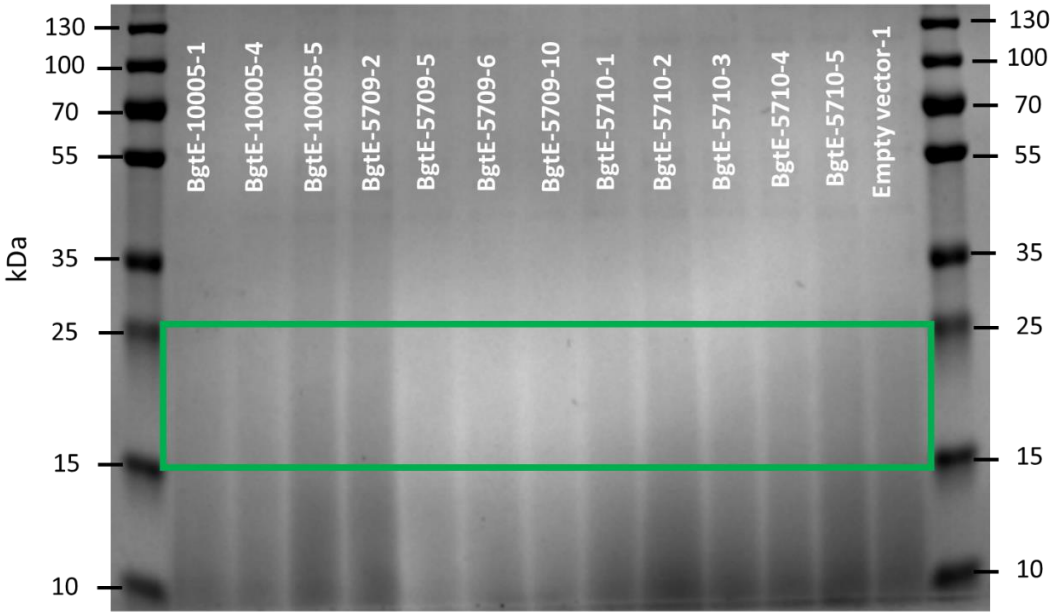

Figure 7. Protein gel with *P. pastoris* cultures with the constitutive *GCW14* promoter and an empty cassette as control. The protein bands were expected within the area of the green square.

3.2 *N. benthamiana* agroinfiltrations

To test if the proteins of the E024 family have a damaging effect on plants, they were expressed in *N. benthamina* through agrobacterium mediated gene transfer. The agroinfiltrations with the constructs for the E024 protein family did not give any observed damage to the leaves of *N. benthamiana*. This was as expected. An unexpected result was that the positive control $Pm3A^{HR}$ did not cause significant damage in all infiltrated spots. In some leaves the infiltration of $Pm3A^{HR}$ led to severe damage, whereas in other leaves it seemed to have no effect (Figure 8). No damage was caused by infiltrations with the negative control pIPKb004. On some leaves there was discolouration in all the infiltrated spots. These spots had a lighter colour than the rest of the leaf.

