Resistance to a non-adapted pathogen of barley is linked to the evolution of the Poales-specific Exo70FX family

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Chapter 1

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Genome assembly and annotation: MM, SH

Subsequent genome analysis: MM, SH

Writing and conceptualisation: SH, MM

Chapter 4

Genome and transcriptome processing: MM, SH

Analysis of Exo70 genes and proteins: SH

Writing and conceptualisation: SH, MM

Chapter 5

Writing and conceptualisation: SH, MM

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1. General introduction

1.1 Thesis Abstract

Three loci contribute to resistance in barley against the non-adapted pathogen *Puccinia striiformis* f. sp. *tritici*: *Rps6, Rps7* and *Rps8*. Using a high-resolution recombination screen, a forward genetic screen, natural variation, and transgenic complementation we identify two adjacent genes on the long arm of chromosome 4H which are both necessary for *Rps8*-mediated resistance. These genes are in genetic coupling and are inherited together as part of a 546 kb In/Del polymorphism between *Rps8* and *rps8* haplotypes. These genes encode an Exo70 protein and an LRR-XII family receptor kinase protein; the Exo70 is a member of the Exo70FX clade which has only been identified in the Poaceae, and the receptor kinase is a member of the LRR-XII clade which is widely conserved across plants and includes the well-studied resistance genes FLS2, EFR and Xa21. We perform an analysis of the Exo70FX clade in the Poales and identify the origins of Exo70FX in the graminid clade. We also catalogue extensive species-specific expansion of Exo70FX subclades throughout the Poaceae as well as sequence-level diversity within and between subclades. We identify the Exo70 at *Rps8* as a member of a novel subclade: Exo70FX12, which is a recent innovation of the Pooideae. To facilitate this analysis, we sequenced and annotated the first genome of *Ecdeiocolea monostachya*, a wild Australian plant which is a close outgroup of the Poaceae, assembling a diploid genome of 1.33 Gbp, covering an estimated 87% of the genome with 84,700 gene annotations.

1.2 Organisation

This thesis is divided into five chapters. **Chapter 1** is a broad introduction to the topics discussed in subsequent chapters, **Chapters 2, 3, and 4** each contain an introduction, results, chapter specific-methods, and short discussion. and **Chapter 5** is a final discussion. A bibliography follows **Chapter 5.**

In Chapter 1, we provide a broad overview of immunity in plants with special emphasis on extracellular recognition mediated by receptor kinase proteins. We also discuss the life cycle

of *Puccinia striiformis*, and summarise its relationship with the grasses. Finally, we give an overview of the function of Exo70 proteins in exocytosis, as well as in other roles.

In Chapter 2, we describe cloning of *Rps8,* a genetic module providing resistance to wheat stripe rust in barley. We fine-map *Rps8* using a high resolution recombination screen derived from the SusPtrit x Golden Promise doubled-haploid population [Yeo 2012]. Natural diversity across a panel of barley accessions, and a forward genetic screen using an M_6 population derived from the reference accession Morex demonstrate that *Rps8* is two genes at a single locus: an *Exo70* (*Exo70FX12a*) and an *LRR-RK* (*LRR-RK-Rps8*). Each of these genes is necessary for *Rps8*, and they are inherited together in a 546 kbp polymorphism. We demonstrate that stable transgenic expression of *Exo70FX12a* is sufficient to complement mutant phenotypes deficient in this gene, but insufficient in the absence of *LRR-RK-Rps8*.

In Chapter 3, we describe the *de novo* whole genome sequencing of *Ecdeiocolea monostachya*. *E. monostachya* is one of three species in the Ecdeiocoleaceae, a family within the graminid clade of the order poales and a critical outgroup of the Poaceae. We assembled a genome with a haploid size of 0.77 Gbp and a heterozygosity of 2.5%. We annotate the *E. monostachya* genome using RNAseq derived from three tissue types: sheath, flower, and root. The annotated genome contains 84'700 gene models, and is 95% BUSCO complete.

In Chapter 4, we investigate the origins of the Exo70FX clade in the Poales. In order to characterise Exo70FX12a more thoroughly, we analysed the predicted Exo70 genes of 45 Poaceae, 16 Poales and two Commelinids, using a combination of reference genomes and transcriptomes. We identify the Exo70FX clade as emerging within the graminid clade, and substantially expanding and diversifying within the Poaceae, after the radiation of the Anomochlooideae. We develop a system for annotating Exo70FX subclades based on homology and synteny. We find that the Exo70FX12 subclade is a recent innovation of the Pooideae, and that the Exo70FX clade exhibits substantial inter-species variation in terms of subclade presence, subclade size, and protein identity.

In Chapter 5 we summarise the results of the previous chapters, and discuss their implications with regards to the specialisation of *P.striiformiis* f.sp. *tiritici* and the evolution

and expansion of the Exo70FX clade of genes. We recommend experiments for future investigation of the Exo70FX clade, and specifically to explore the interactions between *Exo70FX12* and *LRR-RK-Rps8*. We provide a brief personal summary of learning experiences over the course of the PhD project.

1.3. The plant immune system

1.3.1 Plants have innate immunity to most pathogens

Any given plant has an innate immunity to the majority of potential pathogens. Unlike jawed vertebrates, they do not have an adaptive immune system, instead plants have evolved a diverse array of pre-configured mechanisms to both recognise and defend themselves against threats (Ellis, Dodds, and Pryor 2000; Dodds and Rathjen 2010; Jones and Dangl 2006). Plants can attenuate their response based on events occurring over their lifetime. They can recognise when they have been threatened and prepare a more effective defence against future attacks (Benhamou 1996; Galis et al. 2009; Crisp et al. 2016). They can signal to one another, and they can make decisions about when an interaction goes from benign, to neutral, to harmful and respond appropriately (García-Garrido and Ocampo 2002; Schäfer et al. 2009).

In the majority of cases, innate barriers to infection such as a waxy cuticle, a thick or toughened cell wall, and antimicrobial compounds combine with varying tissue and cellular organisation between species to prevent would-be pathogens from colonising a plant species to which they are not adapted (Jones and Dangl 2006). In a host system, a pathogen is capable of overcoming these obstacles and taking nutrients from the host plant in order to complete its life-cycle (Stahl and Bishop 2000). In host systems, whether the pathogen is successful will depend on a combination the interactions between the plant's immune responses and the tools available to the pathogen to overcome or avoid that response. Resistance genes are genes which provide a plant with the ability to recognise and respond to specific molecular signals associated with pathogens such as extracellular pathogen-associated ligands, intracellular non-self molecules, or by monitoring for plant-derived molecules which have

been disturbed or damaged in some way (Tsuda and Katagiri 2010; Katagiri and Tsuda 2010; Dangl, Horvath, and Staskawicz 2013).

1.3.2 Pathogens are specialised to overcome host immunity

In order to infect a plant, pathogens must be able to overcome preformed barriers, to evade or suppress the plant immune response, and to extract nutrients from the plant in order to grow and reproduce. Plant pathogens are often classified according to their method of nutrient acquisition: biotrophic pathogens are parasitic and require a living host to extract nutrients from, necrotrophic pathogens kill host cells in order to feed on them directly, and hemi-necrotrophic pathogens switch from a biotrophic to a necrotrophic lifestyle when certain conditions are met (Panstruga 2003; Glazebrook 2005; Spoel, Johnson, and Dong 2007).

Some pathogens are capable of infecting one plant species under normal conditions, and another under a very limited set of conditions. This additional host can be described as a non-adapted system, and this paradigm is a good descriptor of the interactions between the *formae speciales* of pathogens which are adapted to a particular host but not its close relatives. For example, the powdery mildew fungus *Blumeria graminis* f. sp. *tritici* is specialised to infect bread wheat (*Triticum aestivum*), but it is possible to generate lineages of barley (*Hordeum vulgare*) which are susceptible to infection (Aghnoum et al. 2010; Romero et al. 2018). Similarly, the rust fungi; *Puccinia* spp. Have been characterised as existing on a continuum, whereby they are specialised to infect a primary host, but can infect close relatives of that host under certain conditions (Niks et al. 2015; Niks 1983; Bettgenhaeuser et al. 2014; Bettgenhaeuser et al. 2018).

Pathogens adapted to a plant species are able to form physical structures that are compatible with their host such as infection pegs or specialised intracellular hyphae, and they will also synthesise and deploy molecules such as toxins, catabolic enzymes and effectors which interact with their host to facilitate infection. Effectors are molecules generated by pathogens which perform some operation upon their host. This can include overcoming and supressing immunity, directing the flow of nutrients to the pathogen, otherwise manipulating the host

cell and facilitating the pathogen's life-cycle (Mukhtar et al. 2011; Lo Presti et al. 2015; Ridout et al. 2006; Stergiopoulos and de Wit 2009). As effectors are translocated to the plant cell, they represent ideal candidates for recognition. The pathogen, it is assumed, cannot lose effectors without some penalty to fitness and common effector targets can be monitored by the plant (Montarry et al. 2010; Rouxel and Balesdent 2017). However, pathogens may adapt by evolving additional effectors that inhibit recognition of pre-existing effectors (Bourras et al. 2016).

In the case of biotrophic pathogens, the detection of non-self molecules within the cell requires an immediate response, generally growth of the pathogen is restricted by defence pathways associated with salicylic acid (SA) signalling, leading to a form of localised cell-death called the hypersensitive response (HR) (Jones and Dangl 2006). Host-specific necrotrophic pathogens, however, can exploit this system by mimicking the effectors or effects of biotrophic pathogens in order to induce HR and permit them to feed (Govrin and Levine 2000; Lorang, Sweat, and Wolpert 2007; Lorang 2019). In *Arabidopsis thaliana*, successful plant defence against broad-spectrum necrotrophs generally utilises jasmonic acid (JA) signalling and production of antimicrobials and cell-wall reinforcement, with a corresponding decrease in SA signalling and an inhibition of cell death via the hypersensitive response (Govrin and Levine 2002; Lai et al. 2011).

1.4 Intracellular and extracellular recognition

1.4.1 The plant immune response can be classified by where recognition occurs

Recognition by the plant immune system is mediated by two major classes of immune receptors, which can be classified by the spatiotemporal properties of the interaction. Defence can either be initiated early; at the cell boundary, by membrane-localised receptors, or during infection within the cell by intracellular receptors (van der Burgh and Joosten 2019; Thomma, Nürnberger, and Joosten 2011; Tsuda and Katagiri 2010). This distinction is based on observed differences in both the signalling mechanisms and downstream responses between these interactions, however there is also interaction between these signalling

pathways (Thomma, Nürnberger, and Joosten 2011; Tsuda and Katagiri 2010; Navarro et al. 2004; Ngou et al. 2020; Yuan et al. 2021).

Membrane-bound immune receptors such as receptor kinases (RK), or receptor proteins (RP) recognise non-self molecular patterns associated with microbial activity via direct recognition of epitopes (Newman et al. 2013) such as the bacterial peptide fragment flg22 (Felix et al. 1999), the chitin monomer N-acetylchitoheptaose (Roby, Gadelle, and Toppan 1987; Yamada et al. 1993) or plant-derived peptidoglycan cell wall fragments (Gust et al. 2007) This system permits a single receptor to recognise and respond to a variety of pathogen lineages. However, the approach is vulnerable to subversion by pathogens which take steps to avoid presenting these common epitopes, or to interfere with signalling mechanisms shared between receptors (Abramovitch and Martin 2004; Jones and Dangl 2006; Dodds and Rathjen 2010). Given that plant surfaces are constantly exposed to microbes, insects, and other potential threats they must balance a broad recognition capacity with a moderated response (Huot et al. 2014). In several instances, membrane-bound receptors have been shown to require a co-receptor, which contributes to a multi-faceted downstream signalling response that enables the plant response to be fine-tuned depending on the type and intensity of the input (Felix et al. 1999; Cabrera et al. 2006; Ayres and Schneider 2012), as well as permitting interactions with mutualists that would otherwise trigger a defence response (García-Garrido and Ocampo 2002; Kamel et al. 2017).

1.4.2 Intracellular recognition is primarily mediated by NLR proteins

The majority of cytoplasmic immune receptors belong to the nucleotide binding, leucine-rich repeat (NLR) class of receptor. The intracellular defence response is typically more rapid and intense than extracellular-based responses (Cui, Tsuda, and Parker 2015; Jones, Vance, and Dangl 2016). While the down-stream regulatory pathway of NLR proteins remains unknown, considerable work has established diverse accessory proteins involved in recognition and signal transduction. While some NLRs such as L (Dodds et al. 2006), RGA5 (Cesari et al. 2013), Pik-1 (Maqbool et al. 2015), and RPP1 (Krasileva, Dahlbeck, and Staskawicz 2010), have been shown to directly interact with a pathogen effector protein, others have been found to recognise perturbation of a guarded host protein such as RIN4 by RPS2 (Mackey et al. 2003), SUMM2 and CRCK3 (Zhang et al. 2012), or PBL2 by ZAR1 (Wang, Sun, et al. 2015). A subset of

NLRs may also contain one or more non-canonical integrated domains (ID), which are also hypothesised to act as targets of pathogen effectors by mimicking *in planta* targets (Kroj et al. 2016; Bailey et al. 2018).

1.4.3 Physical properties and mechanisms of NLR proteins

NLR protein structure can vary immensely. The most basic components are a central nucleotide binding (NB-ARC) domain followed by a tandem array of leucine-rich repeats (LRR), each generally 24 residues long (Liu, Du, et al. 2017). N-terminal variation may include a TIR or coiled coil domain, and may also include a full or partial integrated domain (ID) derived from another protein class, such as a WRKY, kinase, HMA, or Exo70 (Eitas and Dangl 2010; Takken and Goverse 2012; Jacob, Vernaldi, and Maekawa 2013; Bailey et al. 2018). In some cases, only one or two of these domains are detectable in the protein, although the majority will contain both an NB and LRR domain.

Activation of NLRs is hypothesized to occur through a transition from inactive to primed state, where the inactive state is maintained through binding ADP, which prevents interaction with downstream signalling components (Tameling et al. 2002; Kim et al. 2005; Maekawa, Kufer, and Schulze-Lefert 2011; Williams et al. 2016). After activation, whether by directly binding a particular ligand or some other signal, the protein switches to an active configuration, releases ADP, binds ATP, and initiates an appropriate response, often induced cell death (HR) (DeYoung and Innes 2006; Collier and Moffett 2009; Wang, Hu, et al. 2019; Wang, Wang, et al. 2019). An alternative model, the equilibrium model, was proposed by Bernoux et al. (Bernoux et al. 2016), who observed that effector binding in the L-AvrL567 system was reduced in NLR isoforms with a preference for the ADP-bound state, but increased in isoforms with a preference for the ATP-bound state. In this model, NLRs exist in equilibrium between the active and inactive states, and pathogen detection stabilises; rather than induces, the active state, permitting downstream signalling.

1.4.4 Intracellular recognition prompts the hypersensitive response

While HR is a common outcome in intracellular defence, there appear to be multiple mechanisms by which it can occur, and exhibits characteristics that overlap with several forms of programmed cell death (Dalio et al. 2020). There is evidence that TIR-NLRs execute HR in an autophagy-dependent manner (Hofius et al. 2009), and through a conserved system of oligomerisation along with helper molecules to form a porin-like structure known as a resistome (Wang, Hu, et al. 2019; Lapin et al. 2019). CC-NLRs also induce cell-death, but in a manner independent of autophagic machinery and without apparent formation of a resistome (Hofius et al. 2009). Other outcomes from NLR activation include Ca^{2+} signalling, transcriptional changes leading to upregulation of plant defences and intracellular signalling, especially through increased SA production, often in a way that amplifies or extends an earlier response driven by extracellular receptors (Abdul Malik, Kumar, and Nadarajah 2020; Pandey and Somssich 2009; Moscou et al. 2011; Maekawa et al. 2012; Jacob, Vernaldi, and Maekawa 2013; Tsuda and Katagiri 2010). Numerous domain swap and truncation experiments indicate that the N-terminal (ie, TIR or CC) domain is necessary for HR in a full length NLR protein, and when present as a single domain can often induce HR without an elicitor (Collier, Hamel, and Moffett 2011; Bai et al. 2012; Cesari et al. 2013; Adachi et al. 2019; Horsefield et al. 2019; Wan et al. 2019).

1.4.5 Receptor kinases in extracellular recognition

Extracellular recognition occurs at the boundary to the cell, generally via membrane-bound receptor (RP) or receptor kinase (RK) proteins; which are classified based on whether the receptor has an integrated kinase domain for downstream signalling (van der Burgh and Joosten 2019; Zipfel and Oldroyd 2017). Epidermal and stomatal plant cells are exposed to the majority of microbes and other threats such as insects, and must be able to recognise and respond to their presence without overreacting to harmless or even beneficial organisms.

