Non-host and induced resistance in the *Taphrina-Arabidopsis* model system

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Tiivistelmä — Referat — Abstract

Plant pathology is an important area of research for maintaining food security and ecosystem services. Many model systems have been developed to study plant diseases in a more practical environment such as in *Arabidopsis thaliana*. However, among the ten most important pathogens for research or economy, dimorphic plant-associated yeasts and pathogens of trees are missing. Plants are constantly challenged with a huge number of potential pathogens but the development of disease remains the exception. This complete resistance against a pathogen is termed non-host resistance. Some yeasts are capable to induce resistance in plants against other pathogens. This has potential for biological control measures.

Our aim was to develop a model system for studying non-host and induced resistance with the dimorphic birch pathogen *Taphrina betulina* in Arabidopsis.

We studied the mechanistic details conferring non-host resistance to *T. betulina* by challenging stress signalling mutants with the pathogen and observing symptom development, root growth, fungal establishment *in planta* and phototoxic stress. The results suggest that type I non-host resistance protects Arabidopsis against *Taphrina*. This is dependent on the EIN2 protein and mediated through an antagonism between the salicylic acid and jasmonic acid defence signalling pathways.

Pre-inoculation with *Taphrina betulina* surprisingly increased growth of *Pseudomonas syringae*. We suggest that this is due to the activation of jasmonic acid and ethylene signalling which antagonise the salicylic acid defences required for Pseudomonas resistance.

In this study, we showed that Arabidopsis is a non-host for *Taphrina betulina* and that this pathosystem can be used as a model for studying the mechanisms of non-host resistance. These insights can give information about possible properties leading to susceptibility in the host. There is a large scope of opportunities for further study into this model.

Avainsanat — Nyckelord — Keywords
Plant pathology, non-host resistance, induced resistance

Säilytyspaikka — Förvaringsställe — Where deposited

Muita tietoja — Övriga uppgifter — Further information

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Abbreviations

ASM	acibenzolar-S-methyl
Avr protein	avirulence protein
BABA	β-aminobutyric acid
COI1	CORONATINE-INSENSITIVE 1
CTR1	CONSTITUTIVE TRIPLE RESPONSE 1
DIR1	DEFECTIVE IN INDUCED RESISTANCE 1
ET	ethylene
ETI	Effector-Triggered Immunity
ETR1	ETHYLENE RECEPTOR 1
HR	hypersensitive response
Ile	isoleucine
ISR	induced systemic resistance
JA	jasmonic acid
JAZ	jasmonate ZIM-domain protein
MAMP	microbe-associated molecular pattern
NRPS	nonribosomal peptide synthase
PAMP	pathogen/pattern-associated molecular pattern
PKS	polyketide synthase
PR protein	pathogenesis-related protein
PRR	pathogen recognition receptor
Pst	*Pseudomonas syringae* pv. *tomato*

PTI	Pattern-Triggered Immunity
R protein	resistance protein
SA	salicylic acid
SAR	systemic acquired resistance
TF	transcription factor

For defence signalling protein abbreviations, see materials section.

Introduction

As integral members of the ecosystem, plants have always existed in dynamic relationships with other organisms and under the influence of the environment. In co-evolutionary processes, they developed symbiotic and parasitic relations with microorganisms while environmental processes obliged them to adapt to demanding conditions. The properties of these factors and the interaction between them determine the type of biotic interaction between the plant and the microorganism. Compatible disease reactions are rare. The host has to be susceptible to an infection by the microorganism, the microorganism has to be pathogenic to the host and the environmental conditions have to be favourable for an infection in order for a disease to occur. This equilibrium is summarized in the disease triangle (**Fig. 1**).

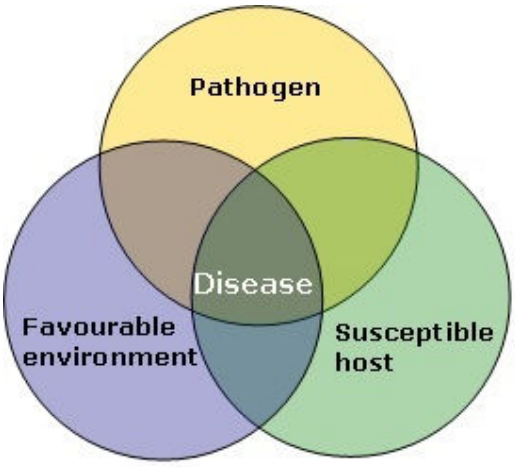

Fig.1 The disease triangle. Schematic diagram of the factors which influence disease outcome. The overlapping area represents those properties of the factors which lead to full disease development.

The domestication of plants for use by humans brought about the discipline of plant pathology, which is the study of plant diseases caused by biotic but also abiotic agents. It is important for establishing food security and conserving ecosystems. These areas have become more threatened by diseases through unsustainable

management practices such as monoculture plantations and the global anthropogenic movement of pathogens and their hosts. Forests are as much affected by these threats as crop plants. History has shown this, for example in the loss of billions of American chestnut trees due to the accidental import of the fungal pathogen *Cryphonectria parasitica* from Asia. Forests are also at least as valuable as food crops considering the array of ecosystem services they provide to humans, especially if left in pristine conditions (cf. for example Metla: State of Finland's Forests 2012, revised from Parviainen and Västilä, 2011; Gamfeldt *et al*. 2013).

The study of how plants can be protected against diseases is not new and attempts to increase resistance have been incorporated into conventional breeding programmes. Although the exact properties conferring resistance were not known, observations could identify more resistant or tolerant cultivars, which were interbred to form a homogenous line of a resistant breed. However, molecular biology made it possible to pinpoint specific genes and molecular mechanisms that confer resistance to diseases.

In the following, mechanisms and models of plant defences against microbial pathogens will be elucidated, leading to the reasoning why we use a fungal tree pathogen as a model organism to study non-host resistance.

Non-host resistance

Plants are constantly confronted with a countless number of microorganisms, which are potentially pathogenic. In relative terms, disease is therefore the exceptional state. Instead, plants can exhibit resistance to a disease to varying degrees. Tolerant plants do not limit the spread of the pathogen but do not incur

severe losses in yield or quality (Schafer 1971). Resistant plants inhibit the growth of the pathogen and development of disease due to an incompatibility of the pathogen and the host. This type of resistance of a host plant is cultivar and/or strain specific and does not confer resistance of the entire plant species or to the entire pathogen species. The major mode that prevents disease against the vast array of pathogens that a plant is constantly confronted with is, however, non-host resistance. This is the resistance of the whole plant species to the pathogen.

Two types of non-host resistance are suggested by Mysore and Ryu (2004): In type I non-host resistance, no visible symptoms appear. This is because the pathogen is unable to surpass preformed plant barriers (first layer or defence) or the active defences induced upon pathogen recognition (second layer of defence). Both preformed and induced defences can be composed of physical and chemical blockages such as papilla formation, cell wall lignification or phytoalexin production (Brown 1998; Dixon 2011; Heath 1997; McLusky *et al*. 1999). Type I non-host reactions are considered the more common type of non-host resistance (Mysore and Ryu 2004) and confers a broader range of resistance to the challenged organism, as a very specific infection mechanism would be required by the pathogen to overcome this barrier. Callose deposition alone results in complete resistance against powdery mildew infections in Arabidopsis (Ellinger *et al.* 2013). From the point of view of the plant, non-host resistance is often quantitative and multi-layered (Fan and Doerner 2012). Many of these primary physical and chemical barriers and how fungi have evolved to respond to these have been reviewed by Łaźniewska *et al*. (2012).

There is also a sound evolutionary reason why the non-specific type I resistance is more common. Pathogens have evolved alongside their hosts which required increasing specificity in the pathogenecity mechanisms in order to keep up with the

co-evolution of plant defences against them. As different plants evolved in different biotic and abiotic environments, this led necessarily to an evolutionary divergence between the pathogen and those plants with which it interacted little during its evolution. Non-host defences can therefore be assumed to be non-specific, arisen only due to a chance divergence of basic physical and chemical properties away from those that would lead to susceptibility (Antonovics *et al.* 2013).

In type II non-host resistance, the plant activates rapid cell death in the area surrounding the infection to limit the spread of the invader. This is termed hypersensitive response (HR) and is elicited by secreted or surface proteins of the pathogen once it has succeeded in surpassing the first layers of defence. This type of resistance requires the pathogen to be able to overcome physical obstacles and avoid preformed plant metabolites which may be toxic. Resistance in the plant is dependent upon the recognition of, for example, detoxifying enzymes or other avirulence (Avr) proteins by plant resistance (R) proteins (Osbourn 1996). Type II non-host resistance can therefore be considered more specific.

Unless preformed barriers prevent the successful infection by the pathogen in the first place, some mode of recognition is necessary for the plant to activate appropriate defence responses, both in non-hosts and resistant hosts. The routes to plant resistance comprising recognition and reaction are illustrated in a zigzag model (**Fig. 2**). The first point of recognition is via Pattern-Triggered Immunity (PTI). This is elicited by the recognition of molecules either present on the pathogen surface or excreted by it. If these bind to a plant receptor and are identified as foreign, the plant activates inducible defence responses which act to delimit the spread of the pathogen or the development of disease. To counteract this response, the pathogen may produce effectors which block the activated plant defences, for example by

interfering with signalling pathways or degrading anti-microbial toxins. These effectors can also be recognised by the plant, leading to Effector-Triggered Immunity (ETI). The plant can either directly interfere with the virulence proteins by binding to them or blocking the target of these proteins (Hammond-Kosack and Parker 2003). This type of immunity is gene-dependent, as it relies on the interaction between Avr and R genes and is associated with HR (Jones and Dangl 2006; Shabala 2012). This concept of recognising pathogen elicitors and inducing appropriate defences, which in turn prompt the pathogen to produce different compounds, can theoretically continue. Eventually, there will either be a compatible interaction in which the plant no longer recognises the pathogen or the pathogen successfully compromises the response, or an incompatible reaction in which the plant can respond adequately to the pathogen. The former leads to disease, the latter to resistance.

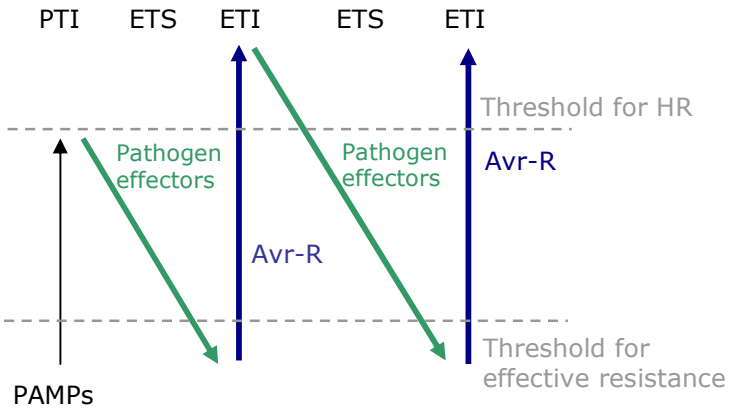

Fig. 2 Zig-zag model of Pattern-Triggered Immunity (PTI), Effector-Triggered Susceptibility (ETS) and Effector-Triggered Immunity (ETI) in plant defence responses. The model illustrates the dynamic interaction between recognition of the pathogen, activation of defences and avoidance of defences by the pathogen. Avr-R: recognition of Avirulence proteins (pathogen effectors) by R proteins. It is shown that HR is associated with ETI.
(after: Jones and Dangl 2006)

Induced resistance

An additional type of resistance to pathogens not mentioned previously is induced resistance. Induced resistance is the mechanism by which abiotic or biotic elicitors activate physical or chemical plant resistance barriers (Kloepper *et al*., 1992; Lyon, 2007). The possibility to induce the natural resistance mechanisms of plants bears a promising alternative to using toxic pesticides or gene modifications to decrease the susceptibility of staple crops to diseases which affect yield or quality. These are often controversial as they may affect other biota, apply strong selection pressure on the pathogen, which could render it resistant to the treatment and are associated with ethical issues. Induced resistance, in contrast, only leads to a reduction in disease by 20 to 85%, enabling the pathogen to survive without adapting to overcome plant defences, while maintaining a satisfying amount of healthy crop (Walters and Heil, 2007). The variation in effectiveness is mostly caused by differences in the genotype within host or pathogen species, the variable costs of allocating resources to defence compound production and interactions with the abiotic environment (reviewed in Walters 2013). Furthermore, induced resistance decreases the resource cost of defence compared with, for example, genotypes with constitutively active defences which are unnecessary.

Two types of induced resistance are currently recognized and will be elaborated on in the following: Systemic acquired resistance (SAR) and induced systemic resistance (ISR).

SAR can be activated by pathogens which cause necrosis either as a symptom or an HR, by non-pathogenic microorganisms or by chemicals (Durrant and Dong 2004; reviewed in Sticher *et al.* 1997). Molecularly, the response is characterized by the

local and systemic expression of pathogenesis-related (PR) proteins and salicylic acid (SA) (Durrant and Dong 2004; van Loon *et al*. 2006). Biotic stimuli may be perceived through the aforementioned PTI or ETI recognition models and the signal is mediated through the NON-EXPRESSOR OF PR-GENES 1 protein (NPR1), which acts as a co-activator of PR genes (Tsuda *et al*. 2008; Mishina and Zeier 2007; Dong 2004). It is now established that one of the systemic signals is transduced by the lipid transfer homolog protein DIR1 (DEFECTIVE IN INDUCED RESISTANCE 1) in Arabidopsis, since *dir1* mutants exhibit normal levels of local resistance but do not express SAR or PR proteins systemically (Maldonado *et al*. 2002). It is thought that this mobile signal may regulate methyl-salicylic acid synthesis which is an inactive precursor of SA (Liu *et al*. 2011).

Induced systemic resistance is conferred by beneficial mycorrhiza or plant-growth promoting rhizobacteria like some *Pseudomonas* species (van Loon *et al*. 1998; Pozo and Azcon-Aguilar 2007; Bakker *et al.* 2007). The microorganisms are perceived by microbe-associated molecular patterns (MAMPs) on their surface, resulting in the systemic activation of immune responses (Van Wees *et al*. 2008; Bakker *et al.* 2007). ISR is independent of SA and PR protein production and is instead regulated by the ET and JA pathways which prime the defence responses (Pieterse *et al*. 1996; Pieterse *et al*. 1998; Conrath *et al.* 2006). A further distinction from SAR is the specificity observed in some ISR responses, while SAR is often effective across a broad range of plant species (Van Wees *et al*. 2008).