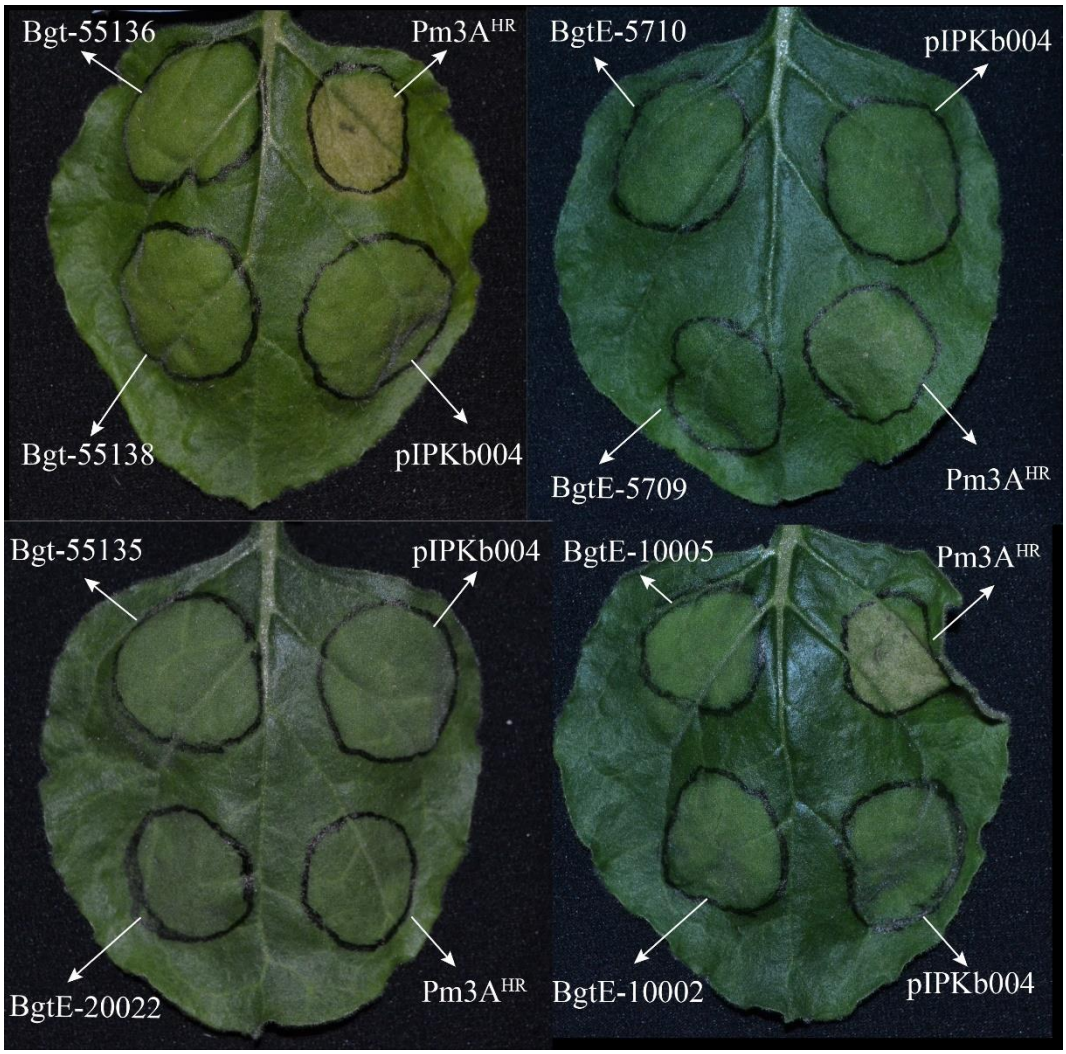

Figure 8. Agroinfiltrations in *N. benthamiana* with the eight effector proteins of the E024 family and pIPKb004 as a negative control, and Pm3A^{HR} as the positive control.

This difference of reactions to Pm3A^{HR} can be caused by variation of the location of the infiltration, the leaf position and between plants (Bashandy et al. 2015). The weak or absent reaction to the agroinfiltration was mostly observed on the lower infiltrated leaf, which is older. As the leaves of *N. benthamiana* get older, they become less suitable for agroinfiltrations (Leuzinger et al. 2013). Another thing to consider is that staining with trypan blue could have visualised cell death more clearly (Ma et al. 2012).

The hypothesis that the effectors of the E024 family from *B.g. tritici* have an antagonistic effect on *P. nodorum* and *Z. tritici*, could not be tested because the proteins could not be purified.

3.3 Wheat infiltrations

The aim of the wheat infiltrations was to gain a better understanding of the susceptibility to SNB in several wheat cultivars. The first infiltrations with 22 isolates were used to select the isolates of *P. nodorum* that cause symptoms on most cultivars. This resulted in choosing four isolates that caused the most damage on 'Bobwhite', 'Fielder' and 'Chinese Spring'. These four isolates were Sn001, Sn002, Sn005 and Sn021 (Figure 9). 'Fielder' had severe leaf damage from all isolates. 'Chinese Spring', which has the *tsn1* gene and therefore is insensitive to ToxA (Liu et al. 2006), still developed necrosis from infiltration with Sn001 and Sn005. This indicates that the pathogen secretes effectors other than ToxA. It is known that *P. nodorum* can secrete seven other proteinaceous toxins. If the genomes of the isolates of *P. nodorum* are known, then with a BLAST search it can be found if they produce effectors that are already known to exist, or if perhaps an unknown toxin causes the necrosis.