1.4.6 Ectodomains of receptor proteins

Membrane-bound receptor proteins are categorised by their exogenous domain. There are 14 common exogenous domains found in green plants, as well as various noncanonical

integrations, and generally kinases and their ectodomains have evolved in tandem (Shiu et al. 2004; Shiu and Bleecker 2003). The general mechanism of receptor proteins involves the binding of a particular ligand or ligands to the ectodomain, which can induce a conformational change in the receptor protein to permit co-receptor binding and signalling, or can permit binding to a co-receptor by presenting an appropriate binding interface (Zipfel and Oldroyd 2017; Couto and Zipfel 2016).

Classes of ectodomains are also specialised towards particular classes of ligand. For example, LRR domains primarily associate with peptide-derived ligands (Kobe and Kajava 2001), LysM domains with carbohydrate ligands (Buist et al. 2008) and lectins with lipopolysaccharides (Lannoo and Van Damme 2014). LRR-RK proteins are the largest single class of RK proteins in plants, and LRR domains have been identified in all five kingdoms of life, albeit with structural differences. Protein crystallization of plant receptor kinase LRR domains has found they form a superhelical, coiled, S-shaped structure, rather than the horseshoe structure commonly observed in bacteria and animals (Kobe and Deisenhofer 1994; Jones and Jones 1997; Kajava 1998). The LRR domain in plants is generally composed of up to 30 leucine-rich repeats, which vary from 20 to 29 residues each, and a small 'cap'. It also may contain a small 'island domain' without any leucine rich repeats, which aids in ligand binding (Matsushima and Miyashita 2012; Torii 2004). Crystal structures obtained of LRR domains in complex with receptors indicate that the concave inner surface of the coil is the usual binding site, presumably as it permits the maximum number of possible side-chain interactions (Chakraborty et al. 2019). Glycosylation of LRR domains is likely a conserved feature across land plants (Chen 2021) and plays an important role in their activity, being essential for proper function in FLS2, (Trempel et al. 2016), EFR (Häweker et al. 2010) and the RLP Cf-9 (Piedras et al. 2000; van der Hoorn et al. 2005). It has also been shown to be important for non-LRR receptor kinases such as the LysM-RK NFP (Mulder et al. 2006).

1.4.7 Extracellular recognition involves a co-receptor for downstream signalling

Signal transduction from perception/binding in the extracellular domain to intracellular signalling vary depending on the protein and may encompass elements such as co-receptors, co-ligands, feedback loops or a requirement for a partner kinase to mediate signalling. For example, many RK proteins signal as part of a heteromeric complex with a co-receptor such

as BAK1 /SERK3 (Roux et al. 2011; Schwessinger et al. 2011), or CERK1 in Arabidopsis (Miya et al. 2007), (SERK1 and CEBiP1, respectively in rice). In these interactions, the receptor binds the ligand and then dynamically associates with the co-receptor, rather than the two being permanently in complex together. The formation of the heterodimer can be via direct interactions between all three components, or via ligand-induced conformational changes in the receptor enhancing binding affinity for the mediator (Sun et al. 2013; Wang, Li, et al. 2015).

The stoichiometry of this interaction permits a single co-receptor to interact with several receptors over its lifetime and modulate a response appropriate to the interacting receptor. It also permits RPs without kinase domains to signal via a co-receptor with a kinase domain, for example the *Solanum lycopersicum* RP genes *Cf-2, Cf-4, Cf-5* and *Cf-9* mediate signalling through association with SOBIR family members (van der Hoorn et al. 2005; Hammond-Kosack, Jones, and Jones 1994; Liebrand et al. 2013). Finally, given the irreversible nature of ligand-receptor binding, it provides a mechanism whereby the receptor can be degraded after use without compromising the co-receptor, permitting co-receptor degradation to trigger an immune response similarly to an intracellular recognition event (Shan et al. 2008; Yamada et al. 2016). Conversely, signalling-deficient BAK1 mutants such as *bak1-5* attenuate the immune response of their partner receptors without an increase in SA signalling or hypersensitivity (Yasuda, Okada, and Saijo 2017). BAK1 is regulated by a set of BRI proteins (BRI1 to BRI4) which compete with immune receptor proteins for BAK1 binding and are outcompeted by immune receptors that have bound a ligand (Halter et al. 2014; Ma et al. 2017; Imkampe et al. 2017).

1.4.8 Extracellular recognition requires homeostatic regulation

After receptor-ligand-co-receptor complex formation and signalling, the receptor is decoupled from the co-receptor via ubiquitination and reclamation of the receptor-ligand complex, and the co-receptor is "reset" such that it can accept another partner. In the FLS2/BAK1 interaction this proceeds via BAK1 mediated phosphorylation of the ubiquitin ligases PUB12 and PUB13 (Robatzek, Chinchilla, and Boller 2006; Lu et al. 2011) as well as a PP2A-complex-mediated dephosphorylation of BAK1 (Segonzac et al. 2014; Couto and Zipfel 2016). Other receptor/co-receptor pairs are believed to undergo similar events. The

reclamation of a given immune receptor (e.g. FLS2) also provides for a desensitisation of the plant cell towards its ligand, as at lower than homeostatic receptor concentrations, signalling will be attenuated (Smith et al. 2014).

1.4.9 Kinase activity is required for downstream responses to extracellular recognition

While RK signalling pathways are complex, two of the better described components are calcium-dependent protein kinase signalling (CDPK) (Romeis 2001), and mitogen-associated protein kinase cascades (MAPK) (Ichimura et al. 2002). The kinase domain in the receptor or its signalling partner is activated by auto- or reciprocal- phosphorylation upon formation of the holoenzyme complex, which then phosphorylates an appropriate intermediate such as BIK1 (Veronese et al. 2006; Ma et al. 2020). This is the first step of a "phosophorelay", whereby phosphorylation serves to activate a protein kinase domain, which then phosphorylates and activates another kinase protein, and so on. A MAPK cascade is composed of a relatively conserved set of protein classes that signal in this manner: first phosphorylation activates a MAP-kinase-kinase-kinase (MAPKKK) protein, which may then activate a specific MAPKK (or set of MAPKKs), and from there a particular MAPK(s) which go on to phosphorylate regulators of transcription (Zhang and Klessig 2001; Asai et al. 2002; Ekengren et al. 2003; Meng and Zhang 2013). This arrangement permits a single receptor to effect multiple signals within the plant, allowing fine-tuning of the response to the threat presented (Asai et al. 2002). Within the *A. thaliana* genome, for example, Ichimura et al. (Ichimura et al. 2002) identified 20 MAPK genes, 10 MAPKK genes, and at least 60 putative MAPKKK genes, with MAPK3, MAPK4 and MAPK6 being especially relevant to plant-pathogen interactions (Petersen et al. 2000; Asai et al. 2002; Colcombet and Hirt 2008).

In CDPK signalling, the signalling pathway is less well understood but certain family I, III and IV CDPKs have each been found to play a role defence signalling (Boudsocq et al. 2010; Boudsocq and Sheen 2013). CDPKs have been shown to induce RBOHB-mediated ROS bursts, induce transcriptional reprogramming and hormone signalling (Ludwig, Romeis, and Jones 2004; DeFalco, Bender, and Snedden 2010; Boudsocq and Sheen 2013), and to prepare the cell to initiate the hypersensitive response (Romeis 2001). Ca^{2+} influx to the cytoplasm is also one of the first signals deployed in response to contact with microorganisms (Keinath et al.

2015) whether pathogenic or mutualistic, although not all CDPK pathways are dependent on the degree of Ca^{2+} in the cytoplasm (Boudsocq and Sheen 2013). CDPKs have been found to work co-operatively with the MAPK pathway in the *A. thaliana* response to flagellin, as well in providing resistance to *Cladosporium fulvum* in *Nicotiana benthamiana* (Romeis 2001)

1.4.10 LRR-XII is a subfamily of LRR-RK predominantly associated with defence

LRR-RK proteins are the predominant class of extracellular recognition receptor (Morillo and Tax 2006; Fischer et al. 2016) and can broadly be divided into two major categories based on their evolutionary history: Lineage-specific, expanded and orthologue-unambiguous, nonexpanded. These two classes are also associated with function: lineage-specific, expanded LRR-RKs are associated with immunity and tend to exist as tandem repeats at particular loci, whereas the more conserved, orthologue-unambiguous, nonexpanded class are associated with developmental functions (Shiu et al. 2004; Tang et al. 2010) although a minority of LRR-RKs do not adhere to either classification. LRR-RKs can also be divided into specific clades although their diversity throughout the plant kingdom based on their protein kinase domain. Shiu et al. (Shiu and Bleecker 2003; Shiu et al. 2004; Lehti-Shiu et al. 2009) categorised the RLK / PELLE genes of Arabidopsis and Rice into over 60 families and subfamilies, of which 15 families were LRR-RK-specific (LRR-I, LRR-II, LRR-III, LRR-IV, LRR-V, LRR-VI, LRR-VIIa, LRR-VIIb, LRR-VIII-1, LRR-VIII-2 LRR-IX, LRR-Xa, LRR-Xb, LRR-Xc, LRR-XI, LRR-XII, LRR-XIIb, LRR-XIIIa, LRR-XIIIb, LRR-XIV, LRR-XV). Their proposed nomenclature for RK protein phylogeny has informed most further analyses.

Of special note is the LRR-XII subfamily, which is dramatically expanded in Rice (>100 members) compared to Arabidopsis (10 members), and which contains several of the most studied plant defence LRR-RK genes, including FLS2 and EFR in dicotyledonous plants and Xa21 in the Oryzoideae. Liu et al. (Liu, Du, et al. 2017) performed an expanded analysis of the LRR-RK proteins of an additional eight species including: *Physcomitrella patens, Selaginella moellendorffii, Citrus clementina, Glycine max* and *Populus trichocarpa*. They identified a phylogenetic structure which broadly agrees with that of Shiu and Bleecker, although certain clades were divided into multiple sub-clades, indicating that innovation generally progresses within these subclades, rather than through the generation of entirely novel, species-specific LRR-RK protein clades. They identified three classifications for subclades, describing the

degree of gene structure conservation: Category A, in which gene structure is conserved between vascular plants and brophytes, Category B; in which gene structure is conserved only within vascular plants, and Category C; in which gene structure is only conserved between homologues in more closely related plants. They also identified 16 LRR 'motifs', which varied from being universal across LRR-RK subclades to being entirely unique. They also found that related subclades tend to exhibit related LRR motifs, although LRR domains are too variable to provide an informative phylogenetic analysis of RK proteins as a whole.

1.5. Barley and *Puccinia striiformis*

1.5.1 Barley is a diploid cereal crop closely related to wheat

Barley (*Hordeum vulgare*) is a member of the Triticeae, a tribe within the subfamily Pooideae in the family Poaceae and order Poales. It is estimated to be the world's fourth most cultivated cereal crop after maize, rice, and wheat (FAO 2019) and is mainly used for animal feed and malting. It is inbreeding, diploid, and grows well in a variety of climates. The barley genome is approximately 5.1 Gbp in length and is highly repetitive (Mascher et al. 2021) and a pan-genome describing 20 accessions of barley was recently published (Jayakodi 2020). In addition to its role in agriculture, barley provides an excellent genetic resource for comparative studies within the Poaceae, due to its overall tractability and close relationship with wheat.

1.5.2 *Puccinia striiformis*

1.5.3 Life cycle of *Puccinia striiformis*, the causal agent of stripe rust

P. striiformis, like many rusts, has a complex five stage lifecycle. When infecting the primary host (such as wheat), an urediniospore (2N), or aeciospore (2N) makes contact with and adheres to the leaf surface through a combination of hydrostatic forces and rapidly secreted cellulase enzymes (Stubbs, Roelfs, and Bushne 1985; Chen, Wellings, et al. 2014; Schwessinger 2017; Chen and Kang 2017). The spore then germinates, developing a germ tube which seeks out a stomata. If successful, the tip of the germ tube develops into a penetration peg. This structure forcibly enters the leaf through the stomatal opening, and once inside undergoes differentiation into a sub-vesicle from which hyphae grow between

the surrounding mesophyll cells. The hyphae will also generate specialised haustorial mother cells outside the cell wall of host mesophyll cells, which proceed to first eliminate a section of the plant cell wall, then invaginate the plant cell membrane to form a large and roughly spherical or tubular feeding structure called a haustorium on the other side of this neck through the plant cell wall. The haustorium is in close or direct contact with the host cell membrane and is the interface for nutrient exchange between the host and fungus. The fungus will also develop runner hyphae, which do not generate haustorial mother cells but instead travel perpendicularly to the leaf axis until they reach a vascular structure, then multiply within that structure to spread further across the leaf, giving rise to the characteristic striped patterning of the eventual uredinia. After 7-12 days of growth within the leaf in this manner, the fungus will begin to create one or more pustule beds: areas with a high concentration of mycelia that develop into uredinia – specialised structures just underneath the leaf epithelium (on either side of the leaf) which produce huge numbers of bright orange, clonally produced urediniospores. Each colony may produce several uredinia and each uredinium may produce tens of thousands of urediniospores (Katsuya and Green 1967). The optimum conditions for uredinial development and spore release are damp (e.g. light rain or dew) and between 7-12˚C, however adaption to other climates across the globe has been reported (Mboup et al. 2012). After 10-15 days the uredinia erupt from under the leaf epithelium and begin to release their spores which may be carried by the air, in water droplets, or upon passing organisms, and which may repeat the process of infection on other leaves or other susceptible plants, sometimes hundreds of miles away if blown by a favourable wind.

After a longer period of growth, or under higher growth temperatures the fungus will begin to develop telia. Telia generate teliospores, which are a dark brown or black colour and more rectangular than urediniospores. Crucially, teliospores are diploid and consist of two binucleate cells. The teliospores remain physically attached to the fungal colony, and can endure for several months, often upon leaf detritus after harvest. Under favourable environmental conditions the nuclei of the teliospores fuse, (N+N to NN) and undergo meiosis. The teliospores then germinate, giving rise to four haploid daughter basidiospores. These spores are specialised to the alternative or secondary host. In the case of *P. striiformis* the alternate hosts are *Berberis* spp. (barberry plant) (Jin, Szabo, and Carson 2010). Upon the alternative host, infection proceeds in a similar manner to the primary host (Rodriguez-Algaba

et al. 2014), except that colonies develop pycnia, rather than uredinia. Pycnia are specialised structures which generate a large number of pynciospores and secrete a nectar within which pycniospores may travel across or between leaves under external forces such as water splashes or via insects. When pycniospores are exposed to the receptive hyphae of a pycnium belonging to a colony of another mating type, they fuse together and the nuclei within undergo sexual recombination. The colony then develops dikaryotic mycelium, becoming a spore bed and eventually a single aecium, which erupts from the leaf in a similar manner to the uredinum and releases thousands of genetically identical aeciospores which may infect the primary host.

1.5.4 The *formae speciales* of stripe rusts

P. striiformis can be further divided into *formae speciales* which exhibit different degrees of species specificity. Jacob Erikkson (Eriksson 1894, 1898) first identified that rust urediniospores isolated from a given plant species did not germinate on other species. Since then, *P. striiformis* isolates have been categorised into a *formae speciales* depending on their primary host, although not all are limited to a single species. *Puccinia striiformis* f. sp. *tritici* is generally a pathogen of wheat and is rarely observed to infect cultivated barley. For example in Australia, while barley accessions known to be susceptible to *Puccinia striiformis* f. sp. *hordei* have been exposed to *Pst* inoculum for over 60 years, a host-jump event has not been recorded. Despite this, *Pst* is capable of infecting and reproducing on certain barley accessions, primarily landraces and wild barley, as well as the hypersusceptible line SusPtrit (Niks 1983; Jacobs 1989; Yeo et al. 2014; Niks et al. 2015; Dawson 2015).

The *formae speciales* of species in genera such as *Puccinia* (rusts), *Fusarium* (scabs), and the order *Erysiphales* (mildews) are adapted to particular hosts. The resistance of related species to a non-adapted *forma specialis* is durable, meaning that it "remains effective during prolonged and widespread use in an environment favourable to the disease" (Johnson 1979; Bettgenhaeuser et al. 2014; Lee, Whitaker, and Hutton 2016; Panstruga and Moscou 2020) in contrast with the "boom and bust" cycle associated with gene-for-gene or race-specific resistance (Robinson 1976; McDonald and Linde 2002). However, it should be remembered that durability is defined by the dynamic relationships between plants and pathogens in their environment (Brown 2015; Morris and Moury 2019), in contrast to concepts like intracellular

recognition or adaptive immunity which are physical properties inherent to the components of an immune response. This thesis will focus on the interactions between *Puccinia striiformis* f. sp. *tritici* and *Hordeum vulgare*.