Several chemical agents were shown to induce resistance such as acibenzolar-S-methyl (ASM) which is effective against rust and ascochyta blight in faba beans until several weeks after application (Sillero *et al*. 2012), or β-aminobutyric acid (BABA) which induces resistance in lettuce against *Bremia lactucae* even when applied two

days after infection (Cohen *et al.* 2011). The mode of action of these chemical activators is usually through the accumulation of defence-related enzymes, proteins or phenolic compounds such as lignin or flavonoids (see for example: Zhang *et al.* 2011; Yoshioka *et al.* 2001; Šašek *et al*. 2012). Some, however, promote resistance in a more interactive manner. For example, phosphite directly inhibits pathogen growth and biochar enhances overall plant fitness by facilitating nutrient retention and promoting formation of mycorrhizal associations (Daniel and Guest 2005; Chan *et al*. 2007; Warnock *et al*. 2007).

More interesting are probably the biological elicitors as many of them are already natural components of the plant environment. Mycorrhizal fungi are associated with the majority of plants and well-known for generally increasing plant fitness through nutrient exchange and competition for space and resources with potential pathogens. Arbuscular mycorrhiza in particular also induce systemic plant defence regulators and enhance PR protein expression, thereby inducing resistance against microbial pathogens but also parasitic nematodes or angiosperms (Campos-Soriano *et al*. 2012; Hao *et al.* 2012; Lopez-Raez *et al.* 2011). Rhizobacteria such as *Pseudomonas fluorescens* are also capable of suppressing diseases, mediated for example through ethylene (ET) or jasmonic acid (JA) signalling pathways *(Weller et al*. 2012). Other elicitors are, for example, ulvans from algal extracts or the fungal genus *Trichoderma*, which is commercially available as a biocontrol agent *(Cluzet et al*. 2004; Woo *et al.* 2006). Lastly, the yeast *Saccharomyces cerevisiae* was found to induce resistance against the pathogens *Pseudomonas syringae* and *Botrytis cinerea* in *Arabidopsis thaliana*, which is a non-host for the yeast (Raacke *et al.* 2006). Autoclaved suspensions of *S. cerevisiae* induce the SA-responsive PR genes and camalexin and reduced the growth of both pathogens (Raacke *et al.* 2006). The

protective effect of the yeast suspension against *P. syringae* was not seen in mutants of the SA pathway, indicating that this pathway is necessary for induced resistance against the pathogen, while JA and camalexin mutants were similarly susceptible to disease as wild-types (Raacke *et al*. 2006). Similarities between non-host and basal resistance have previously been established for *P. syringae* (Delaney *et al.* 1994; Zimmerli *et al*. 2004; Raacke *et al.* 2006; Henry *et al.* 2012). This study shows that elucidating the pathways responsive to non-host resistance may give insights into the basal defence mechanisms involved against a pathogen. Yeasts are especially suitable as biological control agents in agricultural applications, as they are non-toxic, biodegradable and cost-effective (Raacke *et al*. 2006).

Inducible plant defence signalling pathways

Plants have evolved several physical and chemical defences which are constitutively expressed and often protect against a broad range of pathogens. These include the cuticle, waxes and compounds of antifungal activities such as phenols. Successful pathogens are able to circumvent these barriers by appropriate penetration mechanisms and avoidance or detoxification of antibiotics. However, infection is the exception and high metabolic costs are associated with the reallocation of resources from growth and development to defences (Barto and Cipollini 2005; Walters and Heil 2007). Furthermore, the diversity of potential pathogens may require a diversity of suitable defences. Therefore, a tightly regulated network of defence signalling pathways has evolved which can be activated as needed. The backbone of this network consists of the SA, JA and ET signalling pathways (Pieterse *et al*. 2009).

SA is thought to be essential for inducing SAR and resistance against biotrophic pathogens (Glazebrook 2005). Upon perception of a pathogen, the EDS1 (ENHANCED DISEASE SUSCEPTIBILITY 1) and PAD4 (PHYOTALEXIN-DEFICIENT 4) proteins dimerize and induce the accumulation of salicylic acid (Brodersen *et al*. 2006; Feys *et al.* 2001). This is the ligand of NPR1, an SA receptor, and causes a change in redox conditions inside the cells, leading to the monomerisation of NPR1. NPR1 binds to TGA transcription factors (TF) and enhances the binding to SA-responsive promoters of PR genes (Loake and Grant, 2007; Dong 2004). The PR proteins include, for example, PR1, α-Glucanases, Chitinases, Ribosome Inactivating Protein (RIP) and others and exhibit antifungal activities such as enzymatic degradation of the fungal cell wall (α-Glucanases) or inhibit protein translation (RIP) (reviewed in Borad and Sriram 2008).

JA is commonly associated with defence against necrotophic pathogens (Glazebrook 2005). The active JA derivative, JA-Isoleucine (JA-Ile), binds to the CORONATINE-INSENSITIVE PROTEIN 1 (COI1) which promotes the degradation of the jasmonate ZIM-domain (JAZ) inhibitory complex. This releases the repression on JA-responsive transcription factors such as MYC2 and ERF1 which activate JA-responsive defence genes such as the defensin PDF1.2 or the phosphatase VSP2 (Penninckx *et al*. 1998; Liu *et al*., 2005).

ET is mainly thought to act in synergy with JA against necrotrophic pathogens (McDowell and Dangl 2000). Molecularly, ethylene is perceived by plasma membrane receptors such as ETR1 (ETHYLENE RECEPTOR 1) and blocks this receptor, thereby negatively regulating ethylene responses (Kendrick and Chang 2008). In the absence of ethylene, the receptors enable CONSTITUTIVE TRIPLE RESPONSE 1 (CTR1), which is a negative regulator of ETHYLENE-INSENSITIVE

2 (EIN2). In the presence of ethylene, the repression of EIN2 is released and downstream signalling through EIN3 is possible due to an inhibition of EIN3 degradation. EIN3 activates ethylene-responsive transcription factors such as ETHYLENE RESPONSE FACTOR 1 (ERF1), which leads to the expression of ethylene-inducible genes (Pieterse *et al*. 2009; Guo and Ecker 2004).

The most important feature of these signalling pathways is probably their extensive cross-talk which has been studied intensively (for example, Pieterse *et al.* 2009; Kunkel and Brooks 2002; Glazebrook *et al*. 2003; Rojo *et al*. 2003).

In the SA-JA cross-talk, antagonism is most often cited interaction (reviewed in (Koornneef and Pieterse 2008). This negative regulation is exerted by nuclear NPR1 (Dong 2004; Spoel *et al.* 2003). NPR1 regulates the expression of SA-responsive genes which suppress JA-dependent genes such as PDF1.2 (Ndamukong *et al*. 2007). However, synergistic effects have also been reported when Arabdopsis were induced with SA and JA at the same time. This treatment resulted in an elevation of both PR1 and PDF1.2 (Mur *et al*. 2006).

JA and ET are usually considered to act in synergy. For example, expression or PDF1.2 requires the activation of both pathways (Penninckx *et al.* 1998). The transcription factors ERF1 is also induced by both elicitors (Lorenzo *et al*. 2003). A more detailed distinction for the responses to these signalling pathways is made by MYC2. MYC2 is a promoter of JA-responsive genes like VSP2 but a repressor of JA/ET-dependent genes like PDF1.2 which require activation by ERF *(Lorenzo et al.* 2004). This differential gene expression was also shown in infection experiments and suggests that the ET pathway and the interaction between ERF and MYC2 function to elicit the appropriate JA responses.

Lastly, the SA and ET pathways are connected by the EIN2 protein. ET enhances expression of the SA-dependent PR1 gene (Lawton *et al.* 1994), which is repressed in *ein2* mutants. This shows that SA signalling is modulated by EIN2 (De Vos *et al*. 2006).

These three pathways provide the backbone to plant defense signalling. Although some interaction has been touched upon, a lot of the cross-signalling components and feedback mechanisms are probably still undiscovered. Furthermore, as reviewed in Pieterse *et al.* (2009), other phytohormones such as abscisic acid, auxins, cytokinins, brassinosteroids and gibberellins also modulate these main defense responses.

Model systems

In an attempt to understand everything around us out of pure curiosity or the desire to improve our living standards, we have always been limited by ethical and practical constraints. For example, we cannot justify testing medication with yet completely unknown side-effects on humans or requiring an individual to take part in a mock accident in order to test the safety standards in a car, instead of using a dummy. Likewise, future climate change scenarios and their effects cannot simply be estimated without considering complex interactions between abiotic and biotic phenomena. For this reason, science has always relied upon model systems which enable us to make predictions based on effects observed in more ethical systems or more practical computer algorithms. Plants themselves have served as models for many discoveries in molecular biology which are valid across all ranges of organisms, such as RNA silencing (Baulcombe 2008). Some parallels have also been

drawn from plant pathogen research to human diseases (reviewed in Jones *et al.* 2008), besides the enormous contributions plant models like Arabidopsis have made to improving food quality and quantity.

Although ethical issues are seldom associated with plant pathogen research as long as precautions are taken to prevent contact with natural systems, there are many practical issues which may limit the study of specific plant diseases. Plant pathogens live in a complex environment and often require host or environmental factors to exhibit virulence which may not be given under laboratory conditions. Furthermore, many host plants such as trees have long lifecycles with annual disease cycles, making their research costly in time. For some hosts, the space requirement may be too large to allow the study at institutions which possess other necessary facilities such as laboratories and where the host-pathogen system can be disconnected from natural systems. Lastly, there are few published genomes of both host and pathogen species relative to their abundance and modified genotypes are often non-existent. This greatly limits the elucidation of any molecular mechanisms of interaction.

There are some basic requirements for each model to be considered as a potential model system. They must be tractable in several areas such as genetics, development, transformation and culture (reviewed in Mandoli and Olmstead 2000). A short lifecycle, small size, developed methods for controlled crossing and cheap and easy maintenance are also important. In the special issue *Emerging Model Systems in Plant Biology* of the *Journal of Plant Growth and Regulation*, model systems were selected according to their evidenced viability as a model and their novelty in a branch of phylogeny not well covered by better established systems (Mandoli and Olmstead 2000).

Arabidopsis thaliana is a model plant species related to mustard and has been used in numerous plant-microbe interaction models. Its advantageous properties for studying molecular plant genetics have already been acknowledged over 25 years ago by Pang and Meyerowitz (1987). It was the first plant to have its genome sequenced by the Arabidopsis Genome Initiative in 2000 and nowadays The Arabidopsis Information Resource (TAIR) maintains an entire database solely dedicated to genetic and molecular data collection for this species (http://www.arabidopsis.org/).

A. thaliana has been used as a model plant for many plant-pathogen systems. Some particularly well-studied ones include, for example, *Pseudomonas syringae* pv. *tomato* (Whalen *et al.* 1991), *Botrytis cinerea* (Gonzalez *et al*. 2006) or *Fusarium oxysporum* (Diener and Ausubel 2003). If such highly established model systems for plant diseases exist already, why should we endeavour to create more? Approximately 300,000 vascular plant and bryophyte species are currently accepted in The Plant List, a collaboration by several botanical gardens to list all known plant species (www.theplantlist.org). As noted by Mandoli and Olmstead (2000), it is needless to say that no single one of them can encompass a representative fraction of the diverse properties of all of them. This aspect is even more pronounced in fungal diversity. An estimated 5.1 million species of fungi exist today (Blackwell 2011), with many of them still undiscovered, and a mere ten pathogenic ones of them were represented by Dean *et al.* (2012) due to their scientific and/or economic importance. In this list of top ten, only one yeast pathogen is represented (*Ustilago maydis*) despite the small economic impact of the disease which it causes and the advantages of exploiting yeast both as an inducer of resistance and easy to manipulate organism. Furthermore, no forest pathogen seems to have enough importance to make it into

the top ten, even though some of these are able to wipe out an entire tree species in a country, as mentioned previously in the case of chestnut blight. Although forest pathogens might currently be considered of little scientific or economic importance, possibly because of the many indirect and unapparent services which forests provide, threats from translocation of pathogens or changes in pathogen viability and spread due to climate change make it necessary to develop model systems for them. Model systems are especially useful in forest pathology, as the size and longevity of trees prompts more practical ways of studying their diseases.

The birch pathogen *Taphrina betulina*

The genus *Taphrina* compromises approximately 100 species of dimorphic yeast pathogens of plants (Mix 1949). They are early diverging ascomycetes in the subphylum *Taphrinomycotina*, which includes *Schizosaccharomyces*, *Pneumocystis* and *Saitoella* (**Fig. 3**) (Sugiyama *et al.* 2006; Liu *et al*. 2009).

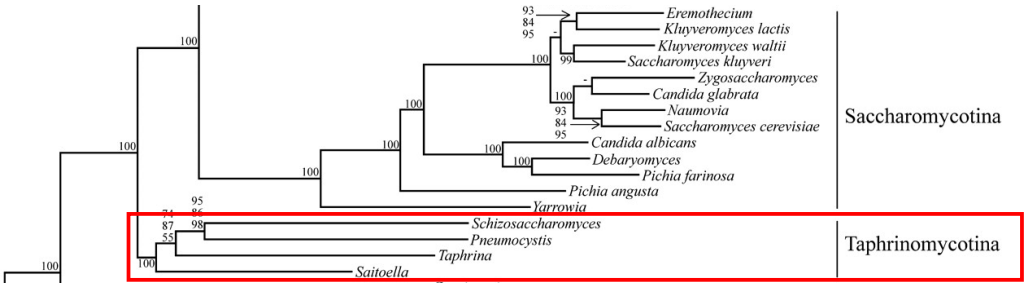

Fig. 3 Excerpt from the phylogenetic tree of ascomycetes showing the relationship between *Taphrinomycotina* and *Saccharomycotina* according to Sugiyama *et al*. 2006 (adapted).

Taphrina affects mostly woody hosts on which it causes malformations such as leaf blisters, leaf curl, stem galls or witches' broom (reviewed in Mix 1949).

Economically the most important species is *Taphrina deformans* which causes peach leaf curl on *Prunus* species like almond, peach and nectarine (Mix 1956). The disease is characterised by curling and puckering of the leaves which turns red and may senesce early. Severe defoliation can lead to yield losses and fruits can directly be affected by drying and cracking (Prakash and Nautiyal 1988; *vonBroembsen et al.* 2004). The disease occurs wherever the peach host is grown with up to 60% of symptomatic fruit reported before in Italy (Commonwealth Mycological Institute 1981; Rossi *et al.* 2005).

All *Taphrina* species form a dikaryotic mycelium intercellularly, subcuticularly or within the epidermal cell walls in plants but grow as yeast cells in culture medium (Mix 1949; Mix 1949). The asci develop from ascogenous cells, which arises from nuclear fusion. The uninucleate, haploid ascospores are produced inside the asci through mitotic and meiotic cell divisions, resulting in four or eight spores per ascus (Martin 1940; Eftimiu 1927). The ascospores can bud to produce blastospores before and after spore release (Mix 1949). These blastospores can survive the winter and cause infection in the next year (Mix 1939).