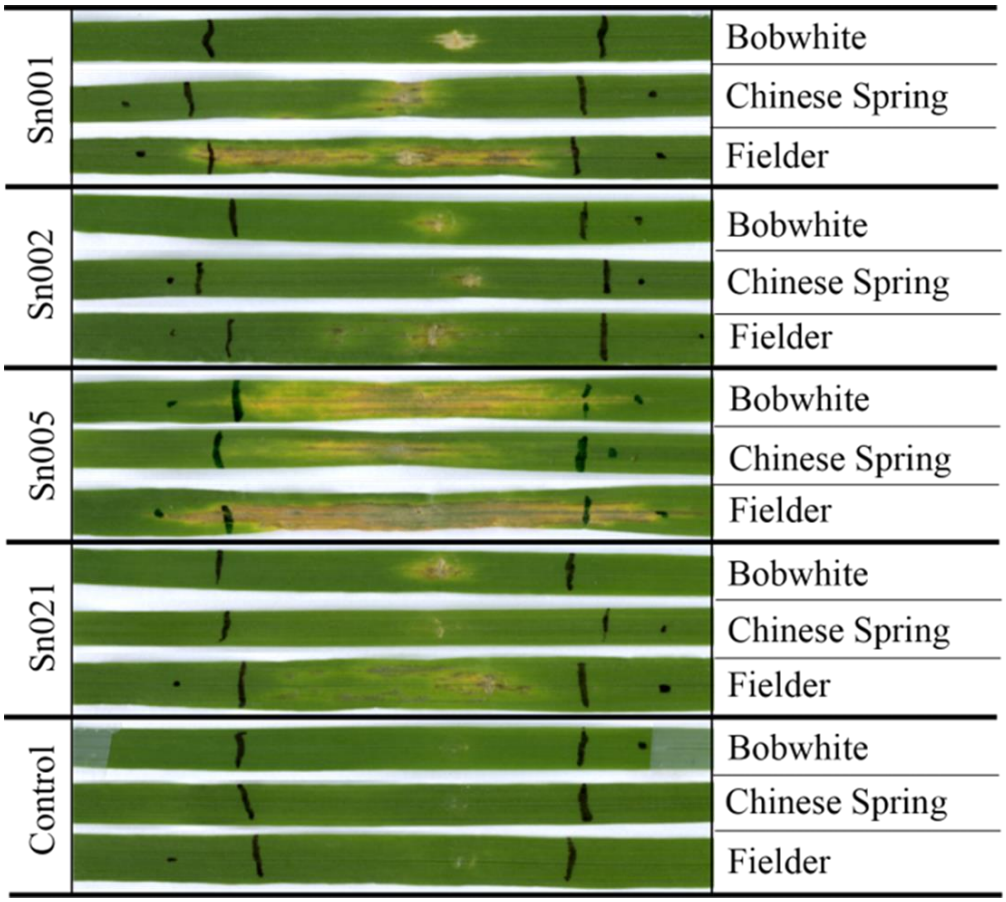

Figure 9. Leaf infiltration assay on wheat cultivars 'Bobwhite', 'Chinese Spring' and 'Fielder'. The *P. nodorum* isolates that caused the most damage were Sn001, Sn002, Sn005 and Sn021. The control group was infiltrated with YMS_{KAN}.

Parental lines were infiltrated with the four chosen isolates to test for their susceptibility. Since these lines are used in breeding programs, it is important to know if they are susceptible to SNB. From the 80 seeds of parental cultivars that were sown, only 49 seeds of six cultivars germinated. For one of the cultivars only one seed had germinated. These low germination rates resulted in only five parental lines of RIL populations that could be compared in infiltration assays. Most of the cultivars were insensitive to the infiltrations with the four different culture filtrates of *P. nodorum*. However, there was one cross where one of the cultivars had symptoms with all culture filtrates, but the other cultivar had no symptoms for any of the culture filtrates (Figure 10). This result indicates a different set of resistance genes between these two cultivars. It would be interesting to do infiltrations on the progeny of this cross. The disease symptoms in SW020 were also weaker than observed in the lab standards. This could indicate that the SW020 has partial resistance and may have the ability to suppress the disease after the HR was activated.

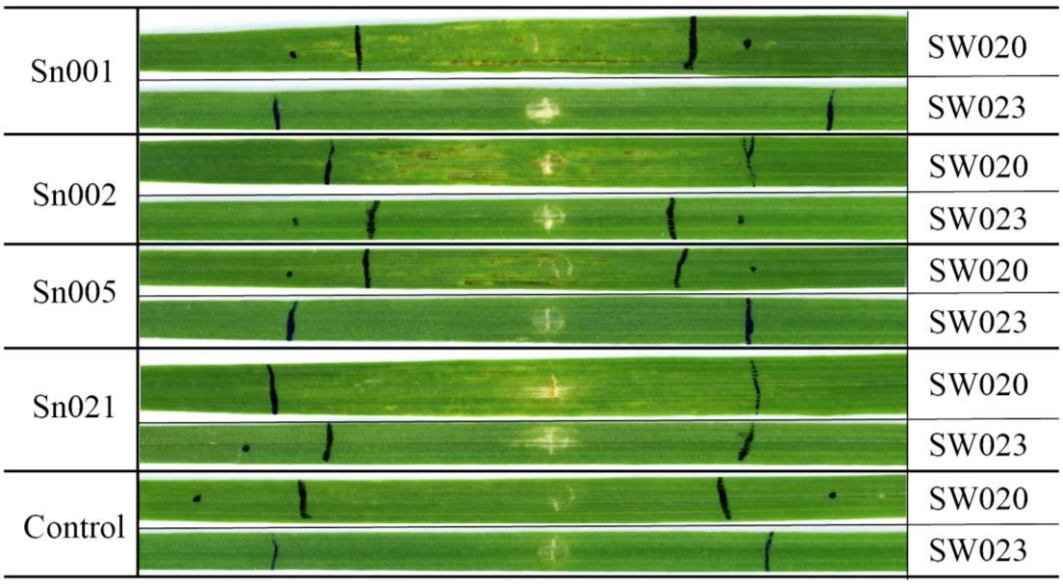

Figure 10. Two lines from a cross, SW020 and SW023, were infiltrated with the same culture filtrates of *P. nodorum* but had different reactions. The brightness and contrast of the image were increased for better visualisation of the disease symptoms.