1.6 Exo70 proteins in eukaryotes

1.6.1 Exo70 proteins facilitate localisation and tethering of vesicles to the plasma membrane

The Exo70 class of proteins are members of the 8-member Exocyst complex along with Sec3, Sec5, Sec6, Sec8, Sec10, Sec15 and Exo84. Exocyst is conserved across eukaryotic life and is primarily responsible for localisation and tethering of secretory vesicles to the cell membrane before exocytosis. Within the complex, Exo70 interacts with Exo84, Sec10 and Sec15 via an N-terminal CorEx domain and with Sec5 via the C-terminal CAT-C and CAT-D domains (Mei et al. 2018). Exo70 also interacts with non-Exocyst partners such as PIP_2, SNARE proteins (Xu et al. 2013), RHO GTPase proteins (Roumanie et al. 2005), RAB GTPase proteins (Robinson et al. 1999; Koumandou et al. 2007), and Arp2/3 (Zuo et al. 2006) via C-terminal domains. Fusion with the membrane and secretion itself involves a variety of additional proteins, primarily components of the SNARE-complex (Sivaram et al. 2005; Yue et al. 2017).

As Exo70 is a class of protein conserved across eukaryotes, it has been extensively characterised in model systems such as yeast and suspended animal cells. Work in plants is generally carried out in the model plants *Arabidopsis thaliana* and *Nicotiana benthamiana* (Žárský et al. 2019). While animal and fungal genomes encode a single copy of the Exo70 gene, plants have evolved numerous copies which can be further divided into ten conserved families: Exo70A, Exo70B, Exo70C, Exo70D, Exo70E, Exo70F, Exo70G, Exo70H, Exo70I, and Exo70FX (or Exo70F-like) (Cvrčková et al. 2012; Synek et al. 2006). These clades are non-redundant and exhibit clear evidence of subfunctionalisation: they are expressed in different tissue types, can localise to different membrane domains, and carry different cargoes (Li et al. 2010b; Žárský et al. 2019; Žárský et al. 2013).

1.6.2 The molecular mechanisms of Exo70 proteins vary across kingdoms

The process of membrane localisation and tethering varies between kingdoms. In yeast, either Exo70 or Sec3 can be responsible for recruiting the Exocyst to the membrane (Boyd et al. 2004), and there is evidence supporting the Exocyst complex assembling at the membrane around this seed protein, or existing as a stable octamer in the cytoplasm (Boyd et al. 2004; Heider et al. 2015). In yeast Exo70 or Sec3 are transported to the appropriate membrane domain by myosins along actin cables (Bendezu 2012).

In animals, Exo70 is the only subunit which can recruit the complex to the membrane (Liu et al. 2007), and evidence suggests that the Exocyst as a whole may exist in the cytoplasm in a dynamic equilibrium between individual subunits, tetrameric subcomplexes, and the full octameric complex (Ahmed et al. 2018). The quarternary structure of the Exocyst as a whole may also vary between animals and fungi, based on differing pleiotropic effects of N- and C-terminal tags on different subunits of the complex (Nishida-Fukuda 2019).

The assembly and recruitment of the plant Exocyst complex is not as well characterised as in yeast, but *Arabidopsis thaliana* Exocyst complex assembly is less attenuated following disruption of actin or microtubule disruption than in yeast or animal cells respectively, implying that plants may have alternative requirements for Exocyst complex assembly at the membrane (Fendrych et al. 2013). Plant Exocyst is also recruited to regions of the cell membrane in a polarised manner, depending on the Exo70 component of the complex, and the biochemical makeup of the membrane domain (Zarsky 2009; Zarsky 2013).

In plants, the Exocyst is specific to plasma membrane tethering, and other complexes are involved in tethering vesicles to other membranes (Hickey et al. 2010; Vukašinović et al. 2017). It is also not the only complex with this role, and TRAPPII (Drakakaki et al. 2012) and EXPO (Wang et al. 2010) have both been linked to PM trafficking and exocytosis. Interestingly, the EXPO body is associated with the Exo70E clade of proteins and sequesters Exocyst subunits upon accumulation, (Wang et al. 2010; Ding et al. 2014). Exocyst has also been associated with a number of other roles associated with the plasma membrane, such as polarised growth, cytokinesis, cell division, cilliogenesis (Gromley et al. 2005; Zhu, Wu, and Guo 2019; Žárský et al. 2019).

1.6.3 Plant genomes encode a variety of non-redundant Exo70 proteins

The Exo70s of plants can be divided into three families: Exo70.1, Exo70.2, and Exo70.3, based on their intron/exon structure and inferred evolutionary history (Cvrčková et al. 2012; Synek et al. 2006). The ancestral family is Exo70.1, which only contains the clade Exo70A. Exo70G and Exo70I are within the Exo70.3 family, and the remainder are members of Exo70.2 (Cvrčková et al. 2012; Elias et al. 2003; Synek et al. 2006).

While clades Exo70A, Exo70B, Exo70C, Exo70D, Exo70E, and Exo70G are well-conserved, the remaining clades exhibit variation between species. Exo70I, which is required for the establishment of mycorrhizal symbioses is not present in species which do not form these associations. Exo70H, which is involved in trichome development and defence is expanded in dicots (6-8 copies) relative to monocots (1-3 copies) (Cvrčková et al. 2012; Kubátová et al. 2019; Kulich et al. 2015; Kulich et al. 2013). Exo70F, conversely is expanded in monocots, and Exo70FX is unique to monocots (Cvrčková et al. 2012; Zhao et al. 2019). However, given the relative paucity of work characterising the Exo70 clades of monocot plants, the evolutionary history of the Exo70FX clade is largely unknown. The evolution of the Exo70FX clade in monocots will be discussed in more depth in **Chapter 4**.

2. Fine-mapping and cloning of *Rps8*

2.1 Chapter Summary

Three *R* gene loci designated *Rps6, Rps7,* and *Rps8* provide resistance to *Puccinia striiformis* f. sp *tritici* across a variety of cultivated barley accessions (Dawson 2015; Dawson et al. 2016). *Rps8* was previously mapped to the long-arm of chromosome 4H using a mapping population derived from SusPtrit x Golden Promise (Yeo et al. 2014; Dawson et al. 2016) we fine-map *Rps8* to a 583 kb locus on chromosome 4H, which exists as a presence/absence variation across barley accessions. Forward genetic screens and transgenic complementation demonstrate that resistance is conferred by the concerted action of two genes at the locus, *LRR-RK* and *Exo70FX12a*, which are both necessary for *Rps8*-mediated resistance.

2.2 Introduction

2.2.1 Leucine-rich repeat receptor kinases in plants

LRR-RK proteins are the predominant class of extracellular recognition receptor (Morillo and Tax 2006; Fischer et al. 2016). LRR-RK proteins are involved in regulating growth and abiotic stress responses, as well as extracellular recognition of pathogens (Shiu et al. 2004; Morillo and Tax 2006; Tang et al. 2010; Fischer et al. 2016). In some cases LRR-RK proteins are involved in multiple signalling pathways that facilitate crosstalk between growth and defence pathways, exemplified by the dual roles of BAK1 in immune and brassinosteroid signalling (Nam and Li 2002; He, Gou, et al. 2007).

LRR-RK proteins have been consistently found to experience species-specific, and subclade-specific selective pressure, in some cases to purify and others to expand and diversify, in a manner consistent with subclade-specific roles in normal growth and stress responses, as well as immunity (Lehti-Shiu, Zou, and Shiu 2012; Fischer et al. 2016; Liu, Du, et al. 2017). This has led to the identification of 15 subfamilies of LRR-RK associated with particular roles (Shiu and Bleecker 2003). The LRR-XII family is associated with defence, and several of the best characterised LRR-RK genes involved in immunity are in this clade, including FLS2, EFR and Xa21.

2.2.2 LRR-XII family members associated with defence

FLS2 is an LRR-RK of the LRR-XII family, identified by Gómez-Gómez and Boller (Gomez-Gomez and Boller 2000) and widely conserved across dicotyledonous plants. FLS2 recognises a 22 amino acid epitope derived from bacterial flagellin (flg22) (Chinchilla et al. 2006). After ligand perception and binding, the protein-ligand complex is able to form a heterodimer with the LRR-II type RK BAK1 (Chinchilla et al. 2007; Schulze et al. 2010) which interacts with both the extracellular bound-ligand and the intracellular membrane-bound components of FLS2 (Sun et al. 2013). This induces BAK1 auto-phosphorylation (Schwessinger et al. 2011) and BAK1 trans-phosphorylation of FLS2 (Chinchilla et al. 2007; Schulze et al. 2010), which promotes downstream signalling through BIK1 phosphorylation (Lin et al. 2014), subsequent RBOH-mediated ROS burst (Yoshioka et al. 2015), CDPK signalling, and MAPK cascade signalling (especially MPK3, MPK4 and MPK6) (Asai et al. 2002; Droillard et al. 2004; Pitzschke, Schikora, and Hirt 2009; Li et al. 2010a; Lin et al. 2013). Importantly, abolishment of BAK1 signalling largely, but not completely, abolishes FLS2-mediated responses to flag22, indicating at least a partial redundancy in extracellular signalling, or a promiscuity on the part of FLS2 in the cell (Chinchilla et al. 2007). Brassinosteroid signalling also appears to be unaffected by BAK1 mutations which compromise immune signalling (Schwessinger et al. 2011; Sun et al. 2013).

EFR is another well-studied LRR-RK in the LRR-XII family, identified Zipfel et al. (Zipfel et al. 2006), which recognises the bacterial elongation factor Ef-Tu, specifically the 18 amino acid peptide fragment elf18. EFR signals via the co-receptor BAK1 and the same downstream components as FLS2 (Zipfel et al. 2006; Meng and Zhang 2013), however upstream components of EFR signalling have also been more thoroughly elucidated than those of FLS2. Proper EFR activity is dependent on a number of post-translational processes collectively referred to as Endoplasmic Reticulum Quality Control (ERQC), and the proteins PSL1, PSL2, and STT3A (Saijo et al. 2009) and SDF2, ERdj3B and BiP (Nekrasov et al. 2009). It is unknown if EFR has more extensive requirements for quality control and PTM than FLS2, or if the equivalent set of proteins have simply not been discovered. EFR has only been identified within the Brassicaceae, and is thought to be a relatively recent innovation, specific to the family.

Xa21 is the third of the well-characterised LRR-RK receptors within the LRR-XII family, identified by Song et al. (Song et al. 1995; Wang et al. 2004) and only present within the Oryzoideae. Similarly to EFR, several ERQC components of Xa21 activity have been identified, including BiP3 (Park et al. 2010) and SDF2 (Park et al. 2013), as well as a number of negative regulators which directly interact with Xa21 at the cell membrane, such as XB15 (Park et al. 2008), XB24 (Chen et al. 2010) and XB10 (Peng et al. 2008). The protein recognised by Xa21 is RaxX: a type-1 secreted peptide of 61 residues which may have effector activity as a mimic of plant growth hormones (Pruitt et al. 2017). The specific minimal ligand is a 20 residue tyrosinated region designated RaxX21-sY (Pruitt et al. 2015). Unlike flagellin and Ef-Tu, RaxX is believed to be specific to *Xanthomonas* spp. (Pruitt et al. 2015). Given that Xa21 has also been found to provide resistance to *Pseudomonas* spp. (Afroz et al. 2011; Holton et al. 2015), Xa21 may also have the capacity recognise other ligands mimicking PSY-family phytohormones. The co-receptor for Xa21 is the AtBAK1 paralogue OsSERK2 (Chen, Zuo, et al. 2014) and signalling occurs through a similar pattern of autophosphorylation and subsequent activation of downstream kinases (Chen, Zuo, et al. 2014). Finally, a fusion of the Xa21 ectodomain with the AtEFR transmembrane and signalling domains is able to provide defence against *Xanthomonas oryzae* in rice (Thomas et al. 2018) indicating conservation of the general mechanism of action within the LRR-XII family of receptor kinases.

2.2.3 Wheat stripe rust (*Puccinia striiformis* f.sp. *tritici*) resistance in barley

The *Pucciniales* are an order of obligate biotroph fungal pathogens, and causal agents of rusts on a wide variety of host plants (McTaggart et al. 2016; Chen and Kang 2017). Particularly relevant to the cereal crops are *P. striiformis* (stripe rust), *P. triticina* (leaf rust), and *P. graminis* (stem rust) (Roelfs and Bushnell 1985). Rusts, like many obligate biotrophs, can be further divided into *formae speciales*: lineages of the fungus which have adapted towards a particular host plant, and into 'races': groups which describe the particular pattern of virulence and avirulence exhibited by a given isolate (Stubbs, Roelfs, and Bushne 1985; Roelfs and Bushnell 1985). *Puccinia striiformis* f. sp. *tritici* (*Pst*) primarily infects susceptible wheat varieties but has been observed to successfully colonise and infect a minority of barley accessions: mainly landraces and wild barley lineages (Dawson et al. 2016).

Previous work identified the genetic architecture of resistance to *Pst* in barley as being conferred by three *R* gene loci in the majority of tested germplasm (Dawson 2015; Dawson et al. 2016). These loci are designated *Rps6, Rps7,* and *Rps8*. *Rps6* and *Rps7* have been cloned and found to encode NLR-type resistance genes on chromosome 7H and 1H, respectively. *Rps8* was identified in several diverse barley accessions and mapped to the long-arm of chromosome 4H using a mapping population derived from SusPtrit x Golden Promise (Yeo et al. 2014; Dawson et al. 2016) Unlike *Rps6* and *Rps7* identified in these studies, *Rps8* has a minor effect on hyphal colonization, but prevented pustule formation (**Figure 2.1**). In this work, we set out to map-based clone *Rps8*. *Rps8*-mediated resistance is conferred by a genetic module, encompassing two genes: an LRR-RK and an Exo70, which are independently necessary, and together sufficient to provide resistance to *Pst* in barley.

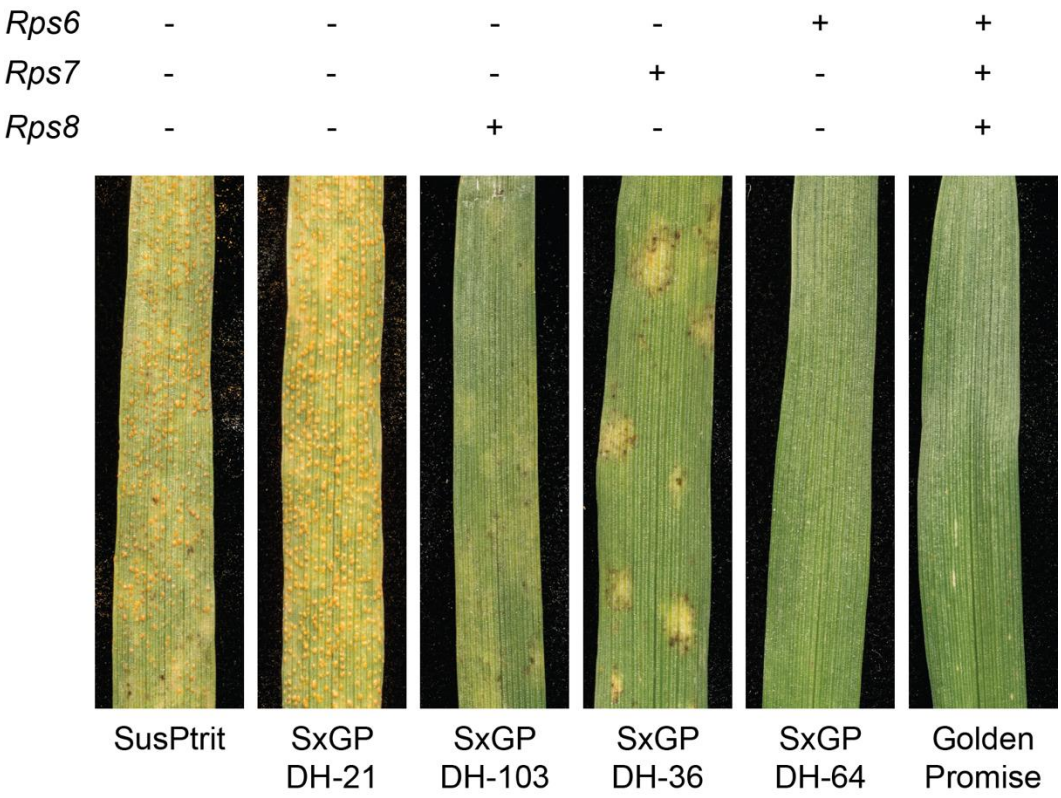

Figure 2.1. *Rps8* prevents pustule formation, but not colonization by wheat stripe rust (*Puccinia striiformis* f. sp. *tritici*) in barley. Top section indicates the resistance genes present in a panel of accessions derived from a Golden Promise x SusPtrit F1 doubled-haploid population. Bottom section displays a representative phenotype from a first leaf of that accession, challenged with *Pst,* at 14 dpi. Any of *Rps6, Rps7* or *Rps8* is sufficient to prevent development of orange pustules, however *Rps6* and *Rps7* permit colonisation and mycelial growth which leads to discolouration of the leaf.