Some *Taphrina* species cause witches' brooms on birch trees such as *T. americana* on *B. occidentalis*. However, other birch pathogens of the Taphrina genus were too similar in their ITS region to separated on the species level and were hence all classified as *Taphrina betulina* (Rodrigues and Fonseca 2003). These isolates of *Taphrina betulina* cause witches' broom on *Betula* spp., including silver birch (*B. pendula*), downy birch (*B. pubescens*) and dwarf birch (*B. nana*) (Mix 1949). This disease is characterized by an overproduction axillary shoots in a cluster at the site of the infected bud (Jump and Woodward 1994) (**Fig. 4**).

Fig. 4 a) Witches' brooms in a *B. pubescens* tree **b)** Cross-section of a broom, tumour approx. 5 cm long. Photos courtesy of Dr. Kirk Overmyer.

In susceptible trees, the symptomatic brooms are highly localized but may reach a diameter of approximately one metre in mature trees. Within a birch stand, susceptible individuals are usually mixed with resistant ones and the size and amount of brooms can vary from tree to tree (personal observations). New shoots produced on older brooms usually die during the second or third winter after emergence (Henderson 1954). Jump and Woodward (1994) described the histology of brooms. The infected bud develops a thick, swollen shoot from which the axillary buds grow in a cluster and may produce new shoots. In older brooms, the centre of the broom forms a thick tumour containing an expanded phloem which resembles callus tissue and inclusions of shoot bases and buds. The broom xylem is disorganised, probably due to an attempt to connect it to all the newly emerging shoots.

There is little information about the effect of *Taphrina* infection on the fitness of birch trees. In a study on managing bird forests to produce quality timber, Cameron found that witches' broom have no significant effect on the growth of affected trees (Cameron 1996). In contrast, Spanos and Woodward (1994) reported a height reduction by 25% in infected birches with younger trees affected by 33% and trees with large brooms close to the base by up to 50%. Distorted stems were also found,

which greatly reduce the quality for timber. Nearly 80% of infected trees also showed low health and growth. Furthermore, leaves in *Betula maximowicziana* infected with *T. betulina* senesce early and show an increase in photosynthetic rates, dark respiration rates and chlorophyll shortly after emergence, followed by a rapid decline (Koike and Tanaka 1986). The rapid metabolic decline and early leaf shedding is probably due to the exhaustion of resources which are reallocated to defence responses. This study shows that the physiological effect of *Taphrina* infection on the individual tree can be quite dramatic.

The choice for using Arabidopsis as a model plant hardly needs to be justified, as it is so well established that very few other plants could compete with the same amount of tractability. We already listed some of the advantages of this model plant above. Since a tree pathogen was studied for this Master's thesis, a model system was required because time limitations would not allow to study the annual cycle of *Taphrina* in its natural host.

In this study, we chose *Taphrina betulina* as a model pathogen for studying plant pathogenic yeasts. Considering this introduction to plant-pathogen systems, several reasons led to the choice of this pathogen.

Plant pathogenic yeasts are under-represented in the major fungal plant pathogens studied currently. We think that the study of dimorphic plant pathogenic yeasts should be promoted, as their distinctive life cycle provide an additional characteristic not found in obligate hyphal parasites. The dimorphic switch requires environmental and/or chemical signals which make it possible to infer host or environmental factors which cause susceptibility. These may apply to other plant-pathogen systems. Furthermore, hyphal growth is required for pathogenecity. The

molecular changes leading to this switch may give insight into pathogenecity factors which, too, may apply to other pathogens.

Forest pathogens are under-studied although they can have much greater aerial effects than for example crop pathogens, due to the extent of forests. Since genetic tools are now being developed to increase the quality of timber in managed production forests, there is a risk of decreasing genetic diversity within monocultures. This could easily lead to outbreaks of diseases like witches' broom, which may not kill the tree population but render them unsuitable for their purpose as timber. On the other hand, the availability of the *Betula* genome, as one of few available tree genomes, allows the derivation of molecular pathways found in a model system by comparison of homologous patterns in the host. Although poplar is now the most widely studied tree species and likewise affected by *Taphrina* (leaf blister caused by *T. populina*), generation times of seven years make it rather impractical to use if one wants to upscale from an Arabidopsis model system to the true host.

The genome of *T. deformans* has also recently been published and efforts to sequence and annotate the *T. betulina* genome are under way (Cissé *et al.* 2013). In combination with the well-studied genome of Arabidopsis and the availability of Arabidopsis mutants, *T. betulina* is suitable for in depth characterisation of a new pathosystems. There is also great novelty in studying this pathogen, as most research on it dates back to the 19th century and more modern publications concentrate almost solely on *T. deformans*. Although this may validate intensification in the study of *T. deformans*, this pathogen only occurs in southern regions where its hosts are found. *T. betulina*, in contrast, is of regional importance in Finland. Northern Finnish Lapland is characterised by moutain birch (*B. pubescens.* ssp. *czerepanovii*) forests.

Mountain birches are a subspecies of the Downy birch and thought to have arisen from a cross between *B. pubescens* and *B. nana*. In this subarctic environment, the climate is particularly demanding on these trees, resulting in high levels of infections. In a changing climate, disease development may be promoted to a level that impacts tree survival, posing a risk to the entire forest ecosystem.

Proposed Investigation

Non-host resistance bears similarities to host defence mechanisms and is the prevalent state against the large majority of pathogens. In the area of plant pathology, dimorphic pathogenic yeasts of forest trees have been under-represented. In this project, we will study the non-host resistance in the *Arabidopsis-Taphrina* model system. Our aim is to investigate mechanisms of non-host defence against this pathogen and evaluate whether this pathosystem can be used as a model for studying plant pathogenic yeasts. Specifically, we will examine how Arabidopsis defence signalling mutants react to challenge by *T. betulina* and whether the pathogen can be visualised *in planta*.

Additionally, we will explore whether *T. betulina* can induce resistance in Arabidopsis against *Pseudomonas syringae* pv. *tomato* (*Pst*) in order to further our understanding of possible defence mechanisms and evaluate whether *T. betulina* could become a tool for biological enhancement of resistance in non-host plants.

Materials

Microorganisms

All our yeasts were acquired from the Portuguese Yeast Culture Collection (PYCC; Caparica, Portugal). We used strains of *Taphrina betulina* (PYCC5889), *Taphrina deformans* (PYCC5710) and *Taphrina robinsoniana* (previously *T. betulina* CBS417.54, but now recognised as *T. robinsoniana,* see (Rodrigues and Fonseca, 2003)) for species identification. Only *T. betulina* was used in subsequent experiments.

The bacterial organism was *Pseudomonas syringae* pv. *tomato* strain DC3000 (Nimchuk *et al.* 2000).

Plants

We used *Arabidopsis thaliana* col-0 plants as a wild-type organism and the following mutants thereof (acquired from University of Nottingham Arabidopsis Stock Center (NASC), http://arabidopsis.info/):

genotype	mutant gene	function of gene product
eds1	ENHANCED-DISEASE SUSCEPTIBILITY 1	Regulator of basal resistance
efr1	ELONGATION FACTOR TU RECEPTOR 1	Receptor for bacterial elongation factor Tu
ein2-5	ETHYLENE-INSENSITIVE PROTEIN 2	Downstream mediator of ethylene signalling
fad3/7/8	FATTY ACID DESATURASES 3/7/8	Synthesis of JA precursor

genotype	mutant protein	function of protein
fls2	FLAGELLIN-SENSITIVE 2	Receptor for bacterial flagellin
jar1	JASMONATE RESISTANT 1	Catalyses conjugation of active JA-amino acids
npr1	NONEXPRESSOR OF PR GENES 1	Co-factor for SA-responsive genes
pad4	PHYTOALEXIN DEFICIENT 4	Involved in SA signalling and phytoalexin production

Methods

Yeast Identification

Yeast cells grown on 0.2X potato dextrose agar (PDA; Sigma-Aldrich PD broth +agar: 4 g/l dextrose, 0.8 g/l potato extract, 20g/l agar) were suspended in sterile distilled water and a 5 µl drop placed onto a glass microscope slide covered with a plastic cover slip for light microscopy.

The ITS region of the genome was amplified with specially designed forwards and reverse primers (Sigma-Aldrich). The polymerase chain reaction (PCR) protocol was 5 min at 94 ℃, (10 s at 94 ℃, 20 s at 50 ℃, 30 s at 72 ℃) x 35 and 5 min at 72 ℃. The restriction enzyme digests for RFLP (restriction fragment length polymorphism) analysis were carried out with AluI and TaqI restriction enzymes (Fermentas) in a 1X digestion buffer (Fermentas Fast Digest) and incubated for 37 ℃ (AluI) or 60 ℃ (TaqI). For DNA gel eletrophoresis, 6 µl of the sample together with a DNA marker ladder (Fermentas GeneRuler 50bp) were run for 1h15 at 120 V.

Yeast Growth

Cultures of *Taphrina betulina* were propagated on round plates (ø 9 cm) containing solid yeast minimal medium (BD Difco™ Yeast Nitrogen Base without Amino Acids). The plates were kept in growth cabinets (Sanyo MLR-350) at defined conditions (18 °C, 12hr photoperiod between 7 am and 7 pm).

Plant Growth

Seeds of *Arabidopsis thaliana* were sown onto well-watered soil (peat:vermiculite 1:1) in 7 cm pots. Initially, the pots were placed into a mini-greenhouse with a plastic cover and kept at +4 °C in the dark for two days in order to synchronize germination of all genotypes. The greenhouses were then transferred into growth chambers with defined growing conditions (23 °C/18 °C day/night temperature, 70%/90% day/night humidity, 12 hr photoperiod between 7 am and 7pm, irradiance approx. 120 μmol $m^{-2}s^{-1}$). When the cotyledons appeared and the seedlings appeared large enough to handle with forceps, they were transferred to new 7 cm pots at one plant per pot. The plants were kept in the growth chambers until they had reached the required size or age for the experiment and watered as necessary (every three to four days).

Taphrina Spray Infection

T. betulina cells were suspended in $MgCl_2$ at $OD_{600}\approx2$. Arabidopsis plants were sprayed with the cell suspension or a control solution of $MgCl_2$ at three weeks old

(approximately 1.5 ml per plant) and kept under a plastic greenhouse cover for eight days. All plants were carefully removed from the pots after the inoculation period and photographed separately. The leaves displaying necrotic lesions were removed from the plant and placed next to the rosette for the photographs. The area of the whole plant and the area of lesions were distinguished by the difference in contrast between green and white/beige and measured with ImageJ software (National Institute of Health, US; http://rsbweb.nih.gov/ij/) which can measure an area according to a difference in contrast from the surroundings.

Maximum Quantum Efficiency

Four-week old, dark-adapted plants were placed under the detector unit of a fluorometer (IMAGINING-PAM *M series* MAXI, Heinz Walz GmbH, www.walz.com). Plants were subjected to a pulse of measuring light (ML) and subsequently to a pulse of saturating light (2800 μmol $m^{-2}s^{-1}$) every 30 s for 4 min. Another ML pulse was emitted 30 s after the last saturating pulse. The fluorescence was measured at each point of light treatment. The average fluorescence at ML was used as the value for minimum fluorescence (F_0) and the average fluorescence at saturating light conditions was used for the F_m value (maximum fluorescence). The variable fluorescence F_v was calculated as the difference between the average F_m and F_0. The maximum quantum efficiency was defined as the ratio of F_v/F_m.

Trypan blue Stain

Rosettes of *Arabidopsis thaliana ein2-5* mutants were placed into 20-30 ml trypan blue stain solution (diluted 1:2 in 95 % ethanol) in a 50 ml Sarstedt tube. The tubes were placed in boiling water for 10 min. After 10 min of cooling down, the trypan blue solution was discarded and the tubes were inverted onto paper towels to drain the remaining solution. The non-permanently stained areas were destained by washing the plants three times in 20-30 ml of chloral hydrate (2.5 g/ml) in two day intervals. The plants were then stored in 60 % glycerol and analysed under a light microscope.

Seed Sterilization

Seeds were sterilized by incubating them on a shaker for 5 min in 300 µl 2 % Triton/70 % ethanol. The seeds were washed three times in 500 µl 70 % ethanol by inversion and finally in 100 % ethanol. They were transferred onto filter paper sterilized with 70 % ethanol and dried for 5 min.

Growth on Yeast Extract

Sterilized seeds of col-0, *efr1*, *ein2-5, fls2,* and *jar1* genotypes were placed in a line on 13 cm square plates containing 50 ml 0.5x Murashige & Skoog medium (2.15 g/l MS basal salt mixture, 0.5 g/l MES hydrate) with 10 g/l sucrose and 10 g/l agarose or the same medium containing dead *T. betulina* cells (OD ≈ 0.2). The progress of latitudinal primary root growth was followed by marking the root tips

after each measurement. Roots length was measured by taking a photograph of the plates and a size marker and measuring the length of the root using ImageJ software.

Hand Inoculation

For the inoculation with *Pseudomonas syringae* pv. *tomato*, approximately 4-week-old plants were used. The control plants had previously been sprayed with a 10 mM $MgCl_2$ solution while the test plants had been spray-infected with 10 mM $MgCl_2$ + *Taphrina betulina* at OD_{600}≈0.2. Four leaves per plant were marked with a water-resistant pen. All plants were sprayed with water and covered with a plastic greenhouse cover for two hours. The bacteria were suspended in 10 mM $MgCl_2$ at OD_{600} ≈ 0.00002 in a 50 ml Sarstedt tube. On day 0, the marked leaves of control plants were inoculated with 10 mM $MgCl_2$ by pushing the solution through a 2 ml needleless syringe on the underside of the leaf until the whole leaf was infiltrated. The other plants were infiltrated in the same manner with the *Pseudomonas* inoculation. On day 0 and day 3, leaves were harvested for bacterial growth curve determination.

After six and 96 hours, a 0.2 cm^2 area of each marked leaf from five plants per genotype was collected. Four discs from five different plants of the same genotypes were pooled into a tube containing 200 µl of sterile distilled water. The tissue was ground using a plastic rod connected to an electrical rotator. The samples filled up to 1ml with sterile water and three (for day 0 samples) or six (for day 3 samples) tenfold serial dilutions were plated in 5 µl drops onto King's B media (10 g/l Proteose Peptone, 1.5g/l anhydrous dipotassium phosphate, 15 g/l glycerol, 15 g/l Bacto-agar) plates containing rifampicin (50 µg/ml), kanamycin (30 µg/ml) and

cycloheximide (25 µg/ml). Individual colonies were counted as soon as they became visible under the microscope.