The infiltrated leaves of the commercial wheat looked different than the infiltrated leaves of the lab standards and parental cultivars. Instead of showing necrosis or brown spots, the main observation on commercial wheat was that the infiltrated area of the leaves became yellow (Figure S1, S2, S3 & S4). Due to the age of these plants, several infiltrated leaves died completely, whereas some leaves turned completely yellow. Even some infiltrations with the control seem to cause chlorosis (Figure S5). This makes it difficult to determine whether damage on the leaf was the result of the proteins infiltrated in the leaf, or simple because of age. There were

some leaves with clear necrosis caused by the culture filtrate. This suggests that some of the bread wheat grown in Sweden is susceptible to SNB. To obtain more convincing results, the experiment should be repeated with the same growth protocol as was used for the lab standards and parental lines.

3.4 Prospective outlook

For further research into the E024 effector family it is important to try other methods of protein production. Different secretion signals can be used in *P. pastoris*, or the parameters in the cultivation can be adjusted. The production of recombinant proteins is a difficult task, and it may require a lot of trial and error before a satisfactory result is obtained. If the proteins can be produced, the functions can be studied. I would have liked to test whether the E024 proteins can inhibit or reduce the germination of fungal spores.

The infiltrations in wheat with the *P. nodorum* population showed that some isolates produce toxins that are not ToxA. It would be very interesting to investigate which other toxins they are. For getting more insight into the heritability of the resistance genes, it would be interesting to perform the wheat infiltrations on the progeny of the SW020 and SW023 cross. It should also be interesting to investigate why the symptoms in SW020 are so weak. This can be important for breeding programs aimed at improving the resistance of wheat against SNB.

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Popular science summary

Wheat is one of the most important crops for the diet of people. It is the third most produced crop and accounts for 20% of the calories and a significant amount of protein consumed worldwide. Diseases on wheat reduce the yield drastically, which have huge impacts given the importance of the crop. To protect the crop against diseases caused by fungal pathogens, the plants can be treated with chemicals called fungicides that weaken or kill the fungi. However, fungi can develop resistance against the fungicides, which reduces the efficiency and makes it a costly disease prevention method. It is therefore important to implement other methods, such as increasing the defence mechanisms of the crop itself. Plants have evolved natural defences against several pathogens. Due to the external protection with fungicides and the focus on larger yields, the genes for defence against diseases have reduced in many commercial wheat cultivars. Resistance genes can be implemented in new cultivars with selective breeding.

One of the fungal pathogens on wheat is *Parastagonospora nodorum*, which kills plant tissue and reduces the growth of the plant. The pathogen makes several proteins which can be poisonous to wheat. Only when these proteins are recognised by the plant, it causes plant cells to die. In this study, we tested the resistance of several wheat varieties. The wheat leaves were infiltrated with liquid containing the proteins of the fungus. In susceptible wheat, we could see cell death in the infiltrated area. We tested 30 commercial wheat varieties from Sweden, that are currently grown, and some of them were shown to be susceptible to these proteins. This will be problematic if the pathogen becomes more abundant in Sweden. However, in wheat that is used for breeding new cultivars, we found that most of them were resistant, and one cultivar may be partially resistant. The resistance genes of these cultivars can be used in breeding programs to develop commercial wheat with resistance to this disease. Complete resistance causes a big selection pressure on the pathogen which can lead to changes in the fungus to overcome the plant's resistance. Partial resistance in wheat might be preferred because it reduces the selection pressure on the pathogen, which means it is less likely to evolve and overcome the resistance. Combined with other disease prevention methods, such as working with clean materials and tools, this can be a sustainable way to grow wheat with minimal damage from pathogens.

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Appendix

Table S1. Composition of the liquid fertiliser that was used for the plants in the phytotron.

Component	**Concentration (mg/L)**
Nitrate	75
Ammonium	37
Phosphorus	22
Potassium	120
Magnesium	3.8
Sulphur	5.5
Calcium	5.5
Manganese	0.42
Boron	0.13
Copper	< 0.029
Iron	0.95
Zinc	0.013
Molybdenum	< 0.016
Silicon	0.15
Sodium	0.73
Aluminium	< 0.17