2.3 Results

2.3.1 Fine mapping of *Rps8* resolved the locus to a 900 kb region on chromosome 4H

Rps8 is located on the long arm of chromosome 4H and prevents the development of pustules of wheat stripe rust, but not colonisation. Inheritance of *Rps8* in an F_2 population generated by crossing SxGP DH-21 (*rps8*) and SxGP DH-103 (*Rps8*) from the doubled-haploid SusPtrit x Golden Promise population (Yeo et al. 2014) found clear segregation for a single dominant gene (**Figure 2.2**). A high resolution recombination screen was performed using additional

markers generated in the *Rps8* region, identifying 127 recombinants from 9,216 evaluated gametes, and mapping *Rps8* to a 0.5 cM genetic interval. This interval corresponds to a 936 kb physical interval in the genome of the reference accession Morex, which contains the *Rps8* haplotype (Mascher et al. 2020) (**Figure 2.3**). Markers co-segregating with *Rps8* are spread across a 664 kb physical interval. Nine recombination events were identified proximal to the markers co-segregating with *Rps8* and fifteen in the distal region (**Figure 2.3**). Despite these regions being much smaller than the *Rps8* interval; 140 kb and 239 kb respectively, no recombinants were identified within the group of markers co-segregating with *Rps8*. To identify candidate genes in the region, RNAseq date derived from Morex and Golden Promise was aligned to the *Rps8* physical region using hisat2 (IBGSC 2012). Gene models were predicted based on spliced alignments using Cufflinks and curated based on expression evidence and existing reference genome annotations. Several protein encoding genes were identified including DUF-1997 protein (DUF), Armadillo-repeat protein (ARM), Exo70 (Exo70FX12), transmembrane receptor protein kinase (LRR-RK), a Pck-like kinase (Kinase), a truncated NLR (NLR), a zinc-finger transcription factor (Zinc-Finger) and a Myb transcription factor (Myb) (**Figure 2.3).**

To evaluate the candidacy of individual genes in the interval: genetic, genomic, and transcriptomic data were evaluated for each gene model. Despite being the most promising initial candidate; the NLR was found to be expressed in roots but not leaves. Furthermore, the NLR was found to not have an intact open reading frame and instead encodes a truncated NLR. To evaluate other genes in the region, we leverage previous genetic analyses that determined the presence or absence of *Rps8* in a panel of 17 barley accessions (**Supplementary table 1.1**). Alignment of RNAseq derived from the same panel of barley accessions revealed the *Exo70* (Subsequently Exo70FX12a) and *LRR-RK* exist as an expression level polymorphism, where both genes are expressed in *Rps8* containing accessions and absent in accessions without *Rps8*. No such pattern was observed for the other genes in the interval. Evaluation of SNPs in other genes in the interval found there were no SNPs relative to Morex and Golden Promise in either candidate gene in any accession known with an *Rps8* haplotype.

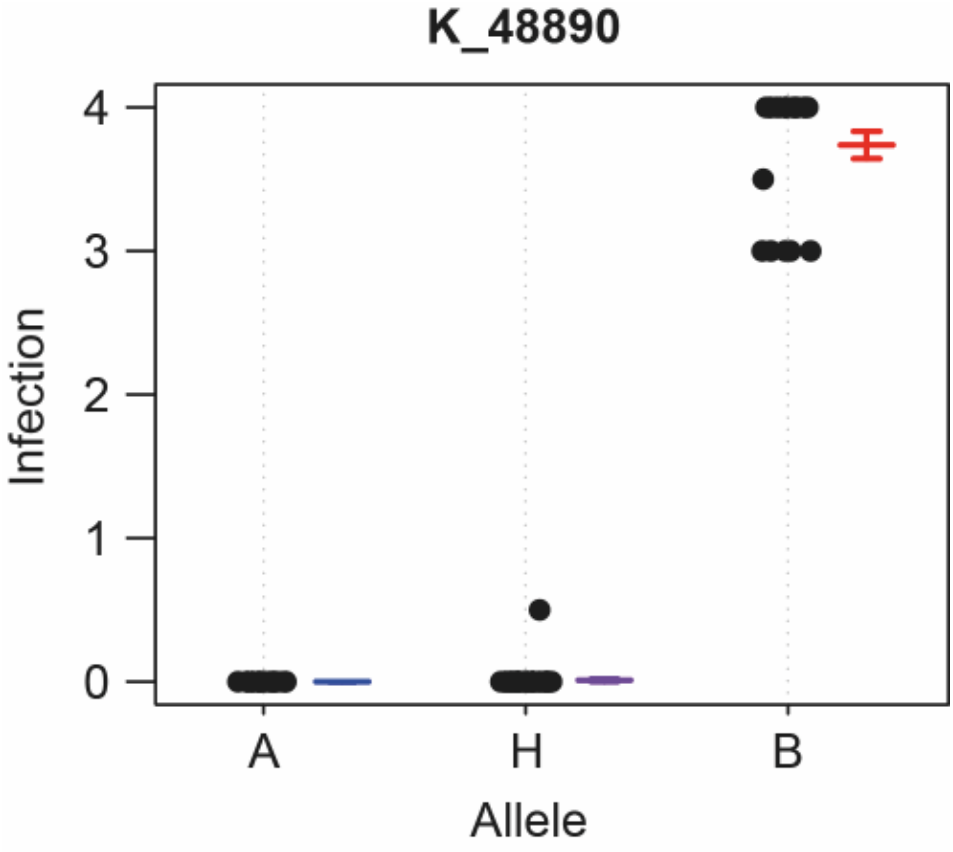

Figure 2.2: Mendelization of *Rps8* in SusPtrit x Golden Promise DH-21 x DH-103 F_2 population. The KASP marker K_48890 cosegregates with *Rps8*. F_2 (n = 94) individuals homozygous for the Golden Promise allele (A) or heterozygous (H) exhibit near-complete resistance to wheat stripe rust, whereas individuals homozygous for the SusPtrit allele (B) are susceptible. Infection was scored using a 0 to 4 scale and reflects the percent of the leaf covered with pustules (range from 0 to 100%).

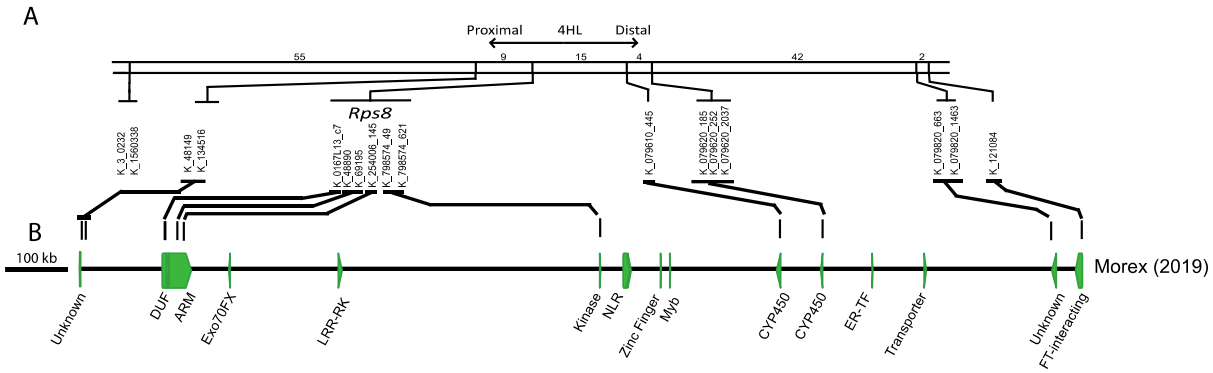

Figure 2.3: Genetic and physical maps of the *Rps8* interval and sample phenotypes of families used in this recombination screen. A: Genetic map of the *Rps8* interval. Numbers above the chromosome indicate individual recombinants obtained between the two adjacent markers and markers are connected to their position on the physical map. **B:** Physical map of the *Rps8* interval in the 2019 Morex genome. Genes are represented with green arrowed boxes and labelled with their predicted protein class. *Rps8* was previously mapped using the markers K_121084 and K_03232.

2.2.3 *Rps8* encompasses a 546 kb InDel

To determine whether the expression polymorphism in *Exo70FX12a* and *LRR-RK* was due to structural variation in the *Rps8* locus between *Rps8* and *rps8* haplotypes, the genomes of Morex (*Rps8*) (Jayakodi et al. 2020), Golden Promise (*Rps8*) (Schreiber et al. 2020), the wild barley accession AWCS276 (*rps8*) (Liu et al. 2020), and a *de novo* sequenced chromosome 4H of accession CI16139 (*Rps8*), assembled using chromosome flow sorting and Chicago-based library construction were compared for structural variation in the region. Sequence identity between the three *Rps8* haplotypes (Morex, GP, CI16139) was extremely high, however a large (190 kb), repetitive area identified in Morex was a breakpoint in the assembly of the CI16139 and Golden Promise *Rps8* locus. Comparing these three haplotypes to the wild barley accession AWCS276 found a 546 kb InDel that encompasses both the *Exo70* and *LRR-RK* (**Figure 2.4)**. The *Rps8* insertion is flanked in both directions by a short duplicated region which is present in the *rps8* haplotype at the location of the insertion. This suggests that the underlying variation observed in accessions lacking *Rps8* involves a deletion of the interval.

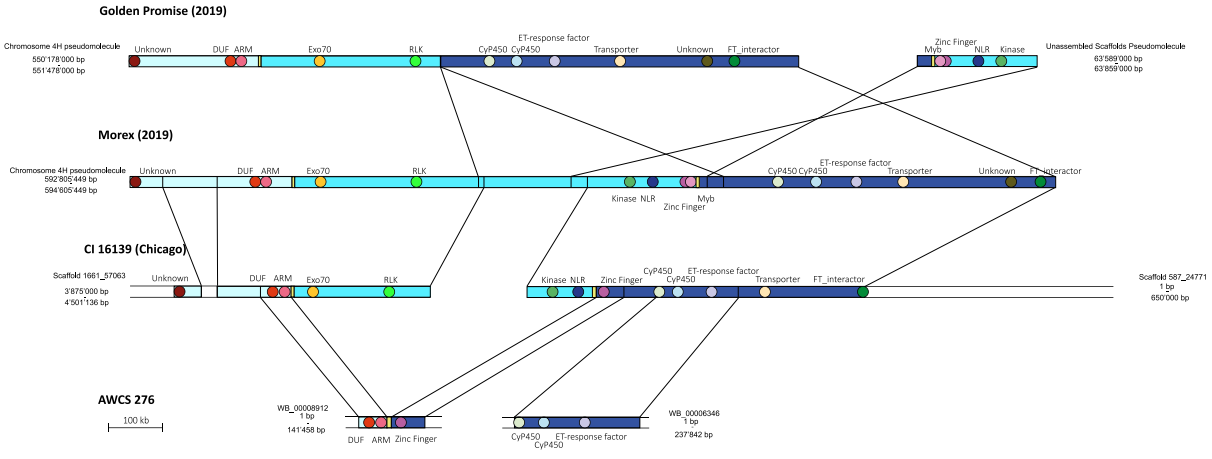

Figure 2.4: Structural variation at the *Rps8* locus between multiple accessions. Physical maps of the *Rps8* locus in Morex, Golden Promise, CI16139, and the wild barley accession AWCS276. Genes are indicated by coloured circles across all four accessions, and labelled with their predicted protein class. Physical sequence is shaded light blue in the area proximal of the *Rps8* InDel, sky blue in the *Rps8* interval, and dark blue in the area distal to the *Rps8* InDel. The yellow block indicates a sequence found at the borders of the *Rps8* interval. The Golden Promise and CI16139 assemblies did not assemble the locus into a single contig, presumably due to the extensive repetitive sequence in the interval. The area of a contig shown is described adjacent to the contig.

2.3.4 Natural and induced variation identifies loss-of-function mutants at *Rps8*

Suppressed recombination in the *Rps8* region, likely due to the presence of an InDel, made further map-based cloning of *Rps8* futile. Next, we interrogated other sources of variation to evaluate candidate genes underlying *Rps8*. RNAseq data from a panel of 40 barley accessions was aligned to *Rps8* and inspected (**Supplementary table 1.1**). Four accessions harboured SNPs in either *Exo70FX12a* or *LRR-RK* (Heils Franken, WBDC008, WBDC013, and WBDC085). Based on RNAseq data from leaf tissue, Heils Franken contains a single non-synonymous SNP in the CDS of *Exo70FX12a* (hereafter *Exo70FX12-HF*, in the haplotype *rps8-HF*) causing a E388K substitution. The wild accessions WBDC008, WBDC013, and WBDC085 each contain 2-7 non-synonymous SNPs in both candidate genes compared to Morex. Whole genome sequencing of Heils Franken and *k*-mer analysis (k=31) of the *Rps8* region identified only nine polymorphisms that differentiate the Morex and Heils Franken haplotypes. The only SNP identified in a gene was the previously identified non-synonymous polymorphism in *Exo70FX12*.

Heils Franken is resistant to *Pst.* To determine whether resistance is conferred by *Rps8*, Heils Franken x Manchuria F_2 and BC_1 populations were generated and inoculated with *Pst* isolate 08/21. Interval mapping using 64 KASP markers identified a single major-effect locus on chromosome 1H providing resistance, and no QTLs on chromosome 4H (**Figure 2.5**). Phenotyping of the population with a marker for *Rps8* and the peak marker identified on chromosome 1H indicates that the *rps8-HF* haplotype does not provide resistance to *Pst* (**Figure 2.6**), potentially due to the mutation present in *Exo70FX12-HF* allele.

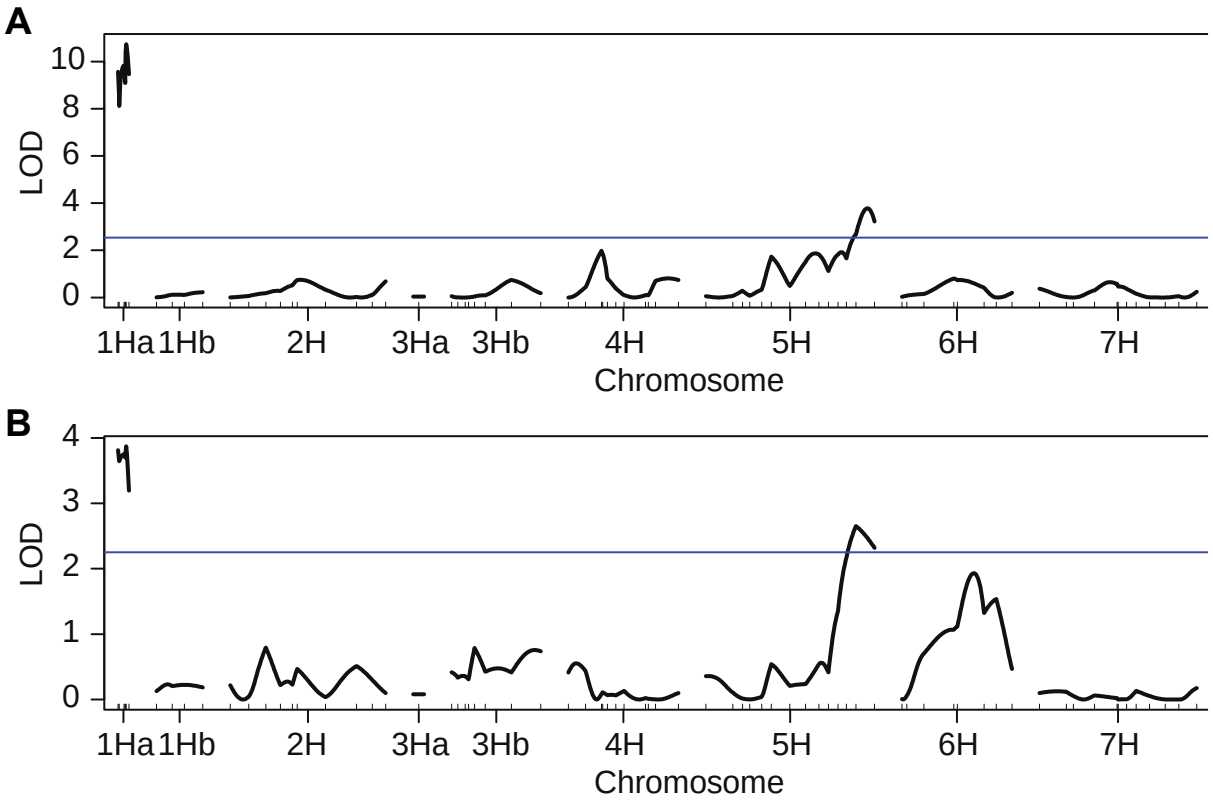

Figure 2.5: Interval mapping using a BC_1 population derived from Heils Franken shows no association between resistance and *Rps8*. A. Interval mapping of the Heils Franken x Manchuria BC_1 population; challenged with *Pst* isolate 08/21. Genetic map was constructed with 64 KASP markers and the phenotype was pCOL; a microscopic phenotype quantifying the proportion of leaf segments with hyphal growth(Dawson 2015), in a BC_1 (n = 94) population derived from an F_1 cross between Heils Franken and Manchuria, backcrossed to Manchuria. **B.** Interval mapping in that same BC_1 population using the phenotype pPUST. pPUST is a microscopic phenotype, where infection is measured by quantifying the proportion of leaf sections where pustules are visible (range from 0 to 1) (Dawson 2015).