Overlay Assay

Taphrina betulina was grown on square plates (13 cm x 13 cm) with 50 ml potato dextrose agar (4 g/l potato starch, 20 g/l dextrose, 2 % agarose; Difco) either as a lawn in 200 µl sterile water spread across the solid surface or as three times five 5 µl drops of sterile water ($OD_{600} \approx 0.2$). After two days, 15 ml of Luria Broth (LB) agar (10 g/l tryptone, 5 g/l yeast extract, 5 g/l NaCl; 2 % agarose) were added to the dishes. After drying, a suspension of *Pseudomonas syringae* pv. *tomato* ($OD_{600} \approx 2$) was plated on the layer of LB. On the plates with a lawn of *T. betulina*, three times eight serial dilutions of *P. syringae* were plated, while a lawn of *P. syringae* (200 µl) at the same concentration was spread onto those plates containing drops of *T. betulina*.

Results

Characterization of *Taphrina* species

The identity of pathogens used in subsequent experiments was assessed by light microscopy study of the cell morphology and investigation of colony appearance on 0.2X PDA plates.

The cells of the three proposed species (*Taphrina betulina, Taphrina deformans* and *Taphrina robinsoniana*) had the typical oval shape of yeast cells and ranged in size from 4 µm to 9 µm, with *T. robinsoniana* visibly smaller at 2 µm to 7 µm (**Fig. 5**). A few cells appeared round with a thicker cell wall and some cells observed were budding.

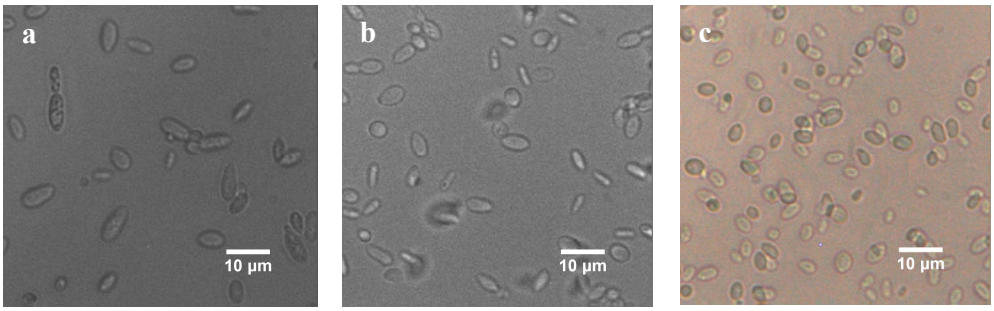

Fig. 5 Light microscope images of **a)** *T. betulina*, **b)** *T. deformans* and **c)** *T. robinsoniana* at 40x magnification.

The colonies were round with a slightly irregular edge and beige in colour for *T. betulina* and *T. deformans*, while *T. robinsoniana* was slightly pink. Confluent circles of colonies appear to grow up evenly in thickness and not spread out beyond the edge of the circle (data not shown).

We then verified the species of fungal isolates by RFLP with Taq1 and Alu1 restriction enzymes and DNA gel electrophoresis of the ITS region. The visualised

bands showed the expected patterns for *T. betulina, T. deformans* and *T. robinsoniana* with this band here and that band there, based on the known ITS sequences for these species (NCBI accession numbers: *T. betulina* AF492080, *T. deformans* AB505447, *T. robinsoniana* AF492116) (**Fig. 6**). For the following experiments only *T. betulina* was used.

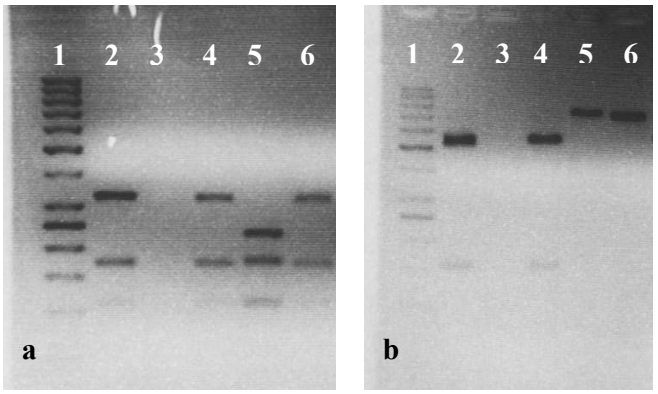

Fig. 6 RFLP of the ITS region in *T. betulina*, *T. deformans* and *T. robinsoniana*. **a)** DNA gel electrophoresis of the amplified Taq1-digested ITS region **b)** DNA gel electrophoresis of the amplified Alu1-digested ITS region.

Identification of mutant *Arabidopsis thaliana* lines

The genotypes col-0*, eds1, efr1, ein2-5, fad 3/7/8, fls2, jar1, npr1* and *pad4* were from well characterized laboratory stocks so their genotype and homozygocity was not confirmed again. LYM1, CERK1 and LECRK-1.9 lines were of further interest but no homozygote plants had yet been identified. Plants of different lines of each of these three genotypes were tested for homozygocity by DNA isolation and amplification of the insertion sequence and the result was visualised by DNA gel electrophoresis. After multiple attempts, the DNA gel showed no bands for any of these genotypes even though the bands for the primers were visible, the enzyme, nucleotide and buffer mixture were proven to be functional in other reactions and the

PCR conditions were varied repeatedly (data not shown). As heterozygous plants are unsuitable for confident conclusions drawn from experimental results, these genotypes were not included in any experiments.

T. betulina infection of *A. thaliana*

We hypothesized that certain stress and defence signalling mutants of *Arabidopsis thaliana* will show disease symptoms to infection by *T. betulina* if the disrupted signalling pathways are involved in the recognition and defence against this non-host pathogen. The selected plant genotypes were sprayed with the fungus and visible and non-visible symptoms were observed and analysed.

Approximately four days after the inoculation, leaf lesions appeared on some plants as brown, necrotic tissue. This development was limited to inoculated plants while control plants did not show such symptoms. After one week of observation, leaves of control plants also showed signs of necrosis (**Fig. 7**). This was to a lesser extent in all genotypes except wild-type plants, which seemed to have similar areas of senescent tissue in both control and infected samples. We hypothesized that if the lesions are a symptom of disease development, susceptible plants will have significantly more necrotic tissue in inoculated samples than in controls.

In order to quantify the extent of lesion development, all plants were harvested and photographed after eight days and the area of necrotic tissue was measured using ImageJ software. Due to the difference in sizes of plants of different genotypes, the affected area was calculated as a percentage of total plant surface, rather than the net area in mm^2. For practical reasons, it was assumed that all plants exhibit a similar degree of overlapping of leaves when measuring total leaf surface area.

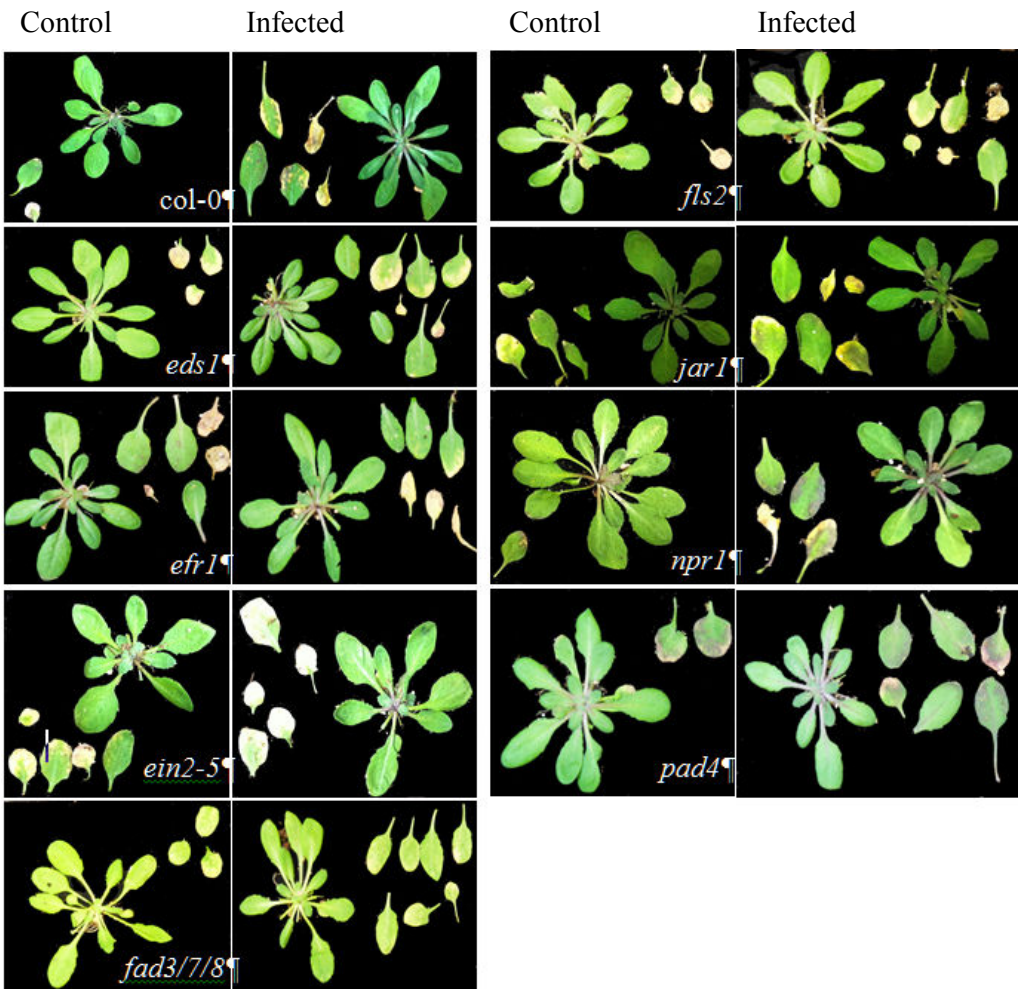

Fig. 7 Lesion appearance on Arabidopsis genotypes sprayed with $MgCl_2$ (control) or a solution of T. betulina (OD≈0.2; infected) eight days post-inoculation. Slight differences in colouring are due to light variation whilst taking the photographs.

The lesion development ranged from 0.2% (*npr1*) to 3.3% (*efr1*) in control plants and from 1% (*jar1*) to 7% (*ein2-5*) in infected plants (**Fig. 8**). The mean percentage of lesions across all genotypes was approximately twice as high in infected compared to uninfected plants (3.1±4.3% compared to 1.5±2.7%). Although the deviations from these means are quite large, the nonparametric Mann-Whitney and Kruskal-Wallis tests confirm a difference of high significance between control and infected samples (p=0.000). There is no significant difference (p=0.07) of lesion

levels across control genotypes, which displayed an unusually high percentage of lesions in the control plants, is excluded from the analysis, while lesion levels between infected genotypes are different (p=0.003).

Among all genotypes, *ein2-5* had the highest percentage of lesions (7%) in infected plants and this was different from *ein2-5* controls (p=0.04) and from infected *jar1, npr1* and *pad4* plants (p≤0.025) (**Fig. 8**).

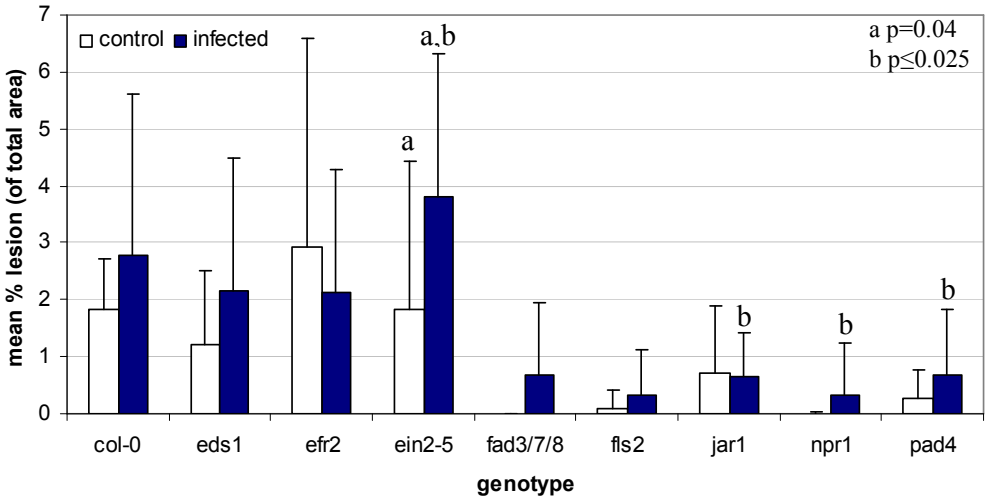

Fig 8. Mean percentage of leaf lesions measured as the amount of necrotic tissue over the total leaf area per genotype. Data taken from three independent experiments. Same letters denote statistical significance. Error bars show one standard deviation. n=18

All results were collated from three independent experiments. A univariate ANOVA with Tukey's and Bonferroni's post-hoc tests was performed and this showed a significant difference in the percentage of lesions in uninfected samples between all experiments (**Fig. 9**) and a significant difference in the percentage of lesions in infected samples between experiment 3 and each of experiments 1 and 2 (data not shown).

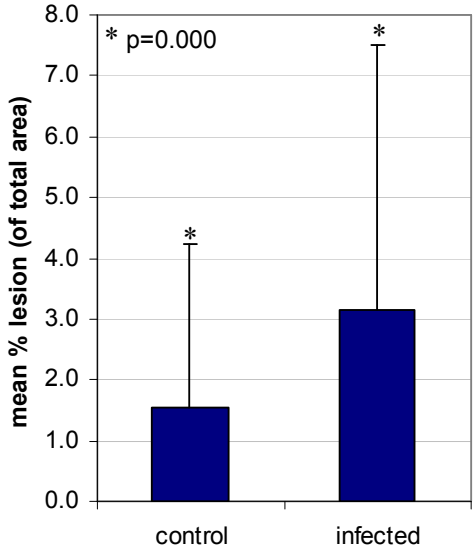

Fig. 9 Mean percentage of lesions measured as the amount of necrotic tissue over the total leaf area for all control samples and all infected samples. Data taken from three independent experiments. Asterisk denotes statistical significance. Error bars show one standard deviation. n=162

Maximum quantum efficiency in infected plants

Pathogens are able to cause phototoxic stress to plants either by directly damaging the photosystem or by activating stress responses (e.g. reactive oxygen species) in the plant which can harm the photosystem (e.g. Manter *et al*. 2007). We hypothesized that genotypes susceptible to infection by *T. betulina* show increased phototoxic stress in photosystem II as a sign of plant stress, characterized by a decrease in the maximum quantum efficiency. The maximum quantum efficiency (F_v/F_m) was measured in dark-adapted plants eight days after inoculation and the mean values of control and infected samples were compared (**Fig. 10**). The mean maximum quantum efficiency of control plants was 0.788 while that of infected plants was 0.789. Interestingly, the infected *ein2-5* mutant had the lowest F_v/F_m ratio (0.779) although this was not significantly different from the *ein2-5* control. There

was no difference in the quantum efficiency between control and infected samples in any genotype and no genotypic effect on the Fv/Fm ratio of infected plants.