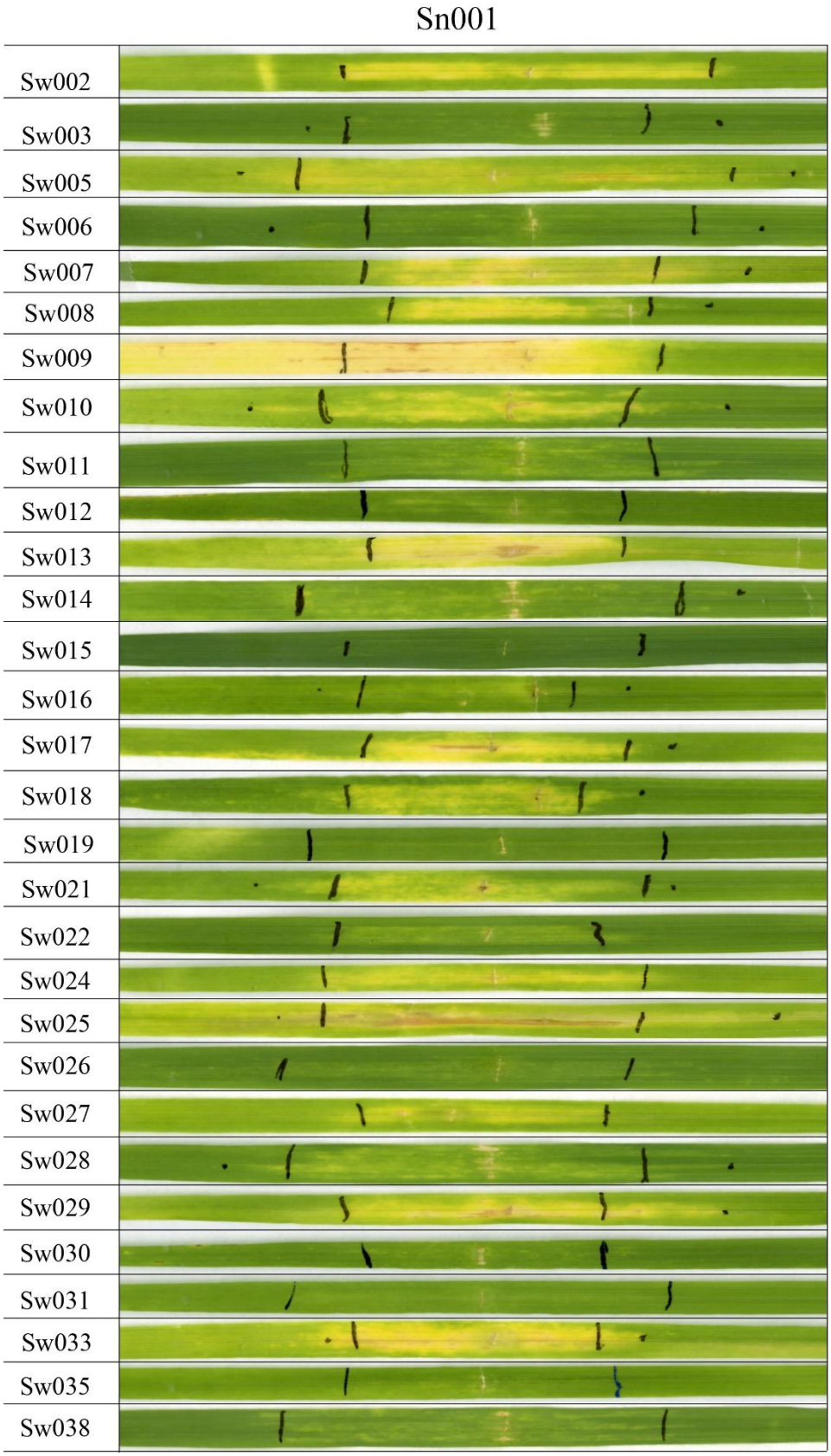

Figure S1. Infiltrations on 30 commercial bread wheat cultivars in Sweden. All leaves were infiltrated with culture filtrate of Sn001.