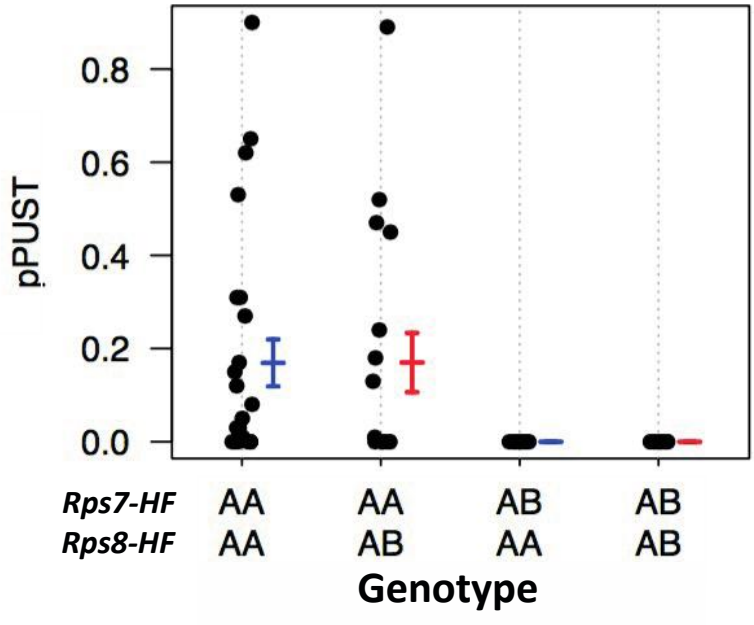

Figure 2.6: The Heils Franken allele of *Rps8* does not provide resistance to *Pst*. BC_1 (n = 94) individuals homozygous for the Manchuria allele (A) of *Rps7* exhibit susceptibility to wheat stripe rust, whereas individuals heterozygous for the Heils Franken allele (B) are resistant. The presence or absence of the Heils Franken allele of *Rps8* confers no effect in the absence of *Rps7. Rps7* is indicated by the KASP marker SCRI_RS_66630_159_R, *Rps8* is indicated by the KASP marker 2_0974_120_F. pPUST is a microscopic phenotype, where infection is measured by quantifying the proportion of leaf sections where pustules are visible (range from 0 to 1) (Dawson 2015).

The lack of further natural variation in the *Rps8* locus indicated that a different approach would be required to generate additional evidence for the role(s) of *Exo70FX12a* and *LRR-RK* in *Rps8*-mediated resistance. In order to determine if *Exo70FX12a* is the sole requirement for *Rps8*-mediated resistance, a forward genetic screen was used to identify genes underpinning additional loss-of-function mutants. A mutant population had previously been generated using the reference accession Morex (TILLMore population), which harbours *Rps8* in isolation from other loci providing *Pst* resistance. The TILLMore population was generated using sodium azide and has been advanced to the M_6 stage (Talamè et al. 2008). Using this population, we screened 1,526 M_6 families with *Pst* isolate 16/035 and identified 35 putative mutants with an infection phenotype (area showing pustule formation) of 1.0 or higher.

Single seed descent of putative mutants and subsequent re-evaluation with *Pst* isolate 16/035 confirmed 9 mutants were susceptible to *Pst* (**Table 2.1**)(**Figure 2.7**). RNAseq was performed on these mutants to identify polymorphisms in genes at the *Rps8* locus as well as other candidate gene families.

RNAseq of confirmed mutants identified two independent mutations in the *LRR-RK* CDS: *rps8-TM90* exhibits a G to A polymorphism at 1,409 bp causing a G432R variation in the LRR encoding region and *rps8-TM98* exhibits a G deletion at 2,504 bp in the kinase encoding region that causes a frame shift and early stop codon. TM3535 was the only line that carried a mutation in the *Exo70FX12α* CDS (*rps8-TM3535*), exhibiting a C to T transition at 388 bp leading to an L130F variation in *Exo70FX12α*. Crosses were undertaken between mutants at the *Rps8* locus in order to identify complementation groups (**Figure 2.7**), and TM 90 and 98 were found to share a complementation group with each other, but not with TM3535.
Six additional mutant lines were identified for which RNAseq did not identify any variation at the *Rps8* locus. The genes mutated in these lines are subsequently referred to as Required for Stripe rust Resistance (*Rsr1, Rsr2* etc.). These results show that mutation in either *LRR-RK* or *Exo70FX12α* at the *Rps8* locus is sufficient to abolish *Rps8*-mediated resistance. *Rps8* is therefore composed of two genes, which function together as a genetic module.

Table 2.1: TILLMore mutants identified as susceptible to *Pst*. A forward genetic screen of 1,525 TILLMore mutants, generated by treating Morex with sodium azide and developed to the M_6 generation, uncovered 9 mutants susceptible to *Pst*, and therefore without *Rps8*-mediated resistance. Infection was scored using a 0 to 4 scale and reflects the percent of the leaf covered with pustules (range from 0 to 100%).

Mutant	Average infection score
TM90	3.0
TM98	3.4
TM181	3.3
TM343	1.5
TM1781	1.5
TM2907	4.0
TM3013	1.8
TM3535	3.9
TM4087	1.1

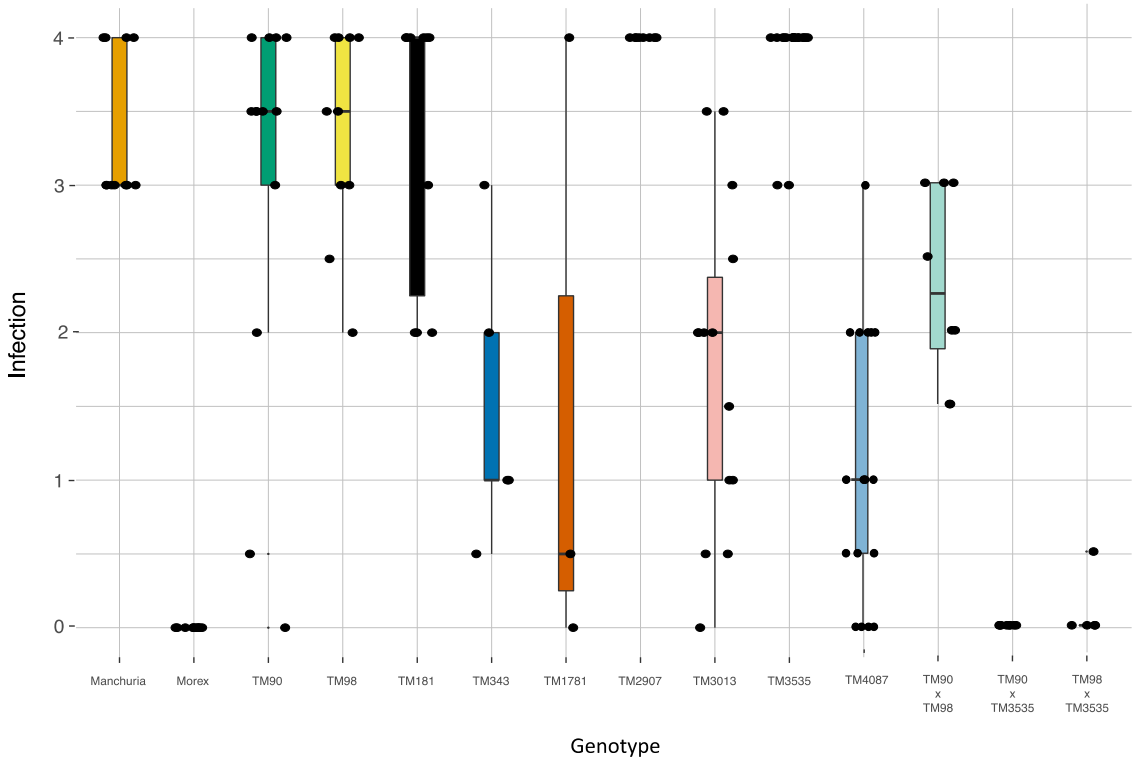

Figure 2.7. Identification of TILLMore mutants with susceptibility to *Pst,* two of which are in the same complementation group. TILLMore mutants were challenged with *Pst* and phenotyped for susceptibility 10 days after inoculation. Mutants TM90, TM98, and TM3535 were found to exhibit mutations in genes at the *Rps8* locus and were crossed together to identify complementation groups. TM90 and TM98 form a complementation group with each other. Morex and Manchuria are included as controls. Infection was scored using a 0 to 4 scale and reflects the percent of the leaf covered with pustules (range from 0 to 100%).

2.3.5 Transgenic complementation shows both *LRR-RK* and *Exo70* are necessary and sufficient to confer *Rps8*-mediated resistance

To confirm the role of *LRR-RK* and *Exo70FX12a in Rps8*-mediated resistance, several T-DNA constructs were designed to express *LRR-RK* and *Exo70FX12a* together and individually. Due to difficulties with cloning the *LRR-RK* gene into an appropriate plasmid, only a T-DNA

construct expressing *Exo70FX12a* under its native promoter and terminator was generated by the time of writing.

Agrobacterium-based transformation of the susceptible accession SxGP DH-47 was used to generate several independent transformed lineages for the construct (Hensel and Kumlehn 2004). T_1 families containing the insert were inoculated with *Pst* isolate 16/035, and found to be susceptible. This suggests that the Exo70FX12a is insufficient to confer *Rps8*-mediated resistance. To determine whether the T-DNA can complement mutants in Exo70FX12a, crosses were performed between homozygous, single-copy transgenics and either TILLMore mutant TM3535 or an *rps8* homozygous backcrossed Heils Franken x Manchuria line (HFxM BC_1S_2) selected for susceptibility and the *rps8-HF* haplotype. All F_1 progeny were resistant to Pst isolate 16/035, whereas the controls SxGP DH-47, TM3535 and HFxM BC_1S_2 were susceptible. Evaluation of F_2 progeny of these crosses found that at least one copy of each gene was required for resistance (**Figure 2.8**). These results demonstrate that a T-DNA containing native *Exo70FX12a* is sufficient to complement both mutants.

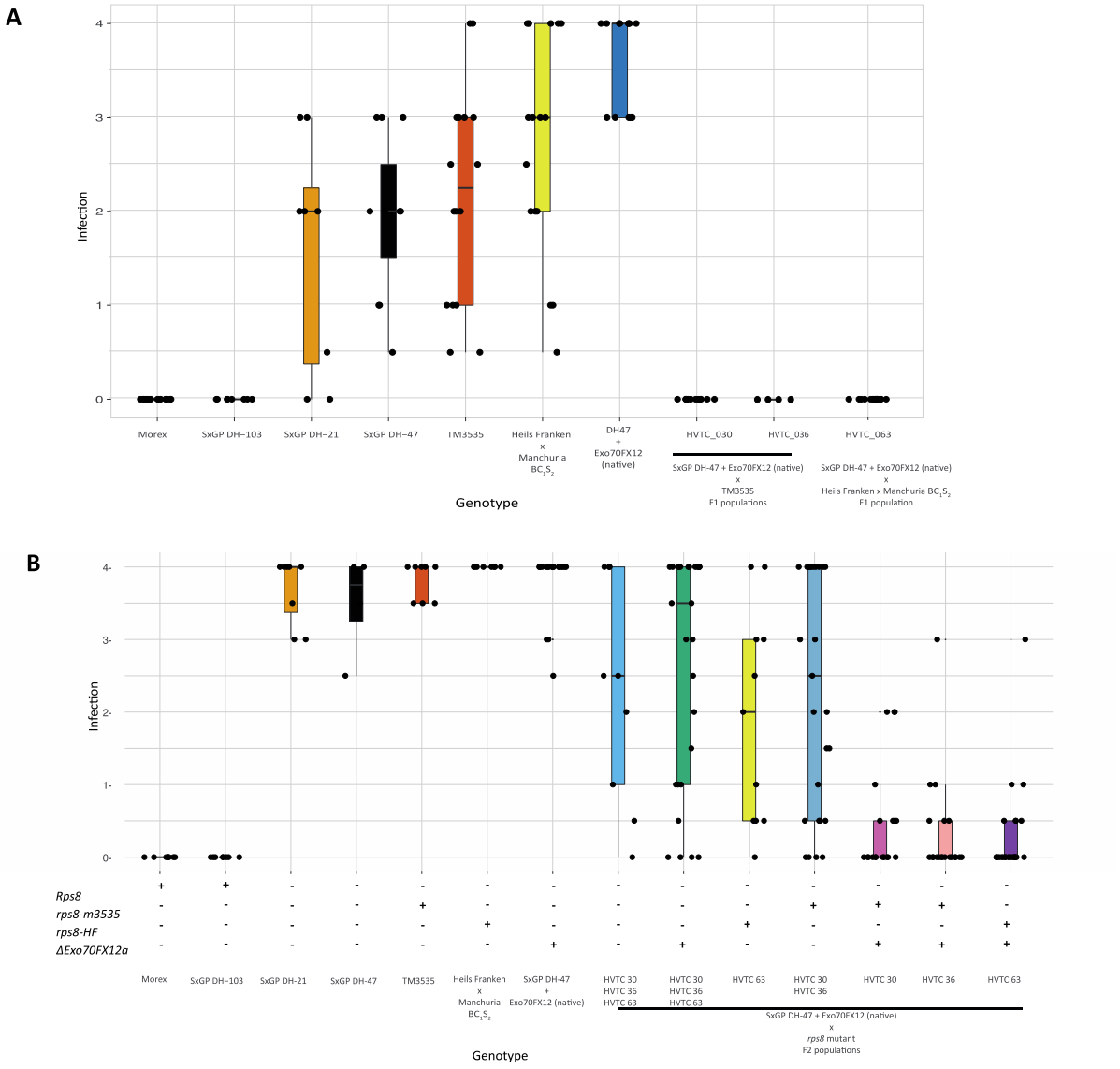

Figure 2.8. Exo70FX12a under its native promoter and terminator is insufficient to provide resistance to *Pst* but complements TILLMore mutant M3535 and the *rps8-HF* allele. **A.** Transgenic SxGP DH-47 individuals with at least one copy of Exo70FX12a are susceptible to *Pst*. F_1 progeny of crosses between these individuals and the susceptible mutant TILLMore M3535 and susceptible lines containing the *rps8-HF* haplotype are resistant to *Pst*. **B.** F2 progeny of crosses between transgenic SxGP DH-47 containing Exo70FX12a and lines with the *rps8-m3535* or *rps8-HF* haplotypes segregate for resistance such that both the transgene and a haplotype containing *LRR-RK* are required for resistance. Presence of T-DNA was verified by RT-PCR, *Rps8* haplotypes were verified by KASP marker analysis. Infection was scored using a 0 to 4 scale and reflects the percent of the leaf covered with pustules (range from 0 to 100%).

2.4 Chapter Discussion – co-operation between an Exo70 and RK in plant immunity.

2.4.1 *Rps8*-mediated resistance is conferred by a genetic module: a receptor-kinase and Exo70

The *Rps8* locus, which provides resistance to *Puccinia striiformis* f. sp. *tritici* in barley has been fine mapped to a 583 kb locus on chromosome 4H, which exhibits presence/absence variation across barley accessions. Resistance is conferred by the concerted action of two genes at the locus, *LRR-RK* and *Exo70FX12a*, which are both necessary for *Rps8*-mediated resistance. Assembly and testing of a construct expressing both genes together; under the control of their native promoters is underway in order to more conclusively demonstrate that the gene pair is sufficient to provide *Rps8*-mediated resistance in an *rps8* background (SxGP DH-47).

2.4.2 Molecular function of LRR-RK in plants

The LRR-RK at *Rps8* belongs to the RLK Pelle LRR-XII family, which includes the bacterial resistance genes FLS2, EFR, and Xa21 (Shiu and Bleecker 2003; Shiu et al. 2004; Sun and Wang 2011). This is the first example of an LRR-XII family RK identified as providing resistance in barley and the first example of an LRR-XII family member providing resistance to a fungal pathogen. The LRR-XII family is expanded in monocots, with >100 members identified in *O. sativa* and 45 in Morex (data not shown), relative to 10 in *Arabidopsis* (Shiu et al. 2004; Sun and Wang 2011).