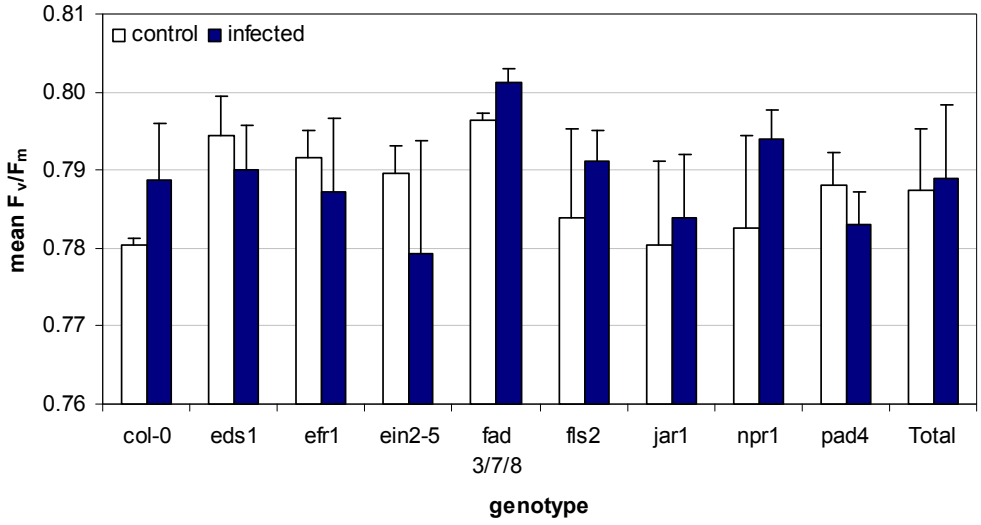

Fig. 10 Average maximum quantum efficiency (F_v/F_m) for *A. thaliana* genotypes eight days after inoculation with *T. betulina* ("infected"; n=4) or with $MgCl_2$ ("control"; n=2). Error bars show one standard deviation.

Visualisation of fungus *in planta*

In order to support the possibility of fungal establishment within the *ein2-5* mutant, an infected plant was stained with trypan blue. This dye stains vasculature, dead plant cells, oomycete and fungal hyphae blue (Strober 2001; van Wees 2008). One healthy-looking Arabidopsis leaf and one showing lesions were visualised under a fluorescence microscope. The green leaf was fairly clear in colour with blue streaks which resemble the structure of the plant vasculature (**Fig. 11a**). In the leaf which had lesions, there was generally a blue background from the stain in addition to dark blue nodules and strings (**Fig. 11b**). The clustering of nodules was higher at the tip of the leaf where the lesion was present (data not shown).

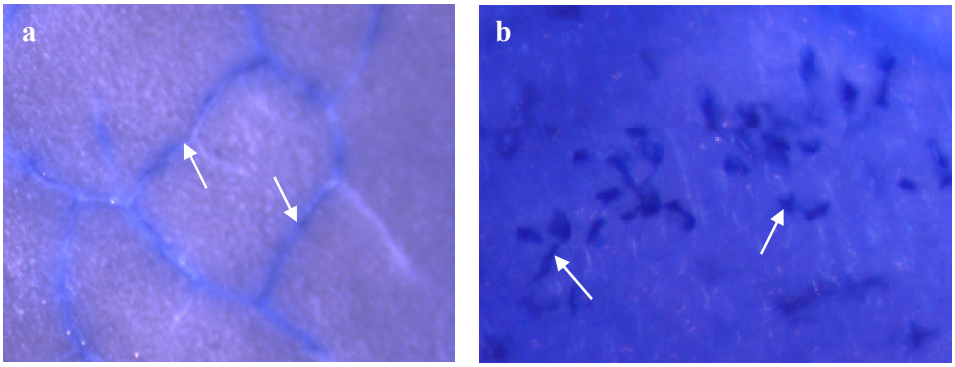

Fig. 11 Trypan blue stain of leaf tissue from ein2-5 mutants infected with T. betulina. **a)** green leaf, **b)** leaf area with lesions. Arrows point to leaf veins in a) and nodules or strings in b).

Root growth assessment

A. thaliana has pathogen recognition receptors (PRRs) which are able to respond to binding by pathogen-associated molecular patterns (PAMPs). PAMPs are molecules contained on the surface of pathogens, giving them a characteristic signature which the plant can recognize. Upon recognition, a signal is relayed intracellularly and an appropriate defence response generated. In incompatible host-pathogen combinations, this leads to the suppression of disease either through a hypersensitive response or a symptomless defence. PAMPs are best studied for bacteria and two commonly studied ones are bacterial flagellin and bacterial elongation factor Tu which are recognized by the FLS2 and EFR1 receptors respectively. Recently, FLS2 has been found to recognise other PAMPs, suggesting a less specific role in pathogen detection (Danna *et al,* 2011). We tested the possibility of recognition of *T. betulina* by these two receptors by growing mutant and wild-type plants on plates containing killed yeast cell extract in the medium and measuring root length as a sign of growth. If either of these receptors is necessary for the successful

defence against *T. betulina*, we would expect that the respective mutant is retarded in its growth on the plates containing the fungal cells compared to control samples. This could be due to a direct growth inhibition of *Taphrina* on the plants or a reallocation of resources to defence compounds rather than growth.

Wild-type plants germinated two days after planting, while the two mutant genotypes required two more days. Measurements began two days after all seeds had germinated. The results are summarised in **Fig. 12**. The delay in germination caused a variation in root depth on the first day of measurement, as some roots had already been growing for longer. Overall, the *efr1* mutants grew the least, with no significant difference between plants plated on control medium and yeast extract medium. In the flagellin-receptor mutants, there was a difference of 6 mm in root length on the first day of measurement, with the control plants having grown more. This difference increased to 10 mm after five days, although this was not significant. Surprisingly, wild-type plants grew on average longer roots on yeast-extract medium, although this difference only precipitated after five days (not significant).

Generally, all plants exhibited a similar trend in growth with a fairly parallel growth rate between treatment types during the first few days after measurements begun and differences only occurring five days later.

In our previous experiment, we showed that *ein2-5* mutants had significantly higher lesions in infected samples. We therefore hypothesized that the ethylene-jasmonate stress response pathway is associated with non-host resistance against *T. betulina*. In order to test whether this is mediated through pathogen elicitors and plant receptors, we additionally grew col-0, *ein2-5* and *jar1* mutants on MS plates containing killed *Taphrina* cells and compared the root length to growth on control.

Germination in all genotypes and both treatments occurred four days after planting. The first measurement was taken two days post-germination. The results are summarised in **Fig. 13**.

Wild-type plants had grown the most on average (≈11.5 mm) when the first measurement was taken. The difference between treatment and control plants was only 1 mm (not significant). This deviation remained approximately the same during the entire measurement period and even decreased to approximately 0.3 mm eight days after germination. This result diverges from the previous experiment in which *col-0* plants were grown with the *efr1* and *fls2* mutants.

The *ein2-5* plants had reached approximately the same length two days after germination but progressively diverged in their growth rate between the treatment groups. On the last day of measuring, there was a difference of 2.5 mm between treatment and control plants, but this was not significant.

The jasmonic acid response mutant was the most clearly affected genotype by the treatment groups. Two days after germination, there was only a small, insignificant difference between control and treatment plants. Subsequently, the rate of growth was delayed in plants grown on yeast extract, resulting in a highly significant difference of approximately 10 mm six days later ($p=0.004$). The shoot development appeared similar to that in control *jar1* mutants.

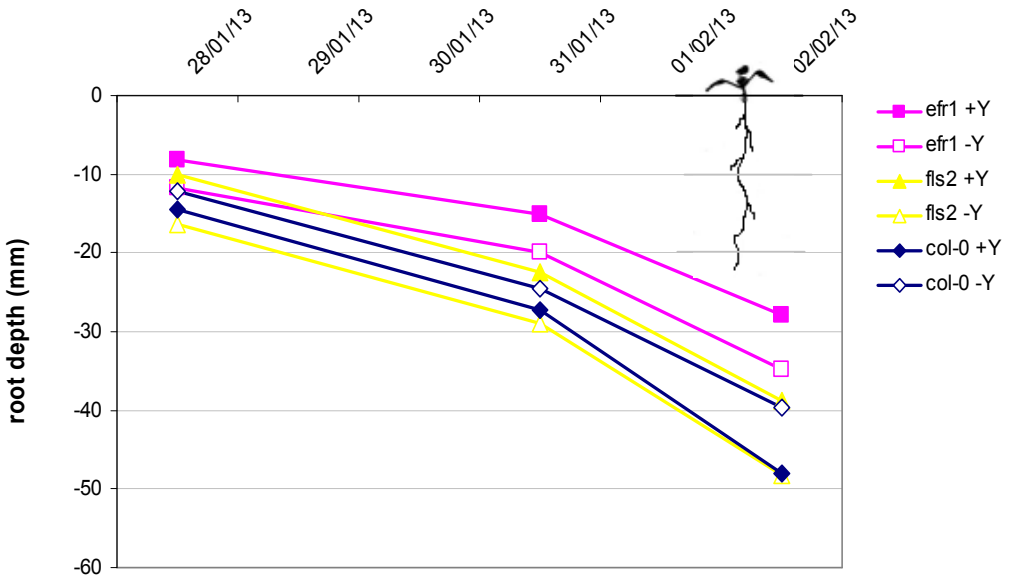

Fig. 12 Average root depth of Arabidopsis wild-type and mutant plants grown vertically on yeast-free 0.5 MS medium (-Y; clear symbols) or 0.5 MS medium supplemented with killed *T. betulina* cells at OD≈0.2 (+Y; filled symbols). Root depth was measured on 28/01, 31/01 and 02/05. n=16 for 28/01 and 31/01; n=7 for 02/02.

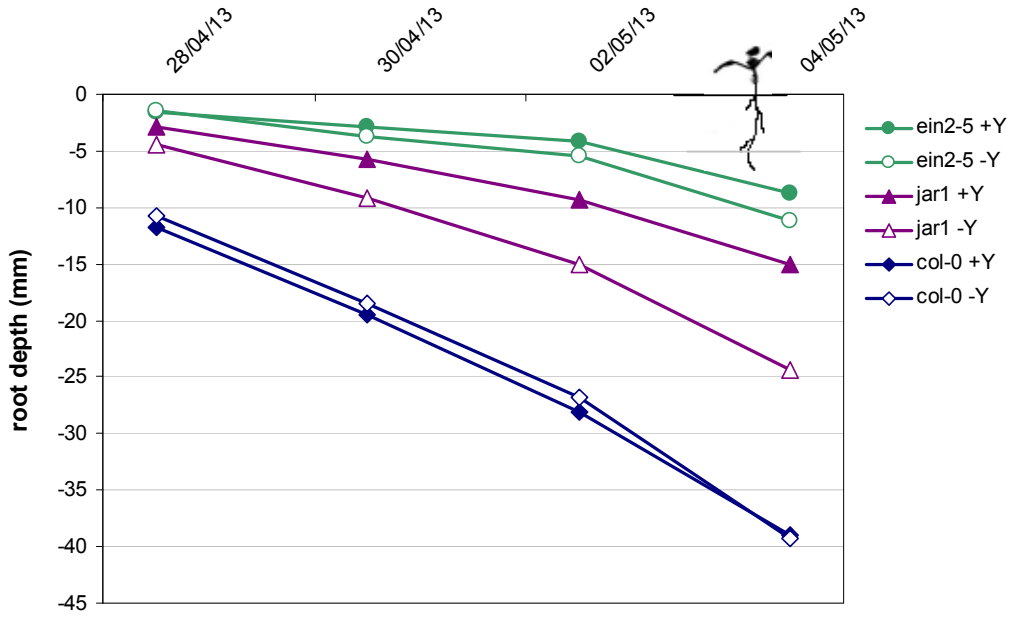

Fig. 13 Average root depth of Arabidopsis wild-type and mutant plants grown vertically on yeast-free 0.5 MS medium (-Y; clear symbols) or 0.5 MS medium supplemented with killed *T. betulina* cells at OD≈0.2 (+Y; filled symbols). n=20

Pseudomonas growth assay

It has previously been shown that yeasts are capable of inducing resistance to other pathogens such as *P. syringae* and *B. cinerea* in *A. thaliana* (Raacke *et al*. 2006). In order to investigate whether *T. betulina* can induce resistance of *A. thaliana* against *Pseudomonas syringae*, wild-type plants sprayed with the fungus (infected plants) or a control solution (control plants) were hand-inoculated with *P. syringae*. The bacterial growth was measured two hours and 72 hours after the spray inoculation with *Taphrina* and hand inoculation of *Pseudomonas*.

Two hours after the inoculation with bacteria, control and *Taphrina*-infected plants contained approximately the same amount of colony forming units (mean=$0.95\pm0.03 \times 10^6$).

After three days, water-soaked lesions could be observed on the leaves inoculated with *Pseudomonas* (data not shown). The number of colonies counted on the plates had risen up to approximately 150. The data is therefore an estimate obtained by counting the colonies in a quarter of the sample circle and interpolating, when the number was too large to count for the whole sample spot with high confidence. The results are summarised in **Fig. 14**. The mean number of colony forming units for control plants had increased by more than three times after three days to 4.9×10^6. In infected plants, an average of 6.5×10^6 cfu's was counted. This is an increase of over 30%. In both treatment scenarios, the growth is significantly different from day 0. There is, however, no significant difference between infected and control samples on day 3. As can be seen in a scatter diagram, the spread of data points is very small on day 0 and very large on day 3 (**Fig. 15**). The results could be replicated similarly in a second experiment (data not shown).