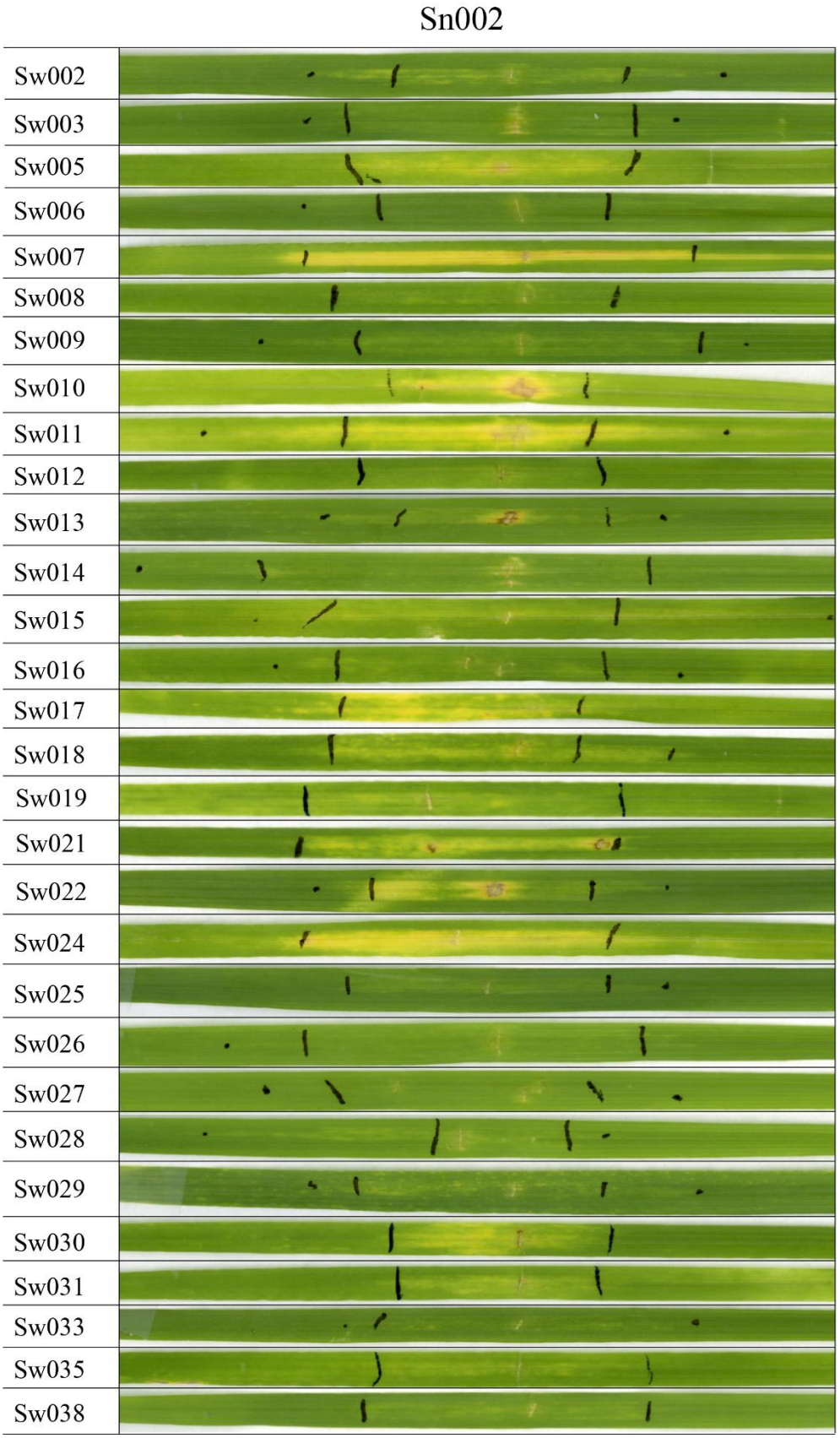

Figure S2. Infiltrations on 30 commercial bread wheat cultivars in Sweden. All leaves were infiltrated with culture filtrate of Sn002.