Given the LRR-XII clade is well-characterised, the major proteins within the clade are an excellent point of comparison for LRR-RK. FLS2 and EFR each recognise well-conserved epitopes found in most bacteria: flg22 and elf18 respectively (Gomez-Gomez and Boller 2000; Zipfel et al. 2004), whereas Xa21 recognises RaxX, a ligand derived only from X*. oryzae* (Pruitt et al. 2017).While the epitope of LRR-RK is unknown, the protein behaves similarly to Xa21 given that it contributes to resistance against a particular *formae speciales* of a single species: *Puccinia striiformis*. One hypothesis to explain the large expansion of LRR-XII genes in monocots is a shift towards recognising ligands which are specific to a particular pathogen, in contrast with the characterised LRR-XII family members of dicots (Shiu et al. 2004; Morillo and Tax 2006). Another is that within the Poaceae, selection pressures have favoured functional redundancy in extracellular receptor proteins to maintain effective resistance against their respective pathogens.

Interestingly, LRR-RK exhibits the requirement for an Exo70 partner in order to function, analogously to FLS2 which requires Exo70B1 and Exo70B2 to function at full effectiveness (Stegmann et al. 2013; Wang et al. 2020). However neither Exo70B1 or Exo70B2 are in genetic coupling with FLS2, unlike *LRR-RK* and Exo70FX12a. Whether Xa21 and EFR also require a specific Exo70 to function is also unknown, however none have been reported in as close physical proximity to those genes as with the *Rps8* locus. It is possible that Exo70 genes involved in other LRR-XII receptor signalling pathways remain to be identified, but have hitherto gone undetected either through functional redundancy, or by presenting with a lethal phenotype when rendered non-functional, given the extensive mutant screens performed using these genes.

2.4.3 Exo70 in plant immunity

The Exo70B clade has been repeatedly implicated in plant immunity, and contains between two and three members in most plants. OsExo70B1 has been shown to be involved in extracellular responses to *Magnaporthe oryzae* in rice, interacting directly with the co-receptor CERK1 (Hou et al. 2020), although the biological role of the interaction is unknown. AtEXO70B1 and AtEXO70B2 have been implicated in resistance to diverse pathogens including *Hyaloperonospora arabidposis Pseudomonas syringae*, and *Phytophthora infestans* (Pečenková et al. 2011; Stegmann et al. 2013). AtEXO70H1 has also been found to contribute non-redundantly to this resistance and to interact directly with EXO70B2 (Pečenková et al. 2011). AtEXO70B1 and EXO70B2 are essential for proper FLS2 homeostasis and trafficking to the membrane (Wang et al. 2020), and AtEXO70B2 is negatively regulated by PUB-18 and PUB-22 following extracellular immune signalling (Stegmann et al. 2013). AtEXO70B1 is also known to be targeted by pathogen effectors in order to disrupt immune signalling (Wang, Liu, et al. 2019; Michalopoulou et al. 2020) and is guarded by the NLR TN2 (Zhao et al. 2015), which initiates a defence response via CDPK signalling (Liu, Hake, et al. 2017). Finally, AtEXO70B1 has been associated with autophagy and transport of cellular components to the vesicle (Kulich et al. 2013; Pečenková et al. 2018), and may play a role in post-recognition reclamation of signalling components.

The Exo70 identified at *Rps8* is a member of the Exo70FX clade. So far only one other member of the Exo70FX clade has been functionally characterised, Exo70FX11-2, which was identified as contributing towards penetration resistance against powdery mildew along with members of the COG complex (Ostertag et al. 2013). Interestingly, Exo70FX andExo70F are the only two Exo70 clades identified as integrated domains in NLR proteins (Bailey et al. 2018) where they take a role in defence as bait or decoy proteins, rather than through a conventional role in exocytosis. The crucial question regarding Exo70FX12a is whether its role is similar to most characterised Exo70 proteins; involved in exocytosis either via the Exocyst complex or another mechanism, or if it has neofunctionalised.

2.5 Chapter-specific methods

2.5.1 Plant maintenance and crosses

Plants were grown in John Innes cereal mix, in glasshouses under a 8/16 Day-Night cycle, watered daily from below.

Crossed were performed manually. At booting stage the spike was emasculated; immature anthers were manually removed, and the spike covered with a paper bag. After approximately 5 days pollen was applied to mature stigma and the spike re-covered and allowed to develop.

2.5.2 Puccinia striiformis infection protocol

We used the methods described in (Dawson et al. 2016):

P. striiformis f. sp. tritici isolates 08/21 and 16/03 were collected in the United Kingdom in 2008 and 2016 respectively, and maintained at the National Institute of Agricultural Botany (NIAB) on the susceptible wheat cultivar Solstice. Urediniospores were stored at 6°C after collection. Inoculations were carried out by sowing seeds in groups of eight seeds per family, and four families spaced equidistantly around the rim of a 1 L pots of John Innes peat-based compost. Plants were grown at 18°C day and 11°C night using a 16 h light and 8 h dark cycle in a controlled environment chamber at NIAB, with lighting provided by metal halide bulbs (Philips MASTER HPI-T Plus 400W/645 E40). Barley seedlings were inoculated at 14 days after sowing, where first leaves were fully expanded and the second leaf was just beginning to emerge. Urediniospores of *P. striiformis* were suspended in talcum powder, at a 1:16 ratio of

urediniospores to talcum powder based on weight. Compressed air was used to inoculate seedlings on a spinning platform. After inoculation, seedlings were placed in a sealed bag and stored at 6°C for 48 h to increase humidity for successful germination of urediniospores. Subsequently, plants were returned to the growth chamber for the optimal development of *P. striiformis* and phenotyped at 10 days post inoculation.

2.5.3 Macroscopic phenotyping

At 10 days post inoculation, plants were scored using a 9 point scale from 0 to 4, with increments of 0.5, for chlorosis (discoloration) and infection (pustule formation). The scale indicates the percentage of leaf area affected by the corresponding phenotype where a score of 0 indicates asymptomatic leaves, i.e. no chlorosis, browning or pustules, and a score of 4 indicates leaves showing the respective phenotype over 100% of the surface area.

2.5.4 Microscopic phenotyping

We use the methods described in (Dawson et al. 2016):

Leaves were harvested at 14 dpi and placed in 1.0 M KOH with a droplet of surfactant (Silwet L-77) and incubated at 37°C for 12 to 16 h. Subsequently, the KOH solution was decanted and leaves were neutralised by washing three times in 50 mM Tris at pH 7.5. A 1.0 mL stain solution (20 µg/mL WGA-FITC in 50 mM Tris at pH 7.5) was applied to the leaves. Leaf tissue was incubated overnight, then washed with water, mounted, and observed under blue light excitation on a fluorescence microscope with a GFP filter.

pCOL estimates the percent of leaf colonised and pPUST the percent of leaf harbouring pustules. Phenotyping was performed by evaluating the leaf surface in equally sized, adjacent portions. Within each field of view, the colonisation of *P. striiformis* was estimated to be less than 15%, between 15 and 50% or greater than 50% of the FOV area and given scores of 0, 0.5, or 1, respectively. The final pCOL score was determined by averaging these scores based on the total number of FOVs evaluated and ranged from 0 to 100%. pPUST was evaluated in a similar manner, but for the clustering pattern of *P. striiformis* pustules. A 5x objective with a FOV of 2.72 mm x 2.04 mm was used.

2.5.5 Nucleic acid extraction

DNA was extracted from leaf tissue using a CTAB based method.

Approximately 6 cm^2 of leaf tissue was harvested from plants, placed in a 96-well plate and lyophilised using a Heto PowerDry LL3000 freeze dryer and Edwards 60X vacuum pump. After lyophilisation, stainless steel balls were introduced to the leaves and a Spex 2010 Geno/Grinder was used to pulverise tissue for approximately 2 minutes at 1500 rpm. Tissue was then immediately suspended in 300µl of a CTAB solution comprising 1.0 g CTAB, 1 ml Beta-Mecarptoethanol, 2 ml 0.5M EDTA (pH 8.0), 14 ml 5M NaCl, 10 ml 1 M Tris (pH 8.0) and 72.8 ml distilled water per 100 ml CTAB solution. Tissue was mixed thoroughly by inversion and then incubated at 65 °C for 45 minutes, with intermittent mixing, then left to stand for 5 minutes at 4 °C. 100 uL of chilled potassium acetate was then added to each sample and mixed by inversion, then incubated on ice for 20 minutes. 150 uL of 24:1 Chloroform:Isoamyl alcohol was then added to each sample and mixed by continuous vigorous inversion for five minutes. The mixtures were then centrifuged for ten minutes at 10'000 RCF, to separate the mixture into two phases. The upper phase was then harvested and transferred to a fresh plate, along with 120 uL propan-2-ol, and mixed by inversion. Nucleic acids were pelleted by centrifugation for 20 minutes at 10'000 RCF and the liquid phase discarded. Pellets were re-suspended in 200 µL of TE + 0.2 mg/mL RNAse and incubated at 65 °C for 10 minutes. Then 300 uL of 7:1 propan-2-ol: 4.4M NH_4Ac was added to the suspension, mixed by inversion and the DNA pelleted by centrifugation for ten minutes at 10'000 RCF. The liquid phase was discarded and the pellets washed in 250 µL 70% ethanol. DNA was re-pelleted by centrifugation for three minutes and the liquid phase discarded. Plates were dried, and incubated at 65 °C for 10 minutes to evaporate all remaining alcohol, before DNA was suspended in TE buffer.

RNA was extracted from the first leaf of 10 day old plants. Tissue was harvested and immediately frozen in liquid nitrogen, before being ground to a fine powder using a mortar and pestle with grinding sand at -80 °C. Ground tissue was suspended in TRI reagent, allowed to incubate for 5 minutes at room temperature before centrifugation for 20 minutes at 10'000 RCF to pellet the lysate. The supernatant was recovered and mixed with chloroform. This mixture was incubated at room temperature for 15 minutes before the phases were separated by centrifugation at 10'000 RCF and the lighter phase was recovered. Nucleic acids

were precipitated from the lighter phase with isopropanol, pelleted via centrifugation, then washed with 75% ethanol and resuspended in water.

2.5.6 KASP Genotyping

For KASP genotyping, SNPs were converted into Kompetitive Allele Specific PCR (KASP) markers using a custom python script (https://github.com/matthewmoscou/QKutilities). KASP primer mix was prepared by mixing 12 µL VIC primer (s1), 12 µL FAM primer (s2), 30 µL reverse primer (r), and 46 µL H2O. KASP PCR reactions contained 2µL gDNA (10-20 ng), 2 µL KASP V4.0 2x master mix, and 0.055 µL primer mix. KASP PCR cycling used an initial incubation at 95 °C for 15 minutes followed by touchdown PCR cycling: 94 °C for 20 seconds followed by ten 25 second cycles of touchdown PCR starting at 65 °C decreasing by 1 °C each cycle. Samples then cycled 30 times at 94 °C for 20 seconds and annealed at 57 °C for 1 minute before being held at 4 °C. KASP assays were performed at the John Innes Centre Genotyping Facility (Norwich, UK).

2.5.7 T-DNA insert copy number testing

T-DNA insert copy number testing was performed by iDna Genetics using a TaqMan Assay. Quantitative real time PCR analysis was used to estimate the numbers of transgene copies in individual plants, similarly to the approach taken by Bartlett et al. (Bartlett et al. 2008) An amplicon from the transgenic Hygromycin insert (with a FAM reporter) and an amplicon from the native Actin gene (with a VIC reporter) were amplified together in a multiplex reaction (15 minutes denaturation, then 40 cycles of 15 seconds 95C and 60 seconds 60C) in an QuantStudio 5 realtime PCR machine. Two replicate assays were run per sample. Fluorescence from the FAM and VIC fluorochromes was measured during each 60C step, and the Ct values obtained. The difference between the Ct values for the Hygromycin and Actin (the DeltaCt) was used to allocate the assayed samples into groups with the same gene copy number.

2.5.8 Chicago sequencing

Chromosome Flow sorting of CI16139 chromosome 4H was performed using the methods described by Doležel et al. (Doležel et al. 2012), after which Chicago Dovetail sequencing of the chromosome (Putnam et al. 2016) was performed by Dovetail Genomics, with initial assembly in Meraculous and final Scaffolding in HiRise.

2.5.9 RNA sequencing

After extraction, RNA was purified, and checked for quality as described in (Dawson et al. 2016) RNA libraries were constructed using Illumina TruSeq RNA library preparation (Illumina; RS-122-2001). Barcoded libraries were sequenced using either 100 or 150 bp paired-end reads. Library preparation and sequencing was performed by Novogene.

2.5.10 *Rps8* fine-mapping

Rps8 was previously mapped to the long arm of chromosome 4H by (Dawson 2015). Additional markers were designed in the *Rps8* interval using the genomes of Barke, Bowman, Morex and Haruna Nijo (**Supplementary table2.1**). These markers (**Supplementary table 2.4**) were used to fine-map *Rps8* using an F_2 population derived from SxGP DH-21 and SxGP DH-103 in a high-resolution recombination screen comprising 9,216 gametes, identifying 127 recombinants in the interval.

2.5.11 Genomic analysis of the physical sequence at the *Rps8* locus

The region delimiting *Rps8* was identified in the 2019 Barley genome (Mascher et al. 2021) using NCBI Blast+. The physical sequence between the flanking markers K_4819 and K_079610_445 was extracted from this genome, and from the same region in Golden Promise 2020 (Schreiber 2020), AWCS 276 (Liu 2020) and from the CI16193 chromosome 4H sequence. RNA from the 40 barley accessions described in **Supplementary table 2.1** was aligned to the reference Morex haplotype using the Bowtie2 aligner, and used to generate *de novo* gene annotations in Cufflinks. The reference gene annotations were also incorporated, to identify gene models which were not expressed in any sequenced tissue type.

For analysis of gene content relative to Morex, paired-end RNAseq reads were aligned to the predicted CDS of high confidence gene annotations extracted from the Morex genome with Bowtie2 and converted into a consensus CDS using custom python scripts (https://github.com/matthewmoscou/QKgenome). The relevant subset of genes were then extracted for further analysis.

2.5.12 Genetic map construction and interval mapping

To assess whether Heils Franken *Pst* resistance was associated with *Rps8*, F_1 progeny of Heils Franken and Manchuria were backcrossed to Manchuria, phenotyped and analysed by interval trait mapping. A genetic map of Heils Franken was constructed using 94 backcross progeny and 64 Kasp markers listed in **Supplementary table 2.4** with the software JoinMap v4.1. and QtlCartographer v2.5. The phenotype of these individuals was used to map resistance to *Pst* using R/qtl v1.48. Individuals with the *rps8-HF* haplotype and no resistance to *Pst* were developed into a BC_1S_2 line homozygous for *rps8-HF.*

2.5.13 Cloning of Exo70FX12

PCR was generally performed by assembling a reaction mix containing 2.5 µl Phusion Master Mix (NEB), 2.5 µl dNTPs (200 µM), 1 µl forward and reverse primer (400 nM each), 1 µl DNA (100 ng gDNA or 10 ng plasmid DNA), and 0.2 µl Phusion Taq polymerase (NEB) per reaction vessel. PCR was performed using a BioRad G-Storm GS4 thermocycler, set to cycle through: 1.5 minutes at 94 °C, then 35 repeats of 30s at 94 °C, 30s at 50 °C, 30s per kb at 72 °C. Finally 5 minutes at 72 °C and a cooling stage of 10 °C. PCR products were run on a 1.5% w/v agarose gel, and excised with a sharp blade, before gel extraction using a Macherey-Nagel NucleoSpin Gel and PCR Clean-up Kit according to the manufacturer's instructions. When desired, DNA was cloned into Oneshot Omnimax (Fischer) competent *E. coli* cells by heat shock: 2 µl of purified plasmid DNA was introduced to Oneshot Omnimax (Fischer) competent *E. coli* cells and incubated on ice for 30 minutes, then subjected to a 30 s heat shock at 42 °C for 30 seconds and recovered on ice for another 2 minutes. 250 µl of L-broth was introduced to the cells, before incubation overnight at 37°C with an appropriate selective marker. The next day, colonies were verified to contain the region of interest by colony PCR, using a similar method

as before but with an initial denaturing step of 10 minutes. DNA was extracted from transformed *E. coli* using a QIAgen miniprep kit, according to the manufacturer's instructions.

Exo70FX12a and its native promoter and terminator were cloned by PCR using the Gibson method (Gibson et al. 2009). One set of primers were designed to bind 2 kb upstream of the predicted start of transcription, and 1.5 kb downstream of the predicted stop codon. Another set of primers was designed to amplify this region while adding an overhang compatible with a matching sequence in the acceptor vector. A final set of primers designed to convert a plasmid vector into a linear sequence featuring compatible overhangs with the Exo70FX12a interval was also designed. PCR was performed using Golden Promise gDNA and the first set of primers to amplify the region of interest. PCR products were extracted and cloned into a TOPO XL-2 vector by adding 4.5 µl purified DNA to 1 µl provided salt solution and 0.5 µl of provided TOPO XL-2 vector, and incubating at room temperature for 30 minutes before cloning into *E. coli*.