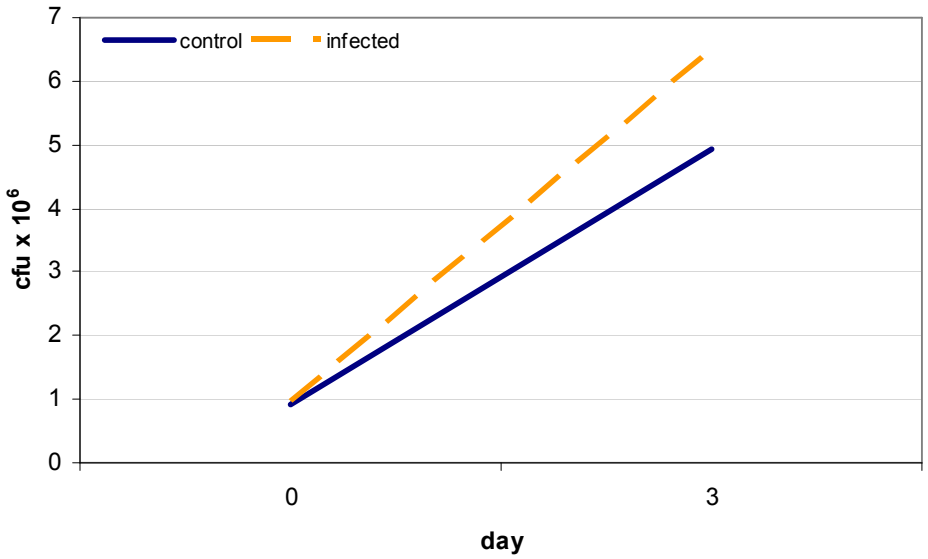

Fig. 14 Mean number of colony-forming units (cfu) of *P. syringae* in plants pre-treated with $MgCl_2$ (control) or a solution of *T. betulina* (infected; OD≈0.2) 0 days and 3 days post-treatment. n=16

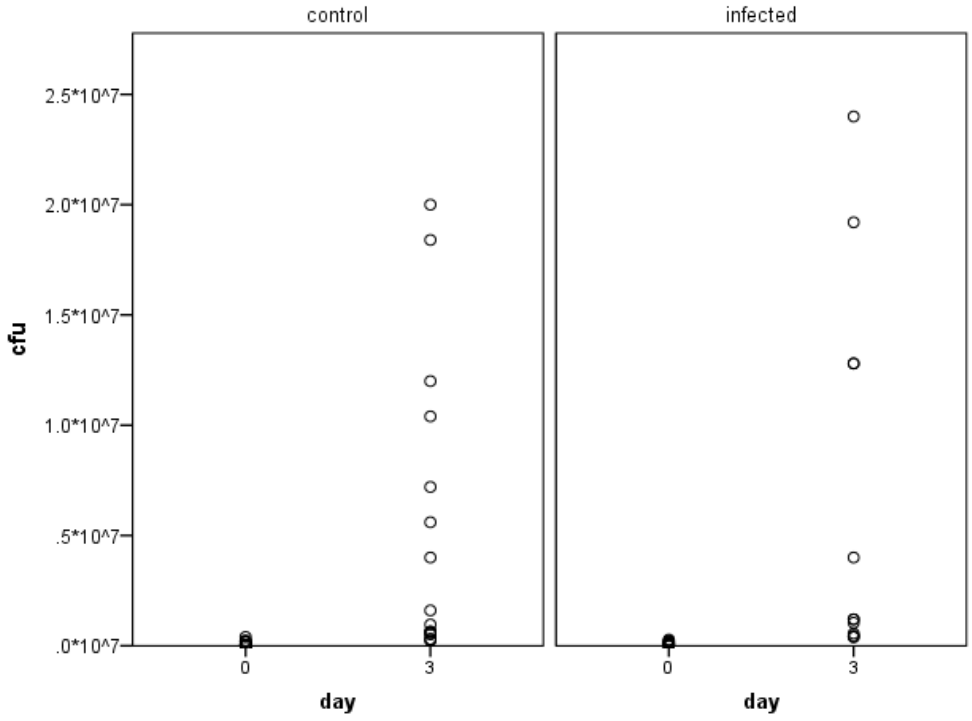

Fig. 15 Scatter diagram showing the variation in numbers of colony-forming units (cfu) of *P. syringae* in plants pre-treated with $MgCl_2$ (control) or a solution of *T. betulina* (infected; OD≈0.2) 0 days and 3 days post-treatment. n=16

Overlay assay

In the previous experiment we observed a trend of increased growth of *P. syringae* in plants previously infected with *T. betulina*. We hypothesized that there is an interaction between the fungus and the bacterium which is either direct or mediated through plant signalling pathways, causing more favourable conditions for growth of *P. syringae*. As *P. syringae* is a known pathogen of *A. thaliana*, increased growth would signify a higher level of disease development and imply changes in the defence mechanisms of the host.

To test whether *T. betulina* has a direct impact on the growth of *P. syringae* in vitro, an overlay assay was conducted. *P. syringae* was grown on a thin layer of LB which was poured on top of PDA. *T. betulina* was grown between the two media layers for test samples. Controls did not contain any fungus.

On a dot plot of *P. syringae*, colonies were visible after two days down to the second dilution. The size and morphology of the colonies appeared the same in treatment and control samples. When *Pseudomonas* were grown on a lawn of *Taphrina*, the mean colony count was $3x10^5$ cfu's/ml. In control samples, the mean was $3.6x10^5$ cfu's/ml (**Fig. 16**). This difference was not significant.

When a lawn of *P. syringae* was grown on top of a dot plot of *T. betulina*, the colonies developed evenly across the plate and after several days the plate was confluently covered by bacteria. No difference in growth pattern could be observed in the regions where *T. betulina* had been grown underneath compared to surrounding areas or control plates (**Fig. 17**).

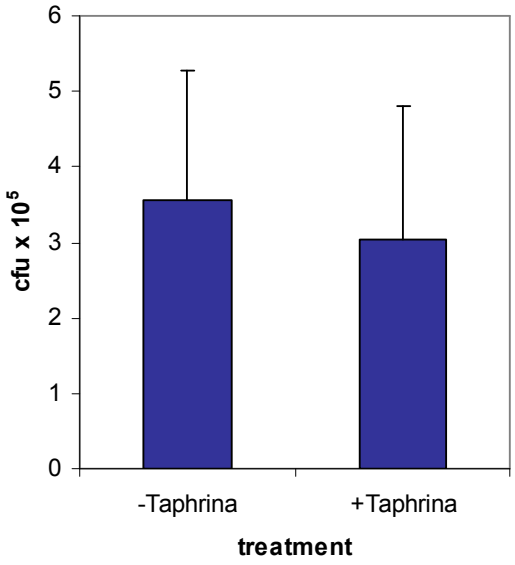

Fig. 16 Mean colony forming units (cfu) of *P. syringae* grown on top of a lawn of *Taphrina* (+Taphrina) which was plated between a layer of LB-agar and PD-agar, or grown on a layer of LB-agar and PD-agar only (-Taphrina). n=11

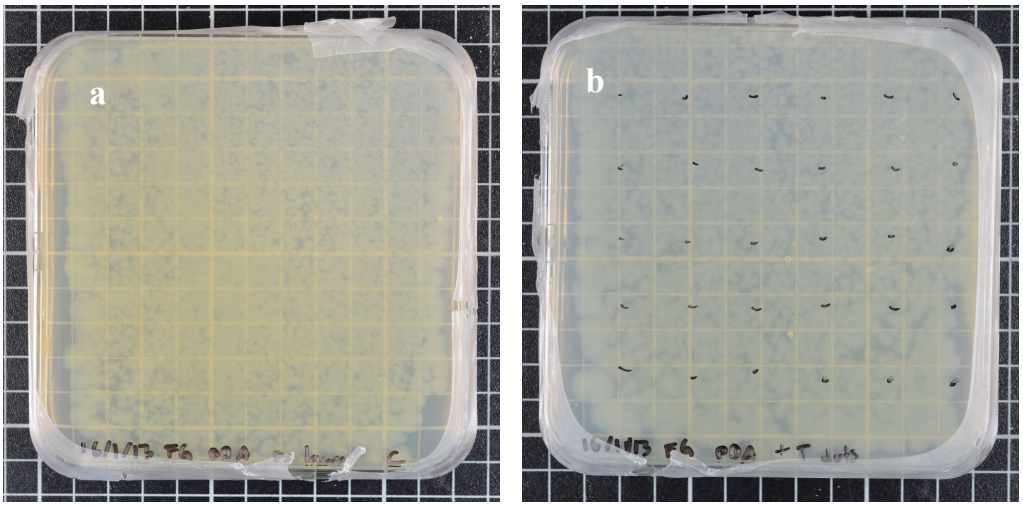

Fig. 17 Appearance of a lawn of *P. syringae* grown on top of a layer of LB-agar and PD-agar (**a**) or a layer of LB-agar and PD-agar with dots of *Taphrina* solution grown in between (**b**). The dots in b) show the location of *Taphrina* colonies.

Discussion

Non-host resistance against *Taphrina betulina*

In this investigation we used Arabidopsis as a model plant to study non-host resistance mechanisms against *Taphrina betulina*.

Several fungal pathogens of *A. thaliana* are known to date, such as *Fusarium oxysporum*, *Botrytis cinerea* and *Magnaporthe oryzae* (Park *et al*. 2009). These cause symptoms such as chlorosis and necrotic lesions on leaves of infected Arabidopsis plants. In our infection experiments, we observed similar lesions on all genotypes in both treatment groups (control and infected plants). It is possible that another stress factor, such as flies which could be observed in the greenhouse during the experiment, was responsible for this, causing a basal level of symptoms on all plants. Although great care has been taken to spray all plants evenly with the control and fungal solutions, it cannot be excluded that this method led to experimental variation. This possibility is supported by the variation seen between independent experiments. The variation of lesion levels in infected plants between experiment three and experiments one and two may have been caused by the difference in time of the year during which the experiments were conducted. Although the day length was controlled in the greenhouse and light supply was artificial, natural day light constantly radiated into the greenhouse room. This may have caused heat and light stress to the plants. Since the intensity of day light varies with the season and weather, it could be one explanation for the observed variations. Furthermore, Arabidopsis has previously been shown to exhibit diurnal variation in its pathogen susceptibility, so potentially the time of the day at inoculation with *Taphrina* in

different experiments could have played a role in disease development *(Bhardwaj et al*. 2011).

The *ein2-1* mutation can act to delay symptom development compared to *col-0* plants, as reported for *Ralstonia solanacearum* (bacterial wilt) infections *(Hirsch et al.* 2002). Completely wilted *ein2-5* plants were only observed two days later than in wild-type samples and after 12 days post-inoculation the number of wilted *ein2-5* plants remained below wild-type levels. Furthermore, colony growth was nearly completely inhibited between six and ten days after inoculation. Such an effect could also be present in our model system considering the low level of symptoms we observed in *ein2-5* plants.

Despite these limitations, a significant difference in the percentage of lesions was found between *ein2-5* control and infected plants. The result was consistent even when all three experiments were analysed separately. This allows us to suggest two main conclusions: Firstly, *Arabidopsis thaliana* is, as hypothesized, a non-host for *Taphrina betulina*, shown by the fact that the wild-type genotype did not vary significantly in lesion levels between treatments. *Arabidopsis* therefore has the potential to be developed as a non-host model plant for studying *Taphrina betulina* pathology in the future. Secondly, the *ein2-5* mutation confers increased susceptibility to lesion development in *Arabidopsis*. As there is already a basal level of lesions in all genotypes and treatments which could account for false positives or other stress factors, we assume that the increase in symptoms in *ein2-5* is caused by the infection by *T. betulina*. We therefore tentatively accept our hypothesis that this defence signalling mutant shows lesions due to an interaction with the signalling pathways involved in the non-host defence against *T. betulina*.

The EIN2 protein is part of the ethylene defence signalling, but it has contrasting roles being involved in both promotion and suppression of disease. Increased symptoms were found in *ein2* mutants infected with *B. cinerea* or *E. carotovora* but a higher resistance was established against *P. syringae* and *X. campestris* (Bent *et al*. 1992; Norman-Setterblad *et al*. 2000; Thomma *et al*. 1999). The nature of the interaction between *T. betulina* and *Arabidopsis* defence pathways can be inferred from the type of non-host resistance which is present in this model system.

We already defined the properties of type I and type II non-host resistance as suggested by Mysore and Ryu (2004).

The observed lesions can be a hypersensitive response by the plant or true symptoms of *Taphrina* infection. Both possibilities need to be investigated in order to derive a mechanism for the non-host signalling pathway induced by *Taphrina*.

Taphrina betulina is a biotrophic pathogen, indicated by the survival of infected plant tissues. Biotrophic pathogens require live plant tissue to survive so that a common defence strategy is a hypersensitive response, also termed type II non-host resistance. This response is elicited after successful penetration of the pathogen into the host by extracellular molecules present on the pathogen surface or secreted molecules. It leads to a usually rapid cell death at and near the point of pathogen entrance. The cell death prevents the spread and survival of biotrophic pathogens effectively.

It is possible that the limited necrotic lesions are a sign of a failed HR. In our experiment, the lesions only became visible after several days post inoculation. Such a slow response could be a sign of a failed hypersensitive response which was suppressed by the pathogen but more successful in the ethylene signalling mutant.

The EIN2 protein is an integral component of the ethylene-mediated plant defence signalling pathway which has been commonly thought of as a response to necrotrophic pathogens (Glazebrook 2005; Pieterse *et al.* 2009), but it has also been involved in defence against biotrophic pathogens and is associated with non-host resistance (Glazebrook 2005; Knoester *et al.* 1998; Zimmerli *et al*. 2004). In the general model representing the cross-talk between the ethylene, jasmonic acid and salicylic acid pathways, the ET pathway interacts positively with JA (e.g. Penninckx *et al*. 1998)). JA activates the COI1 (CORONATINE INSENSITVE 1) protein, which is a repressor of SA responses as will be further explained later. A first mechanism can thus be derived for the development of lesions, assuming that *Taphrina* activates the ethylene response pathway: In the *ein2* mutant, the JA is not activated and hence the SA signalling is de-repressed. This leads to an increase in HR seen in the *ein2* mutant infected with *Taphrina*. It could therefore be argued that a type II non-host resistance is present.

However, there is a caveat associated with this assumption:

In tobacco plants, some defence genes were up-regulated as early as nine hours after inoculation with a non-host pathogen (Oh *et al.* 2006). A type II hypersensitive response would therefore be expected hours after the infection and not days as observed in our experiment. Furthermore, the question would remain open as to why infected plants show no significant difference in lesions if a hypersensitive reaction to *Taphrina* is expected.

We did not see increased necrosis in wild-type plants treated with *Taphrina*. Although this does not necessarily exclude type II HR as a mechanism as argued above, it is unlikely that only one genotype would be affected and the symptoms

would be so mild. In addition, we would still expect a significant difference in lesion levels between treatment and control plants in all genotypes, and lower levels of all infected plants compared to *ein2* infected plants if this was part of a type II HR. As introduced previously, type I non-host resistance is also more common and broad range.

In light of these facts it seems more likely that Arabidopsis exhibits type I non-host resistance against *Taphrina* and we propose that the increased treatment effect seen in *ein2* plants is a discrete symptom of *Taphrina betulina* infection. The lack of any effects in all other genotype can then be assigned to the symptomless type I resistance. The *ein2* mutation completely blocks the ET pathway, so it is possible that the non-host defence to *Taphrina* is solely mediated via this pathway, which is suppressed in the *ein2* mutant plants (Alonso *et al*. 1999).