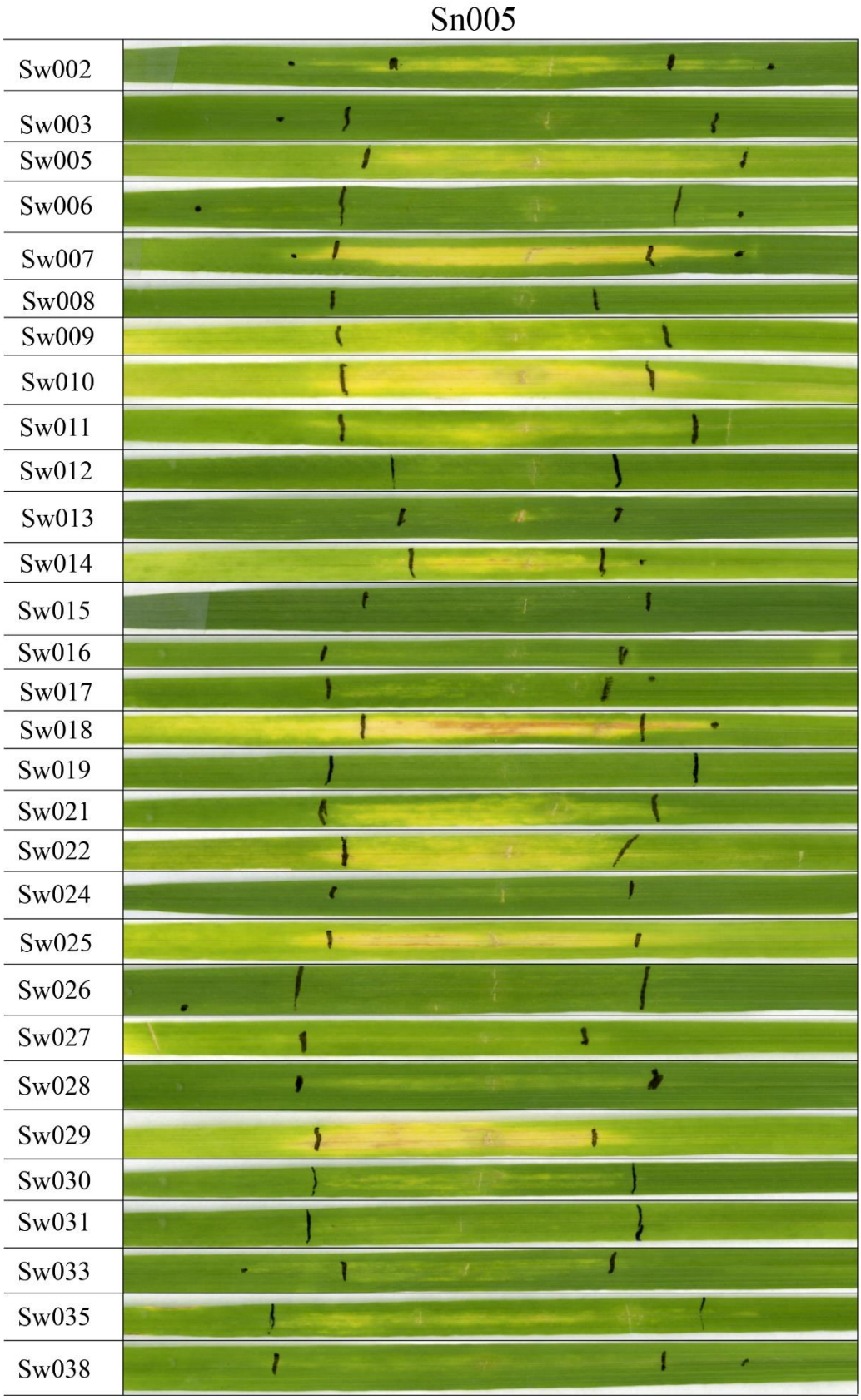

Figure S3. Infiltrations on 30 commercial bread wheat cultivars in Sweden. All leaves were infiltrated with culture filtrate of Sn005.