After plasmid DNA was extracted from *E. coli*, it was used as a template for Gibson cloning with the second set of primers. The barley-compatible T-DNA vector pBract202 was also used as a template to create an acceptor vector using the matching set of primers. Both amplicons were extracted from gel, and verified by sanger sequencing.

To clone the modified *Exo70* interval into the linearised pBract202, 4.6 µl pBract (69 ng) with 0.4 µl Exo70 interval (62 ng) and 15 µl Gibson assembly mix (NEB) and incubated in a thermocycler at 50 °C for one hour. The mixture was then transformed into *E. coli* as before and the assembled plasmid extracted. The plasmid was verified by sanger sequencing, and transformed into electrompetent *Agrobacterium tumifaciens.* 1 µl of plasmid was introduced to 50 µl Agi1 cells, incubated on ice for 1 minute electroporated. Cells were immediately recovered using 500 µl L-media and incubated with shaking at 28 °C for 2 hours. then incubated overnight at 28 °C on L-media plates with appropriate selective markers.

Agrobaterium-mediated transformation of the assembled construct into barley was performed by the TSL transformation team using the methods described by Hensel et al. (Hensel 2004) .

2.5.14 Additional services

Sanger sequencing was performed by Eurofins Genomics

Primers were synthesised by Integrated DNA Technologies

1.5.15 Software used

NCBI Blast+ v2.2.31 (Altschul et al. 1990)

Geneious

MUSCLE v3.8.31 (Edgar 2004)

EMBOSS suite (Rice, Longden, and Bleasby 2000)

JoinMap (Stam 1993)

QTLcartographer (Basten, Weir, and Zeng 1999)

BOWTIE2 (Langmead et al. 2009; Langmead and Salzberg 2012)

Samtools v1.11 (Li et al. 2009)

Cufflinks v2.2.1 (Trapnell et al. 2010)

hisat2 v2.2.1 (Kim et al. 2019)

Python v2.7

Python v3.5.3

R v3.7.0 (Team 2013)

InterProScan v5.20-59.0 (Jones et al. 2014)

2.6 Appendixes

Supplementary Table 2.1: Barley accessions used in transcriptomic analyses. An in-house collection of barley accessions for which RNAseq data is available. **Haplotype** indicates whether the accession does (*Rps8*) or does not (*rps8*) express the two candidate genes at *Rps8*, or whether it has expresses the genes with accession-specific SNPs (*Rps8-X*). ***Rps8* status** indicates whether resistance is known to be provided by that *Rps8* allele (+), known not to be provided (-), or unknown (blank) based on previous mapping work (Dawson 2015)

Accession	Identifier	Type	Haplotype	*Rps8* status
Abed Binder 12	GGCM01000000	Transcriptome	Rps8	+
Aramir	GGCO01000000	Transcriptome	Rps8	
Barke	GGCN01000000	Transcriptome	Rps8	
Baronesse	GGCP01000000	Transcriptome	Rps8	+
BCD12	GGCQ01000000	Transcriptome	Rps8	
BCD47	GGCR01000000	Transcriptome	Rps8	
Betzes	GGCS01000000	Transcriptome	Rps8	+
Bowman	GGCT01000000	Transcriptome	Rps8	
CI 16139	GGCU01000000	Transcriptome	Rps8	+
CI 16147	GFJN01000000	Transcriptome	rps8	-
CI 16153	GFJL01000000	Transcriptome	rps8	-
CIho 4196	GFJK01000000	Transcriptome	Rps8	
Commander	GGCV01000000	Transcriptome	Rps8	
Duplex	GGCW01000000	Transcriptome	Rps8	+
Emir	GGCX01000000	Transcriptome	Rps8	
Finniss	GGCY01000000	Transcriptome	rps8	
Fong Tien	GGCZ01000000	Transcriptome	rps8	-
Golden Promise	GGDA01000000	Transcriptome	Rps8	+
G.Z.	GGDB01000000	Transcriptome	rps8	-
Haruna Nijo	GFJJ01000000	Transcriptome	Rps8	+
Heils Franken	GGDC01000000	Transcriptome	rps8-HF	-
Hindmarsh	GGDD01000000	Transcriptome	Rps8	
HOR 1428	GGDE01000000	Transcriptome	rps8	-
I 5	GGDF01000000	Transcriptome	Rps8	
Igri	GGDG01000000	Transcriptome	rps8	
Manchuria	GFJO01000000	Transcriptome	rps8	-
Maritime	GGDH01000000	Transcriptome	Rps8	
Morex	2017v1	Genome	Rps8	+
Pallas	GGDI01000000	Transcriptome	Rps8	

Q21861	GGDJ01000000	Transcriptome	rps8	
Russell	GGDK01000000	Transcriptome	rps8	-
Sultan 5	GGDL01000000	Transcriptome	Rps8	+
SusPtrit	GGDM01000000	Transcriptome	rps8	-
WBDC 008	GGDN01000000	Transcriptome	Rps8-WBDC8	
WBDC 013	GGDO01000000	Transcriptome	Rps8-WBDC13	
WBDC 085	GGDP01000000	Transcriptome	Rps8-WBDC85	
WBDC 109	GGDQ01000000	Transcriptome	rps8	
WBDC 110	GGDR01000000	Transcriptome	rps8	
WBDC 172	GGDS01000000	Transcriptome	rps8	
WBDC 259	GGDT01000000	Transcriptome	rps8	

Supplementary table 2.2. Reference genomes used in this chapter. Reference genomes were used to compare the physical structure of the *Rps8* interval as well as the coding sequence of candidate genes across barley accessions and wheat. They were also used to develop additional genetic markers in the *Rps8* interval.

Genome	**Species**	**Publication**
Morex (2017)	*Hordeum vulgare*	(Beier et al. 2017)
Morex (2019)	*Hordeum vulgare*	(Mascher et al. 2021)
Golden Promise (2020)	*Hordeum vulgare*	(Schreiber et al. 2020)
AWCS 276	*Hordeum vulgare*	(Liu et al. 2020)
Barke	*Hordeum vulgare*	(International Barley Genome Sequencing et al. 2012)
Bowman	*Hordeum vulgare*	(International Barley Genome Sequencing et al. 2012)
Haruna Nijo	*Hordeum vulgare*	(International Barley Genome Sequencing et al. 2012)
Chinese Spring	*Triticum aestivum*	(Appels et al. 2018)

Supplementary table 2.3: List of Primers used in this chapter

Primer ID	Sequence	Role
Exo70-pBract-p1f	AGAGGTTTCTTGGGTTGAAAGATCCACTAGTTCTAGAGCG	Gibson cloning of Exo70, along with native promoter and terminator to be compatible with right border of pBract vector
Exo70-pBract-p1r	CGCTCTAGAACTAGTGGATCTTTCAACCCAAGAAACCTCT	Gibson cloning of pBract vector to be compatible with Exo70 region
pBract-Exo70-p1f	TAAGCTTGATATCGAATTCCCTACGAAACTGAATATTTAG	Gibson cloning of pBract vector to be compatible with Exo70 region
pBract-Exo70-p1r	CTAAATATTCAGTTTCGTAGGGAATTCGATATCAAGCTTA	Gibson cloning of Exo70, along with native promoter and terminator to be compatible with left border of pBract vector
SH_12 1-1	GGAAGGGAATAACCAACTAG	Forward primer for amplifying Exo70 interval, binds to promoter region
SH_12 1-2	CCATCTGTGGCAATCAAGGA	Reverse primer for amplifying Exo70 interval, binds to terminator region
EXO_REGION_L1	CCGACCCAGCTTTCTTGTAC	Forward primer for sequencing Exo70 interval
EXO_REGION_L2	GCGTTTCCCCTAGCCATTTA	Forward primer for sequencing Exo70 interval
EXO_REGION_L3	ACCCATTATCAAGCCTTGCA	Forward primer for sequencing Exo70 interval
EXO_REGION_L4	TGTGGGTGTGTTTTGATCAGG	Forward primer for sequencing Exo70 interval
EXO_REGION_L5	CTTCTAAAGCCGGAGACCCC	Forward primer for sequencing Exo70 interval
EXO_REGION_L6	CGACCAGGACCAGCTAAAGA	Forward primer for sequencing Exo70 interval
EXO_REGION_L7	CGCTGACTCGAATTTAGCCA	Forward primer for sequencing Exo70 interval
EXO_REGION_L8	ACCGTGCTGTATATGGCCTT	Forward primer for sequencing Exo70 interval
EXO_REGION_L9	GATCTTCGTGAGCTCGTGAG	Forward primer for sequencing Exo70 interval
EXO_REGION_L10	GTCAACATGAAGTACCGGGG	Forward primer for sequencing Exo70 interval
EXO_REGION_L11	TCATAGAATACCTCGTACGACCA	Forward primer for sequencing Exo70 interval
EXO_REGION_L12	ATGCACAGACGTAGCCAGTA	Forward primer for sequencing Exo70 interval
EXO_REGION_L13	GCTTGGCGTAATCATGGTCA	Forward primer for sequencing Exo70 interval
EXO_REGION_R1	TCGTAGGGAATTCGATATCAAGC	Reverse primer for sequencing Exo70 interval
EXO_REGION_R2	ACTCCACTTACATCCCACCT	Reverse primer for sequencing Exo70 interval
EXO_REGION_R3	TGGTAGCTTCGAACTGACTGA	Reverse primer for sequencing Exo70 interval
EXO_REGION_R4	GACGGCGTCTTTAATTTGGC	Reverse primer for sequencing Exo70 interval
EXO_REGION_R5	AGAGGGTCGTGTCAGGTTAC	Reverse primer for sequencing Exo70 interval
EXO_REGION_R6	ACGAAACCGAATACCTCCGT	Reverse primer for sequencing Exo70 interval
EXO_REGION_R7	GTTCTCCAACTCCCCGACG	Reverse primer for sequencing Exo70 interval
EXO_REGION_R8	GGGACAACACTTCACACACC	Reverse primer for sequencing Exo70 interval
EXO_REGION_R9	AAACCCCAAAACCCCGAATC	Reverse primer for sequencing Exo70 interval
EXO_REGION_R10	ACCGGGGAATAATCTATGTGCA	Reverse primer for sequencing Exo70 interval
EXO_REGION_R11	TTTGCTTACCCGATCGATGT	Reverse primer for sequencing Exo70 interval
EXO_REGION_R12	CTTGTCAAGAAGCTACCAACGA	Reverse primer for sequencing Exo70 interval
EXO_REGION_R13	CTTCCGGCTCGTATGTTGTG	Reverse primer for sequencing Exo70 interval

Supplementary table 2.4: List of KASP markers used in this chapter.

KASP_ID	Role	Forward sequence 1	Forward sequence 2	Reverse sequence
1_0420_60_F	HfxM BC_1 Marker 1H	GAAGGTCGGAGTCAACGGATTATTCCATTGAAATGGATGAG	GAAGGTGACCAAGTTCATGCTATTCCATTGAAATGGATGAT	TGAGTGATGATGACCCATAGC
1_1223_120_R	HfxM BC_1 Marker 1H	GAAGGTCGGAGTCAACGGATTAGAAGGGGCCAACACGGTAC	GAAGGTGACCAAGTTCATGCTAGAAGGGGCCAACACGGTAT	GTACAGAGGCGGTAGCGG
SCRI_RS_66630_159_R	HfxM BC_1 Marker 1H	GAAGGTCGGAGTCAACGGATTTATCCAGTTCACTGCCCTCC	GAAGGTGACCAAGTTCATGCTTATCCAGTTCACTGCCCTCT	GGCTCCATCCATACACCTCA
2_1174_120_F	HfxM BC_1 Marker 1H	GAAGGTCGGAGTCAACGGATTACCGGCGCTCGATTAAGTCA	GAAGGTGACCAAGTTCATGCTACCGGCGCTCGATTAAGTCG	GCATCGCCGGGTGATACA
1_1038_71_F	HfxM BC_1 Marker 1H	GAAGGTCGGAGTCAACGGATTCACTACACAGGCCCCCTTTA	GAAGGTGACCAAGTTCATGCTCACTACACAGGCCCCCTTTG	CCTCTGATGAAGGCTGGGC
1_0854_120_F	HfxM BC_1 Marker 1H	GAAGGTCGGAGTCAACGGATTCAGTGAACAAGAGAATGCTG	GAAGGTGACCAAGTTCATGCTCAGTGAACAAGAGAATGCTT	ACTGCCTTGACATACCAGGG
2_0625_120_F	HfxM BC_1 Marker 1H	GAAGGTCGGAGTCAACGGATTGGCTTGCATGTTGATCCACC	GAAGGTGACCAAGTTCATGCTGGCTTGCATGTTGATCCACT	AGCAAAGACCTGCCTGTGT
2_0475_120_F	HfxM BC_1 Marker 1H	GAAGGTCGGAGTCAACGGATTATGAGCTGTTGTGCTGTGTA	GAAGGTGACCAAGTTCATGCTATGAGCTGTTGTGCTGTGTG	AGATGCTGCGGTGCACTT
1_1059_120_F	HfxM BC_1 Marker 1H	GAAGGTCGGAGTCAACGGATTAGCTCAACGCGCCGCTTAAC	GAAGGTGACCAAGTTCATGCTAGCTCAACGCGCCGCTTAAT	ACGGGTAGTCGGTGTAACC
1_0943_120_F	HfxM BC_1 Marker 2H	GAAGGTCGGAGTCAACGGATTGTTATTACTCCTTGTCGCCC	GAAGGTGACCAAGTTCATGCTGTTATTACTCCTTGTCGCCG	GCACTTGTCACTCACAGCG
2_1015_120_F	HfxM BC_1 Marker 2H	GAAGGTCGGAGTCAACGGATTTTTGGAGAAGAGCAGGCCTA	GAAGGTGACCAAGTTCATGCTTTTGGAGAAGAGCAGGCCTG	CGCTAGTCGGTCCTCGAG
1_0837_120_F	HfxM BC_1 Marker 2H	GAAGGTCGGAGTCAACGGATTCCTACGACACTAAACCCAGG	GAAGGTGACCAAGTTCATGCTCCTACGACACTAAACCCAGT	TGCGTGCCCCTACCTTTG