A molecular pathway for the increased susceptibility of *ein2* mutants can also be deduced from the cross-talk between the SA and ET signalling cascades. The salicyclic acid pathway is positively modulated by ethylene signalling, specifically through EIN2 (**Fig. 18**). Ethylene enhanced SA-dependent resistance against Turnip crinkle virus in Arabidopsis and augmented PR1 gene expression and this synergism was repressed in the *ein2* mutant (De Vos *et al*. 2006).

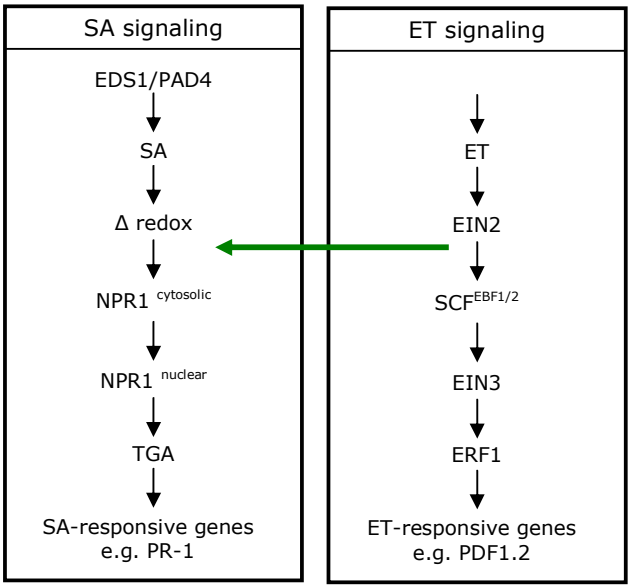

Fig. 18 Synergistic cross-talk between SA and ET signalling pathways.
According to Pieterse *et al.*. (2009).

We propose that non-host resistance against *T. betulina* in *Arabidopsis thaliana* is mediated by EIN2-dependent activation of the SA defence pathway. We suggest a model in which *Taphrina* is capable of penetrating the plant surface (first layer response) and subsequently elicits inducible defence responses through surface protein interactions with plant receptors. These responses activate EIN2-dependent SA pathways. The augmented defences suppress further infection in a symptomless type I non-host resistance response. In *ein2-5* mutants, the defence signalling through both the SA and ET pathways is severely impaired, leading to disease development (**Fig. 19**). The symptom caused by *Taphrina* in Arabidopsis is tissue necrosis which occurs as a delayed response post-inoculation when the pathogen has depleted the cells of nutrients and spread to the surrounding live cells.

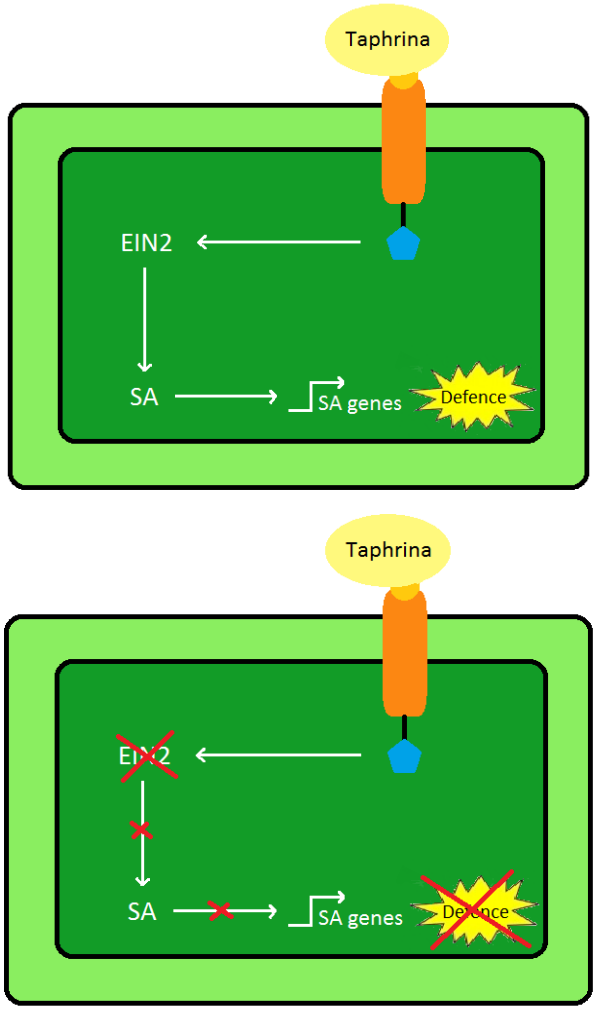

Fig. 19 Proposed signalling pathway for non-host resistance against Taphrina in Arabidopsis.
a) In wild-type plants, Taphrina is recognized by a receptor which is an upstream activator of EIN2. EIN2 activates SA which leads to the transcription of SA-dependent defence genes. This results in resistance.
b) In *ein2* mutants, there is no activation of SA by EIN2. The SA-dependent defence responses are suppressed, resulting in increased susceptibility.

Extracellular perception of *T. betulina*

We germinated and grew wild-type and Arabidopsis mutants *fls2* and *efr1* on MS agar and MS agar containing dead yeast extract in order to test whether either of the two receptors functions in perceiving *Taphrina* and relaying downstream defence signals. If either of these receptors was important in recognition and activation of defences, we would have expected a significantly stunted growth in the respective mutant. This was not the case. It is therefore unlikely that the FLS2 and EFR2 receptors have a dual function in recognising molecular patterns present inside or on the surface of *Taphrina* cells. It is unclear why control plants would grow better on plates containing the fungus.

In contrast, *jar1* mutants displayed a significant decrease in root growth when grown on *Taphrina* extract. This implies mechanisms for the defence signalling pathway involved. The protein lacking in this genotype is the jasmonic acid-amido synthetase. It catalyses the conjugation of jasmonic acid to the amino acid isoleucine (Ile), thereby synthesising the active compounds JA-Ile. In the mutant, the JA signalling is inhibited due to the lack of this enzyme. This may interfere with SA or auxin responses. As we noted earlier, jasmonic acid can act antagonistically to salicylic acid through the MYC2 transcription factor. In *jar1* mutants, JA-Ile is not produced so there is no relief of inhibition of JAZ on MYC2 and other transcription factors. When MYC2 is repressed, SA is deregulated. In a scenario where a *Taphrina* induces SA responses via PAMP recognition, signalling through this pathway would be increased in mutants grown with yeast extract. The resource allocation shift from growth to defence of this up-regulated defence could be the cause of the stunting. Furthermore, hormone homeostasis could be disrupted. Salicylic acid is a negative

regulator of the growth hormone auxin, so when *Taphrina* increases the SA-dependent responses which are not controlled in *jar1* mutants, the growth stimulation by auxins is inhibited.

Effect on maximum photochemical efficiency (F_v/F_m)

In our study of the photosystem function in control plants and plants sprayed with a *Taphrina* solution, we could not find any significant difference between treatments or genotypes. We therefore reject our hypothesis that *Taphrina betulina* induces phototoxic damage as a sign of plant stress.

The reduction in the F_v/F_m ratio in infected plants compared to controls was highest in the *ein2-5* mutant. Although this result was not significant, this trend may suggest that the EIN2 protein confers slight protection against phototoxic stress induced by non-host pathogens.

In our experiment, we only measured the F_v/F_m ratio across the whole plant surface. Due to the small size of the lesions, it is possible that maximum quantum efficiency was affected locally around the areas of the lesions (and in the lesion areas, due to the death of the cells), but that this effect was too small to be seen in the average ration of the entire plant. The F_v/F_m ratio was not measured separately for the necrotic tissues. Nevertheless, one conclusion can be drawn from this experiment. The result obtained indicates that there is no systemic damage to the photosystem as this would be expected to have a significant effect on the maximum quantum efficiency of the whole plant.

Fungal establishment inside the plant

There are two limitations to restricting the analysis of susceptibility to the development of symptom-like effects: Firstly, the cause of the observed leaf lesions has not been proven and so we cannot yet confirm that they are truly symptoms caused by *Taphrina betulina*, a hypersensitive response or simply due to environmental or physiological factors such as plant age. Secondly, even if the lesions are symptoms of a successful infection, their extent does not necessarily correlate to the establishment and spread of the pathogen. A direct visualisation of the pathogen inside the plant would therefore provide additional support that the lesions are caused by the growth of *Taphrina* inside the plant.

We hypothesized that if *ein2-5* is susceptible to infection by *Taphrina*, it would be possible to detect the fungus inside the leaf tissue with a suitable dye.

The regions of the leaf which appeared clear after the Trypan blue staining consist of live cells, as these are unable to absorb the dye and therefore do not appear blue. The leaf veins were clearly visible in blue in these areas because the xylem partly consists of dead tracheids. In the leaf area with necrotic lesions, dark nodules were visible. These are most likely dead cells, inferred from their identical appearance to dead cells reported elsewhere in Arabidopsis (cf. for example Vorwerk *et al*. 2007). The dark blue string-like structures in the plant tissue resemble the hyphae reported in other fungal stains in Arabidopsis (e.g. Todesco *et al*. 2010). However, these strings were usually short in our sample and difficult to see, so their nature is ambiguous.

Trypan blue staining may not be the most adequate method to detect the pathogen inside the host tissue. Even if the results had been clearer, it would have

been difficult to quantify the extent of hyphae in infected plants or between different genotypes. Different sensitive techniques are available for measuring pathogen presence both by its growth and the reactions it causes inside the plant. As has been done with the *Pseudomonas* growth assay, one possibility would have been to re-isolate *Taphrina* from infected plant tissues. If this was successful, this method would also contribute to confirming Koch's postulates which designate a pathogen. For a molecular approach, DNA could be isolated from the plant and amplified for a fungal-specific gene. The presence of *Taphrina* could be confirmed, for example, by a restriction fragment digest as we carried out earlier to identify our *Taphrina* species. The amount of fungal growth between different genotypes could be assessed with a quantitative PCR (qPCR) assay. This would be a direct way of measuring proliferation *in planta*.

From the perspective of the plant, the presence of the pathogen could be inferred from its effect on the host. Changes in hormones or gene expression levels can suggest a response specific to a pathogen, although care must be taken in the interpretation, as other (biotic and abiotic) stress factors can influence these levels, too. If the growing and experimental conditions are tightly controlled, any differences in the expression of hormones can be attributed to the treatment variable. As we described earlier, three main hormones – SA, JA and ET – are involved in the major defence signalling pathways. Although hormone levels can be measured directly, this is a laborious task (cf. e.g. Engelberth *et al*. 2003). Instead, reverse transcription and qPCR for these hormones or hormone-responsive marker genes (e.g. PR1 as a response to SA) enable detection of changes in expression levels. In combination with the known mutations, this would also give further insight into the signalling pathways involved and which proteins are crucial for elevating defences.

These additional methods are the next logical step in furthering our understanding of the non-host defences against *Taphrina* in Arabidopsis.

Pseudomonas growth

Pseudomonas syringae pv. *tomato* is a known pathogen of *Arabidopsis thaliana* (Whalen *et al*. 1991).

In a preliminary experiment, *Pseudomonas* could be isolated on day 0 but no colonies were seen on day 3. In a second independent experiment, the magnitude of colonies on day 3 was lower than expected by a factor of 100, although the observed trend in the results was the same (data not shown). This demonstrates that inoculation and/or establishment of *Pseudomonas* in Arabidopsis in hand inoculation experiments is not always successful. Due to the low OD required, which cannot be measured by the spectrophotometer with sufficient accuracy, the desired concentration has to be obtained from serial dilutions of a known OD. In order to achieve greater success rates, humidity should be high during the inoculation to ensure opening of stomata and the success of the initial inoculation should be confirmed by re-isolation.

In the data presented, the amount of colonies isolated on day 0 corresponded to the number expected from the optical density of the original inoculum (OD=0.0002). We could therefore confirm that the inoculation had been successful.

The increase in bacteria and characteristic water-soaked blisters observed after 72 hours indicate that *Pseudomonas* had effectively infected the leaves. However, the growth seen is by several magnitude of ten smaller than would be expected. Other *Pseudomonas* growth assessments in *Arabidopsis thaliana* have reported magnitudes

of 10^8 after three days, even with a lower initial concentration (e.g. Bent *et al.* 1992; Cao *et al.* 1998). This discrepancy to our experimental results was present in both control and *Taphrina*-infected samples, so that a treatment effect on the generally low growth can be excluded. It is possible that growing conditions for *Pseudomonas* were less favourable due to environmental conditions or that the error incurred from estimating the large number of colonies greatly reduced the accuracy of the data. Furthermore, a diurnal variation in the infection capacity as mentioned in the *Taphrina* infection experiment could have caused the low growth (Bhardwaj *et al.* 2011).

The variance in day 3 samples of both treatment types is large, giving a clearer picture of the success of the infection. The variance is systemic, meaning that conditions found in both treatment types are the cause. One explanation may be the viability and vigour of the *Pseudomonas* isolate used in the experiment. When plating *Pseudomonas* initially on selective antibiotic King's B medium, it did not grow. It was necessary to initiate the culture on antibiotic-free LB medium, probably due to the stress caused by the presence of antibiotics in addition to the stress of resurrection. Although the colony used for the infection was transferred to fresh medium and grown up two days before inoculation, it still did not grow very vigorously. This could have been caused by an old stock or too much stress or a combination of the two. A problem with high variance was also observed in Fusarium infection of Arabidopsis by Chen *et al.* (2006) and a more reproducible bioassay was developed using detached leaves to study resistance and susceptibility mechanisms.

Nonetheless, a difference was observed between mean colony forming units of the two treatments after three days. Although this was not significant, it shows a

trend towards increased growth of *Pseudomonas* in plants pre-infected with *Taphrina betulina*. This opens up interesting possibilities for interpretation.

Clearly, our expectation that *Taphrina* can induce resistance in Arabidopsis against *Pseudomonas* could not be verified. If this had been the case, we would have predicted a decline in *Pseudomonas* growth in *Taphrina*-infected plants due to an increased resistance in these. The trend we observed, however, indicates another type of relationship between the two microorganisms. The presence of *Taphrina* in Arabidopsis appears to promote growth of *Pseudomonas*. This may be due to an indirect effect through a change in the plant environment caused by *Taphrina* or a direct interaction between the organisms.

Indirect changes to the plant environment can be due to changes in chemical and physical properties. It has already been shown that changes in the plant can lead to different responses by other organisms. In one study, elevated CO_2 levels decreased resistance to the cotton bollworm *H. armigera* in tomato plants and this was caused by suppression of the jasmonic acid defence pathway (Guo *et al*. 2012). Although these conditions do not exactly apply to our experiment, an interesting conclusion from this study is that plant defence can not only be induced but likewise negatively regulated by elicitors.