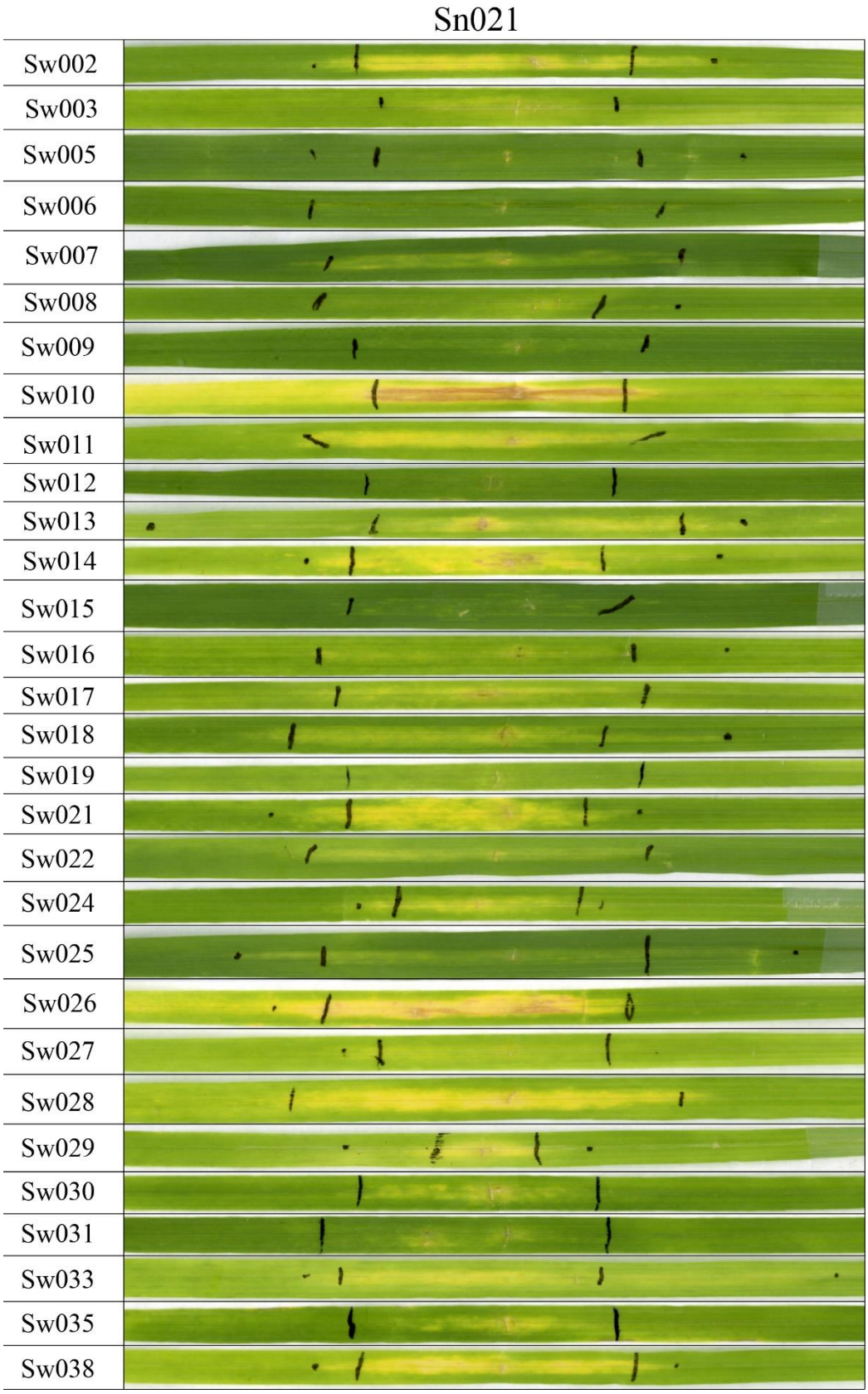

Figure S4. Infiltrations on 30 commercial bread wheat cultivars in Sweden. All leaves were infiltrated with culture filtrate of Sn021.

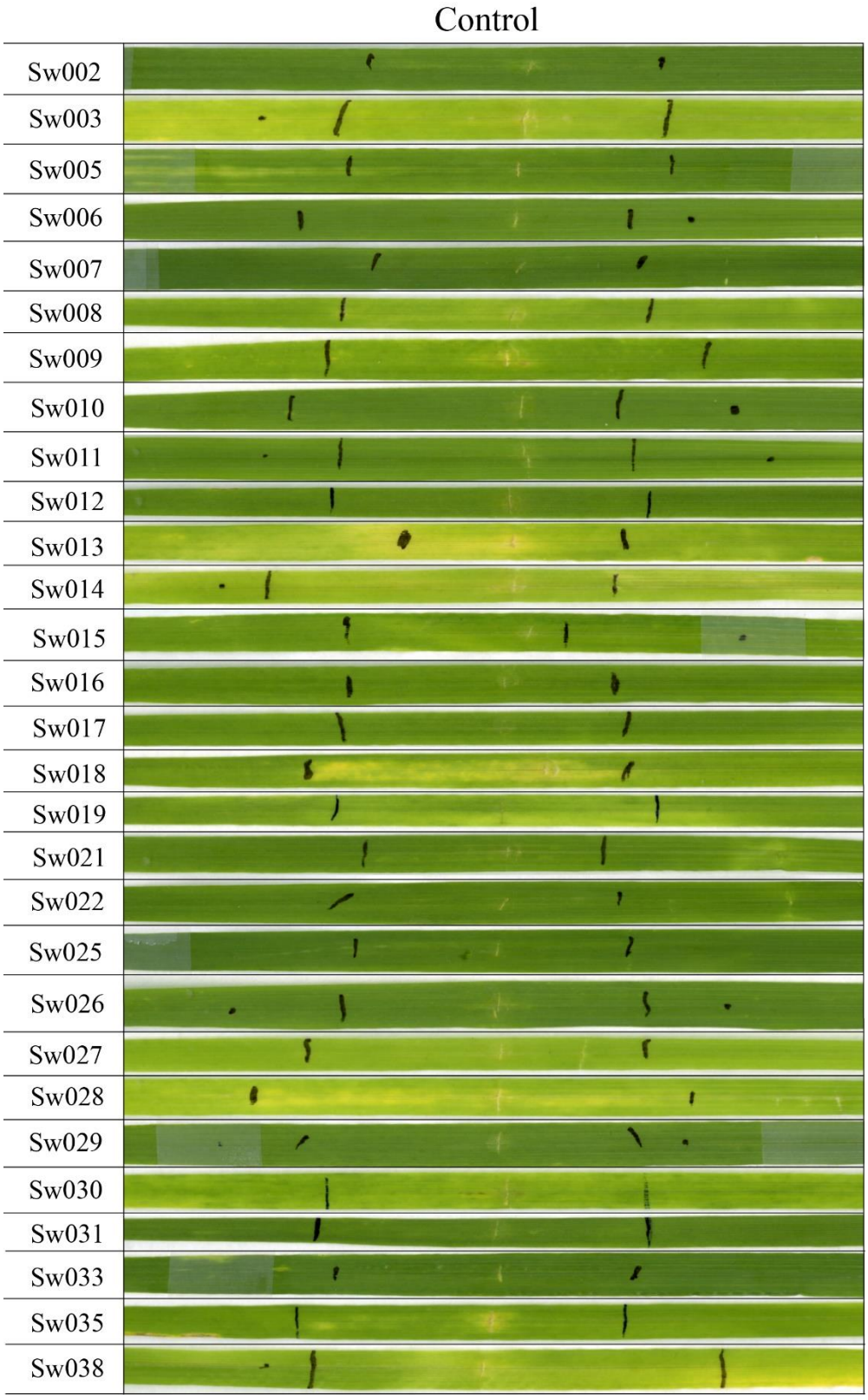

Figure S5. Infiltrations on 30 commercial bread wheat cultivars in Sweden. All leaves were infiltrated with YMS$_{KAN}$.

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