Direct fungal-bacterial interactions can take on many forms (reviewed in (Kobayashi and Crouch 2009), including parasitism in which the bacteria benefit by sequestering nutrients from the fungal host (de Boer *et al*. 2005) or facilitated dispersal of bacteria by fungal mycelium (Ingham *et al*. 2011). Although this type of relationship has seldom been reported, it cannot be excluded immediately in this scenario. Previously, bacteria have also been found to promote growth of fungi in vitro through the release of volatile organic compounds, although the exact

mechanism has not been elucidated (Alharbi *et al*. 2011; Kai *et al*. 2008; Wheatley 2002). It is conceivable that fungi, which also produce a large range of volatile organic compounds and other secondary metabolites (Morath *et al.* 2012), can likewise affect bacterial growth through chemical interaction. As reviewed by Effmert *et al.* (2012), these molecules are an important means of communication between microorganisms in shared environments.

The plant-bacteria-fungi community is very complex and here we only listed interactions which could explain our observed results. It should be noted that in many instances the bacterial and fungal communities live in relative neutralism with each other and that likewise fungi can negatively impact bacterial growth for example through the targeted production of antibiotics.

We investigated the nature of interaction between the bacteria and the fungus which led to the increase in bacterial growth in fungi-treated plants.

In our bacterial overlay assay, we assumed that secondary compounds produced by *Taphrina* would diffuse through the layer of LB-agar and reach the bacteria grown on top. If such compounds are produced by *Taphrina*, we could not see any effect on them on the growth of *Pseudomonas*. We would have expected a negative effect of the presence of *Taphrina* which would have produced clear plaques in the lawn of *Pseudomonas* where the *Taphrina* dots are present underneath, or no growth of *Pseudomonas* on top of a lawn of *Taphrina*.

There is no published genome sequence available yet of *T. betulina*, but partial sequencing and preliminary assembly and annotation work has been carried out (Overmyer *et al*., unpublished). Gene clusters for NRPS (nonribosomal peptide

synthase), PKS (polyketide synthase) or a NRPS/PKS hybrid were identified (unpublished data). Furthermore, the genome of the related species *Taphrina deformans* has been published at the time of writing this thesis (although it was not yet available when the experiments were conducted) (Cissé *et al.* 2013). The genes for a PKS and NRPS were identified. It is therefore legitimate to assume that *T. betulina* possesses similar secondary metabolite pathways as those suggested in *T. deformans*. These enzymes families are involved in secondary metabolic pathways, including those that produce immunosuppressive (cyclosporine), anti-tumour (bleomycin) and antibiotic (vancomycine) peptides. Some subfamilies of the NRPS are specific to fungi (Bushley and Turgeon 2010). The metabolic products may further be involved in microbial interactions. A whole separate database has been compiled with the functional and structural properties of over 700 nonribosomal peptides, underlining their significance in microbial research (Caboche *et al*. 2008; http://bioinfo.lifl.fr/norine/).

The absence of any effect of such metabolites in our experiment could be a true negative, meaning that there is no antagonist or amensalistic effect of *Taphrina* on *Pseudomonas*. This interpretation would be in accordance with our results, which indicated that the presence of *Taphrina* in Arabidopsis does not decrease *Pseudomonas* growth. In contrast, the experimental setup could have produced a false negative of the effect on *Taphrina* growth below a layer of *Pseudomonas*. Any substances produced by *Taphrina* may have mostly diffused downwards into the PD agar, diffused laterally into the PD and LB agar layers, or the LB agar may not be permeable to them, at least not sufficient to reach the surface and get into contact with *Pseudomonas*.

It can therefore not be stated whether there is no direct interaction between the microorganisms which is mediated remotely by chemicals. Other common interaction assays could be carried out for further analysis, such as a visual agar plate assay where microorganisms are grown side by side and the growth pattern in response to the presence of the other organism can be studied. This would require a medium on which both organisms can grow, or at least one plate with two different media side by side (although this would exclude one organism overgrowing or out-competing the other).

Due to the temporal limitations of this project, interactions mediated through plant signalling pathways could not be studied. From the negative result obtained in the overlay assay, such an interaction remains an open possibility and the trend we have seen *in planta* supports this. By exclusion of a direct interaction between the microorganisms, we will propose the following plant-mediated interaction.

The defence responses activated against *Pst* are largely salicylic acid-dependent (SA), as has been suggested by several studies showing an increased susceptibility of mutants compromised in SA signalling. These mutations down-regulated SA accumulation (e.g. *eds1* and *pad4* (Aarts *et al.* 1998) or degrade it (*nahG*, *Delaney et al.* 1994).

One of the mechanisms leading to *Pseudomonas* susceptibility is mediated through the jasmonic acid-isoleucine (JA-Ile) mimic coronatine which is produced by *Pst*. Coronatine and JA-Ile are involved in the degradation of JAZ proteins through the promotion of COI1-mediated degradation. This releases transcription factors such as MYC2, which activates a series of NAC TFs. NAC TFs repress the SA synthesis enzyme isochorismate synthase (ICS) and activate methyl transferase 1

(BSMT1), which inactivates SA (Zheng *et al*. 2012) (**Fig. 20**). Furthermore, stomatal reopening is induced which facilitates pathogen entry.

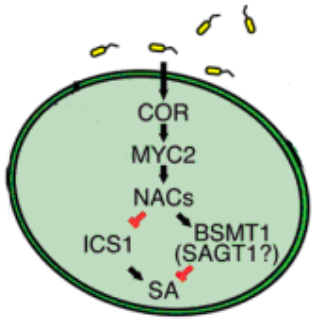

Fig. 20 Coronatine-dependent pathogenecity of *Pseudomonas synringae*. Coronatine is a JA-Ile mimic which promotes the degradation of the JAZ inhibitor of MYC2. MYC2 activates NAC TFs which repress SA synthesis (ICS) and activate SA inactivators (BSMT1). This leads to a decrease in SA.
Simplified from Zheng *et al.*. 2012.

Combining the results obtained from infecting Arabidopsis with *Taphrina* and inoculating *Pseudomonas* into pre-infected plants, a plausible, plant-mediated interaction between the microorganisms can be deduced. In wild-type plants, the EIN2 protein augments SA-dependent defence responses, conferring some level of resistance to *Pst*. The presence of *Taphrina* induces ET-responses which can activate JA responses described above. This may be mediated through ERF1, which links the two pathways and causes reduced SA-dependent resistance to *Pst* when over-expressed (Berrocal-Lobo *et al.* 2002). We therefore propose a pathway by which *Taphrina* indirectly activates JA-responses which act antagonistically to down-regulate SA-dependent defences, creating more favourable growing conditions for *Pseudomonas*.

In this study, we could not observe signs of *Taphrina*-induced resistance. We only tested the induced resistance hypothesis in response to *Pseudomonas*. However, induced resistance relies on the activation of those defences by the elicitor, which protect against the specific pathogen. If the elicitor and pathogen signal through different pathways, the defences elicited are ineffective against the pathogen. Our

results support the idea that defences against *Taphrina* and *Pseudomonas* employ different pathways which work antagonistically.

In order to support the non-host mechanism involved in *Taphrina* defence and the interaction with *Pseudomonas*, experimental conditions need to be optimized and further mutants should be studied. This would also be useful in eliminating the possibility that non-specific metabolic stress caused by *Taphrina* due to a re-allocation of resources to primary non-host defences increased the overall susceptibility of plants to infections. Mutations downstream of *ein2* such as *ein3* and *efr1* and those involved in JA signalling such as *myc2* should be subjected to *Taphrina* infection in order to elucidate whether only the ET pathway is involved in this non-host defence or whether the cross-talk between pathways is of importance. Double mutants would be especially useful in investigating whether a combination of defence pathways is activated. Furthermore, the *coi1* and *ein2* mutants (and double mutants) are a crucial addition to the investigation of how *Taphrina* and *Pseudomonas* defence pathways interact. We proposed a molecular mechanism of interaction based on the growth effects observed, but this can only be supported by the effects of mutations in the suggested pathway. In coronatine insensitive mutants (*coi1*), resistance to *Pst* is increased (Kloek *et al*. 2001). We would expect to see an increase in susceptibility in this mutant when pre-infected with *Taphrina*, if *Taphrina* decreases SA-dependent defences. In fact, virulence has been restored in *Pseudomonas* deficient in coronatine (which mimics coronatine insensitivity) in SA-defective plants (Brooks *et al.* 2005). In the ethylene-insensitive *ein2* mutant, *Pst* grew extensively compared to the wild-type (Bent *et al.* 1992). We have suggested that SA-dependent pathways are reduced in *ein2* mutants so that we would expect to see an increase in growth of *Pseudomonas* in *ein2* plants pre-infected with *Taphrina*

compared to wild-type plants pre-infected with *Taphrina*. However, we also suggested that *Taphrina* antagonises the SA defences through ET/JA synergism. If this is the case, *ein2* mutants may be more resistant to *Pseudomonas* when infected with *Taphrina*, as this antagonism is inhibited. Whichever response would be observed, it could clarify whether EIN2-dependent signalling in *Taphrina* defence cross-talks with the JA pathway and whether this counteracts coronatine insensitivity in double mutants.

Implications for *Taphrina* pathology

The witch's broom caused by *Taphrina betulina* and symptoms caused by other *Taphrina* species as mentioned in the introduction are a hypertrophy of the plant tissue. Auxin is a plant growth hormone often involved in hypertrophic diseases due to its ability to stimulate growth (reviewed in Yamada 1993 and Woodward and Bartel 2005). Pathogens may either produce auxin themselves, activate plant auxin synthesis or desensitize auxin responses in the plant cells. *Taphrina deformans* and other *Taphrina* species have early been shown to produce the auxin indole acetic acid (IAA) (Crady and Wolf 1959; Perley and Stowe 1965; Yamada *et al.* 1990). The recent publication of the *Taphrina deformans* genome revealed that IAA biosynthetic genes are present in this species and we confirmed auxin production in *Taphrina deformans* by Salkowski staining (Sippilä, unpublished; after Pilet and Chollet 1970). It is likely that *T. betulina* contains similar biosynthetic genes. The question remains whether the hypertrophy is specifically induced by *Taphrina* to promote its survival or a side-effect of auxin production. Auxin is implicated in disease development by down-regulating plant defence responses and inhibiting HR

(Dominov *et al*. 1992; Robinette and Matthysse 1990). This effect is probably due to an auxin-SA antagonism, as SA is able to counteract auxin-mediated disease susceptibility to biotrophic pathogens such as *Pseudomonas syringae* by repressing auxin-related genes globally (Wang *et al*. 2007) and auxin receptor mutants have increased levels of SA and pathogenesis related genes (Iglesias *et al*. 2011). We suggested previously that non-host resistance in Arabidopsis against *Taphrina betulina* is mediated by a type I SA-dependent response. This has implications for the disease mechanism in the true host, *Betula pubescens*, if we consider the interaction between auxin and SA. It suggests that *Taphrina* does, in fact, not produce auxin specifically to stimulate hypertrophy, but simply to suppress SA-dependent defence responses. A side-effect is the tree's response to locally increased auxin levels which stimulates growth. This is seen in the development of hypertrophic branch formation. The same could be true for other *Taphrina* species and their hosts.

We established that non-host resistance against *Taphrina* in Arabidopsis is via the type I mechanism. In Arabidopsis leaves we have seen possible signs of fungal establishment, meaning that physical barriers are probably surpassed and the non-host resistance elicited on a pattern recognition level. As *Taphrina* is a fungus which grows extracellularly, the recognition likely occurs at the plant cell wall. Due to the absence of hypersensitive cell death, the downstream signalling elicits PTI defences rather than ETI, leading to the development of resistance. Applying this concept to the true host *B. pubescens*, the PTI may be circumvented and fungal effectors released into the cells, for example for the acquisition of nutrients. These may be recognised by intercellular plant resistance genes. In a compatible interaction, *Taphrina betulina* may degrade or block these gene products, finally resulting in a

successful infection. The suggestion for an intracellular ETI interaction which is overcome by *T. betulina* is supported by the fact that this species can grow on ethanol extracts of *B. pubescens* buds, but the species *T. deformans* and *T. robinsoniana* are inhibited, probably because they cannot inhibit the effector triggered responses (unpublished data).

Taphrina-Arabidopsis – a good model system?

This is the first instance that *Taphrina* has been studied in a model plant. There are few recent studies on this pathogen genus and most are concerned with the forecasting and disease ecology of the disease but none have considered this as a model pathogen. Fungi are the major causal agents of tree diseases and some of the most devastating forest diseases are caused by fungi, such as ash dieback (caused by *Chalara fraxinea*), which is currently spreading throughout Europe and has reached quarantine status in the UK. Fungi are easily spread globally, by humans or in imported nursery trees used in reforestation, and locally by wind or water dispersal. Furthermore, many ecosystem services such as timber production, recreation and ecosystem diversity depend on healthy forests. Fungal tree pathogens should therefore be a priority in forest pathology research. Furthermore, Taphrina is known to overwinter in the tissue of its woody hosts, making it a plant-associated yeast during part of its lifecycle (Mix 1949). This is a novel concept among the major plant pathogens studied today.

Due to the long lifecycle and slow development of tree hosts, and little genetic characterization of many, model systems are an important tool for studying forest

diseases. Criteria for developing *Taphrina-Arabidopsis* as a model system were already mentioned in the introduction.

Conclusion

Here, we showed that *Taphrina-Arabidopsis* can be used as a model system for studying a tree disease. We found that wild-type Arabidopsis is a non-host for *Taphrina*. The study of non-host resistance can aid in elucidating mechanisms responsible for the lack of resistance in host plants. We further showed that defence signalling mutants respond differentially to challenge by *Taphrina*, allowing us to draw first conclusions on the defence mechanisms which protect non-hosts from *Taphrina*. Ethylene-insensitive silver birch mutants are available and could be used for applying our conclusions from the non-host resistance mechanism to the host (Vahala *et al.* 2003). The development of means for genetic manipulation in *Taphrina* and study of other Arabidopsis mutants will also open up a vast array of possibilities for further study. Due to the host specificity of *Taphrina* species, comparison of species effects in Arabidopsis could already provide insights into whether and how different species successfully infect different hosts. Another route of study could be the dissection of the non-host defence layers by studying mutants of primary and secondary defence mechanisms. This could show at which level *Taphrina* infection is prevented in the non-host, hinting at which property may render the host susceptible.

Just like in other areas of scientific research, model systems in plant pathology can never replace the study of the actual pathosystem concerned. No two systems are exactly the same so conclusions are never completely transferable. However, models will always complement and enrich our knowledge of systems and often found the basis of studies conducted on the proper system. They provide a method for quick

screening of possible interactions so that studies in the real system, here the host-pathogen pair, can be targeted to the more likely mechanisms involved.

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