1_0498_120_F	HfxM BC_1 Marker 2H	GAAGGTCGGAGTCAACGGATTGTGGGTGGAGGCTTTGGAAC	GAAGGTGACCAAGTTCATGCTGTGGGTGGAGGCTTTGGAAT	GCTGCCATGTTGTTGCCC
2_0500_120_F	HfxM BC_1 Marker 2H	GAAGGTCGGAGTCAACGGATTTTCGATGCGAATGCGGTGGC	GAAGGTGACCAAGTTCATGCTTTCGATGCGAATGCGGTGGT	TCGGCGCACCAGAAAAGA
1_0213_120_F	HfxM BC_1 Marker 2H	GAAGGTCGGAGTCAACGGATTCAGGAACAGCCTCCTAGCAA	GAAGGTGACCAAGTTCATGCTCAGGAACAGCCTCCTAGCAC	GGGCACTTGGAGCTAAGCT
1_1118_120_R	HfxM BC_1 Marker 2H	GAAGGTCGGAGTCAACGGATTTTAATCTGTACTGATTTTTA	GAAGGTGACCAAGTTCATGCTTTAATCTGTACTGATTTTTG	ACACCTTTCGAGCTGCGAT
2_1440_120_R	HfxM BC_1 Marker 2H	GAAGGTCGGAGTCAACGGATTTTTAGTTGGTCTGTGGCTGC	GAAGGTGACCAAGTTCATGCTTTTAGTTGGTCTGTGGCTGT	CCAGGTGCACAGAGCCAC
2_0895_120_R	HfxM BC_1 Marker 2H	GAAGGTCGGAGTCAACGGATTAGATGGCAACACCTTAGAGC	GAAGGTGACCAAGTTCATGCTAGATGGCAACACCTTAGAGT	CACTGATGAGAAGGCAATGCA
2_1008_120_F	HfxM BC_1 Marker 2H	GAAGGTCGGAGTCAACGGATTGAACTGGCTCAGAGATTGTC	GAAGGTGACCAAGTTCATGCTGAACTGGCTCAGAGATTGTT	CCTTCACGCTTCCCACGT
1_0867_120_F	HfxM BC_1 Marker 3H	GAAGGTCGGAGTCAACGGATTGTGAGATGTGGAATCCGCTA	GAAGGTGACCAAGTTCATGCTGTGAGATGTGGAATCCGCTG	GCTCACAAGCCAACTGCA
2_0023_120_F	HfxM BC_1 Marker 3H	GAAGGTCGGAGTCAACGGATTTGGCATGGCTAACTTCCCCA	GAAGGTGACCAAGTTCATGCTTGGCATGGCTAACTTCCCCG	GAAGGTTAGGCGTGCCGT
2_0659_120_F	HfxM BC_1 Marker 3H	GAAGGTCGGAGTCAACGGATTGTAGCTATTGTACACTTCAC	GAAGGTGACCAAGTTCATGCTGTAGCTATTGTACACTTCAT	GCTGAACTGGAACATCCGC
2_0115_120_F	HfxM BC_1 Marker 3H	GAAGGTCGGAGTCAACGGATTTCTTCATGCATAATTCTTTC	GAAGGTGACCAAGTTCATGCTTCTTCATGCATAATTCTTTT	ACCTCCTTCAATGATCCCAGA
2_0931_120_F	HfxM BC_1 Marker 3H	GAAGGTCGGAGTCAACGGATTACGCTAACCTGTAATCCTAC	GAAGGTGACCAAGTTCATGCTACGCTAACCTGTAATCCTAT	CCCTTCAAGGCAGAGGTGG
1_1124_120_F	HfxM BC_1 Marker 3H	GAAGGTCGGAGTCAACGGATTCTAACCCAGGGTGCTCCAAC	GAAGGTGACCAAGTTCATGCTCTAACCCAGGGTGCTCCAAT	ACCATGGAAGGGCCCTTG
2_1533_24_F	HfxM BC_1 Marker 3H	GAAGGTCGGAGTCAACGGATTGAACGCATCGCCTGAGGCCC	GAAGGTGACCAAGTTCATGCTGAACGCATCGCCTGAGGCCG	AACGGCCCATCCAAGTGG
2_0013_120_F	HfxM BC_1 Marker 3H	GAAGGTCGGAGTCAACGGATTGTTTCAAGCCACGCGATTCC	GAAGGTGACCAAGTTCATGCTGTTTCAAGCCACGCGATTCT	GAAGCACCAACCCCGTCA
2_0974_120_F	HfxM BC_1 Marker 4H	GAAGGTCGGAGTCAACGGATTTGTCCTTGTAGGCGGTCAGA	GAAGGTGACCAAGTTCATGCTTGTCCTTGTAGGCGGTCAGC	TTTCAACGCCAGGACGCA
1_0510_120_F	HfxM BC_1 Marker 4H	GAAGGTCGGAGTCAACGGATTCGGATAGGCCAAAATCAATC	GAAGGTGACCAAGTTCATGCTCGGATAGGCCAAAATCAATT	CGCGATCTCAAGCCGGAA
2_0454_120_F	HfxM BC_1 Marker 4H	GAAGGTCGGAGTCAACGGATTCCACCCGCATCCCCGCCAGC	GAAGGTGACCAAGTTCATGCTCCACCCGCATCCCCGCCAGT	GGCGAAGTCGACCCTCTG
1_0588_120_F	HfxM BC_1 Marker 4H	GAAGGTCGGAGTCAACGGATTCTTTACTCTGGTCAGCAGCA	GAAGGTGACCAAGTTCATGCTCTTTACTCTGGTCAGCAGCG	GGAATGGATTGGACGGAGTGA
2_0670_120_F	HfxM BC_1 Marker 4H	GAAGGTCGGAGTCAACGGATTGAATCTCACTGATTGGTGCC	GAAGGTGACCAAGTTCATGCTGAATCTCACTGATTGGTGCG	CGGACGTGACACGGAGAA
2_0451_120_R	HfxM BC_1 Marker 4H	GAAGGTCGGAGTCAACGGATTAACCATACAACCATAGTGGC	GAAGGTGACCAAGTTCATGCTAACCATACAACCATAGTGGT	GGCCAACCAAGCGATCGA
2_0482_120_F	HfxM BC_1 Marker 4H	GAAGGTCGGAGTCAACGGATTCGTCCTGCTCCTGCTTTAGC	GAAGGTGACCAAGTTCATGCTCGTCCTGCTCCTGCTTTAGT	GCGGCGGCTAAAAAGAGG
1_0480_120_F	HfxM BC_1 Marker 4H	GAAGGTCGGAGTCAACGGATTGCAACTTTTTCTACCCAAAC	GAAGGTGACCAAGTTCATGCTGCAACTTTTTCTACCCAAAG	GATGCCACTCCAGTGCAGT
2_1397_120_F	HfxM BC_1 Marker 4H	GAAGGTCGGAGTCAACGGATTAAGAGCCGACGGTGCCCACC	GAAGGTGACCAAGTTCATGCTAAGAGCCGACGGTGCCCACT	CGCGCATCTCTTCAACGC
1_0221_120_R	HfxM BC_1 Marker 4H	GAAGGTCGGAGTCAACGGATTAAGAAGCAATACATTTTAAC	GAAGGTGACCAAGTTCATGCTAAGAAGCAATACATTTTAAG	AGCGGTTCAGCCTTCAGA
2_0533_120_F	HfxM BC_1 Marker 4H	GAAGGTCGGAGTCAACGGATTTGGGTGCATCTGGTGGGATC	GAAGGTGACCAAGTTCATGCTTGGGTGCATCTGGTGGGATT	TAAGCAGCGAGAGTGGCT
1_0621_120_F	HfxM BC_1 Marker 5H	GAAGGTCGGAGTCAACGGATTTCGCTCCATATGAGAAACGC	GAAGGTGACCAAGTTCATGCTTCGCTCCATATGAGAAACGT	TGGCGACCTTGAGTGCAC
1_1128_50_F	HfxM BC_1 Marker 5H	GAAGGTCGGAGTCAACGGATTTGACAAATCTAAATGCCTTC	GAAGGTGACCAAGTTCATGCTTGACAAATCTAAATGCCTTT	CTGAGGCAGGCTGTTCTGA
2_1239_120_F	HfxM BC_1 Marker 5H	GAAGGTCGGAGTCAACGGATTGCGCCAACAGGAACCATAGC	GAAGGTGACCAAGTTCATGCTGCGCCAACAGGAACCATAGT	CCCGATATCTTGTTGATGGCA
2_0096_120_F	HfxM BC_1 Marker 5H	GAAGGTCGGAGTCAACGGATTTCCAAGGGGTGAACTGTTGA	GAAGGTGACCAAGTTCATGCTTCCAAGGGGTGAACTGTTGC	CTCTGCAGCTCTCGGTGG
1_0578_120_F	HfxM BC_1 Marker 5H	GAAGGTCGGAGTCAACGGATTCTGGGCACTGGGCCTCGACA	GAAGGTGACCAAGTTCATGCTCTGGGCACTGGGCCTCGACC	AGGTGTGGGGTGCTTTGC
2_1168_120_F	HfxM BC_1 Marker 5H	GAAGGTCGGAGTCAACGGATTTGGCGAAGGTGAGGTTGTGA	GAAGGTGACCAAGTTCATGCTTGGCGAAGGTGAGGTTGTGG	CGGGTTCACGACGGAGTAC
1_1200_120_F	HfxM BC_1 Marker 5H	GAAGGTCGGAGTCAACGGATTGTGTTCAGCCCAGAGGGAGC	GAAGGTGACCAAGTTCATGCTGTGTTCAGCCCAGAGGGAGT	ACCTTTGTTTTGCTTGCAGGT
1_1456_38_F	HfxM BC_1 Marker 5H	GAAGGTCGGAGTCAACGGATTGAAGGCTAGAAGCCCCCCAC	GAAGGTGACCAAGTTCATGCTGAAGGCTAGAAGCCCCCCAT	TGCCATTTTGCGTTTGGACC
1_0705_120_F	HfxM BC_1 Marker 5H	GAAGGTCGGAGTCAACGGATTCCGAGAACAGATCAGTCTCC	GAAGGTGACCAAGTTCATGCTCCGAGAACAGATCAGTCTCT	CCGGAGGTCATGTCGAGC
1_1532_120_F	HfxM BC_1 Marker 5H	GAAGGTCGGAGTCAACGGATTCAACAAATGCCCATAACCTA	GAAGGTGACCAAGTTCATGCTCAACAAATGCCCATAACCTG	GGTCACTGCTTCTCTTGGCT
2_0415_120_F	HfxM BC_1 Marker 5H	GAAGGTCGGAGTCAACGGATTAATCAATTCGCTTCTCAGAC	GAAGGTGACCAAGTTCATGCTAATCAATTCGCTTCTCAGAT	GGCAAGGGAGGTCTCAGG
1_0023_120_F	HfxM BC_1 Marker 6H	GAAGGTCGGAGTCAACGGATTATTGGCTCCCCCTTCCAAAC	GAAGGTGACCAAGTTCATGCTATTGGCTCCCCCTTCCAAAG	ACGACGAGGACCATGACG
1_0994_92_R	HfxM BC_1 Marker 6H	GAAGGTCGGAGTCAACGGATTCCTTCGGGTTGGCCATCTCA	GAAGGTGACCAAGTTCATGCTCCTTCGGGTTGGCCATCTCG	CCCAAAACCCTAGCCCCG
2_0465_120_R	HfxM BC_1 Marker 6H	GAAGGTCGGAGTCAACGGATTTTGCCACGAGAATGGTTGCG	GAAGGTGACCAAGTTCATGCTTTGCCACGAGAATGGTTGCT	CCTTCACCGTCCAGTGGC
1_0124_120_F	HfxM BC_1 Marker 6H	GAAGGTCGGAGTCAACGGATTCTAAAGCAAGACTTCCCAAC	GAAGGTGACCAAGTTCATGCTCTAAAGCAAGACTTCCCAAT	GGTACCAAGGTTGTGGAGGA
2_0379_120_F	HfxM BC_1 Marker 6H	GAAGGTCGGAGTCAACGGATTGGAGGAAACCAAGGATGTGC	GAAGGTGACCAAGTTCATGCTGGAGGAAACCAAGGATGTGG	ACATGCTTGCCAGGGAGA
1_0107_120_F	HfxM BC_1 Marker 6H	GAAGGTCGGAGTCAACGGATTGTCGTGCAAGCTTTAATATC	GAAGGTGACCAAGTTCATGCTGTCGTGCAAGCTTTAATATG	ACCCTGCATGCGGGAATC
2_0537_120_F	HfxM BC_1 Marker 6H	GAAGGTCGGAGTCAACGGATTTAGATGGATAGATTGCCGTA	GAAGGTGACCAAGTTCATGCTTAGATGGATAGATTGCCGTC	CCACAACCGCTACAGCCA
2_0365_120_F	HfxM BC_1 Marker 6H	GAAGGTCGGAGTCAACGGATTAGGTCCAAGACAGAATCTCC	GAAGGTGACCAAGTTCATGCTAGGTCCAAGACAGAATCTCG	GACCGTCTACTGCCGCTC
2_1104_120_F	HfxM BC_1 Marker 7H	GAAGGTCGGAGTCAACGGATTTGCTCGAGTTGAAGTCCATA	GAAGGTGACCAAGTTCATGCTTGCTCGAGTTGAAGTCCATT	ACTGCTTCCGCCAGTCAG
2_0247_120_F	HfxM BC_1 Marker 7H	GAAGGTCGGAGTCAACGGATTCATTTCAAGGTTTTCCATAC	GAAGGTGACCAAGTTCATGCTCATTTCAAGGTTTTCCATAG	TGAATGTTGCGTGGATCCT

2_0103_120_F	HfxM BC_1 Marker 7H	GAAGGTCGGAGTCAACGGATTTGGCGAGCGCCCTGGAGCAA	GAAGGTGACCAAGTTCATGCTTGGCGAGCGCCCTGGAGCAG	GTAGCCGCCGACATCCTC
2_0485_120_F	HfxM BC_1 Marker 7H	GAAGGTCGGAGTCAACGGATTGGGTGTTGCACAGCGCGTCC	GAAGGTGACCAAGTTCATGCTGGGTGTTGCACAGCGCGTCT	CTTCGGCAGCAAGGTCCA
1_0773_120_F	HfxM BC_1 Marker 7H	GAAGGTCGGAGTCAACGGATTAGGCACAAAACTTCATCCAA	GAAGGTGACCAAGTTCATGCTAGGCACAAAACTTCATCCAG	GGAGGTCGCTCGCTCAAG
1_1098_120_F	HfxM BC_1 Marker 7H	GAAGGTCGGAGTCAACGGATTAAATTGCAAGGCGTGTGATC	GAAGGTGACCAAGTTCATGCTAAATTGCAAGGCGTGTGATT	GATGGTAAGCGCTGGGCA
2_1528_58_R	HfxM BC_1 Marker 7H	GAAGGTCGGAGTCAACGGATTGGATAGTCCAGGTGTGCCTA	GAAGGTGACCAAGTTCATGCTGGATAGTCCAGGTGTGCCTT	TGTACAGAGTCCAGGCGC
48149_3381_R	*Rps8* recombination screen	GAAGGTCGGAGTCAACGGATTTGTAAGCACCACACAACAGA	GAAGGTGACCAAGTTCATGCTTGTAAGCACCACACAACAGG	TGTCATGAAACTTGTGCCGG
134516_375_F	*Rps8* recombination screen	GAAGGTCGGAGTCAACGGATTCATGTCCAACTCCTTTTCCA	GAAGGTGACCAAGTTCATGCTCATGTCCAACTCCTTTTCCG	ACAAGATTGGATTGCGACCA
c7_1956_F	*Rps8* recombination screen	GAAGGTCGGAGTCAACGGATTGGAGCATCAATCATCAATGC	GAAGGTGACCAAGTTCATGCTGGAGCATCAATCATCAATGT	TACAGCGTGCCTTACCGC
48890_11948_F	*Rps8* recombination screen	GAAGGTCGGAGTCAACGGATTAAGTAAAACTGGACCATACG	GAAGGTGACCAAGTTCATGCTAAGTAAAACTGGACCATACT	TCGCATTTCGTTTCTGCCA
69195_5316_F	*Rps8* recombination screen	GAAGGTCGGAGTCAACGGATTATGCATCTGTTGACCAGATC	GAAGGTGACCAAGTTCATGCTATGCATCTGTTGACCAGATT	ACACACAGAGGAGACAACCC
49635_5088_R	*Rps8* recombination screen	GAAGGTCGGAGTCAACGGATTGCCACTCGGTGTTCCTATAA	GAAGGTGACCAAGTTCATGCTGCCACTCGGTGTTCCTATAC	TGCACTTATGTTCGCATCAAC
1626625_1054_R	*Rps8* recombination screen	GAAGGTCGGAGTCAACGGATTTGCTGCTTTGGGGGTTATTC	GAAGGTGACCAAGTTCATGCTTGCTTTGGGGGTTATTT	GGGGGAGGTGGTTTGTGG
079610_445_F	*Rps8* recombination screen	GAAGGTCGGAGTCAACGGATTAGCGCTCCGGACGAAATGCG	GAAGGTGACCAAGTTCATGCTAGCGCTCCGGACGAAATGCT	GCTAGCGTCGGAAGTGCT
079620_185_R	*Rps8* recombination screen	GAAGGTCGGAGTCAACGGATTTTAATAAAACTTGCGGTTTA	GAAGGTGACCAAGTTCATGCTTTAATAAAACTTGCGGTTTT	CTGGAAAACAACCGCAAAGGA
079620_252_F	*Rps8* recombination screen	GAAGGTCGGAGTCAACGGATTGAATCTTAATTCGCACATGC	GAAGGTGACCAAGTTCATGCTGAATCTTAATTCGCACATGT	AGGTTTGCGATCGGCCAT
079620_2037_F	*Rps8* recombination screen	GAAGGTCGGAGTCAACGGATTTGGCGGCTGGCCCCACACTA	GAAGGTGACCAAGTTCATGCTTGGCGGCTGGCCCCACACTG	TAAGCAATGGAGGGGGCG
079620_219_F	*Rps8* recombination screen	GAAGGTCGGAGTCAACGGATTATTAAATCAAGCAAATCGAA	GAAGGTGACCAAGTTCATGCTATTAAATCAAGCAAATCGAT	AGGTTTGCGATCGGCCAT
079820_663_F	*Rps8* recombination screen	GAAGGTCGGAGTCAACGGATTAAGAACCTTACTTCACCACA	GAAGGTGACCAAGTTCATGCTAAGAACCTTACTTCACCACC	TCCGTGCCCAACCAGTTC
079820_1463_F	*Rps8* recombination screen	GAAGGTCGGAGTCAACGGATTGGGCAAACTCTGTCCGGCCG	GAAGGTGACCAAGTTCATGCTGGGCAAACTCTGTCCGGCCT	CCTGGTGGTGAGGGTTGC
121084_sp_1669_F	*Rps8* recombination screen	GAAGGTCGGAGTCAACGGATTACAATGGACGTACTACTGCC	GAAGGTGACCAAGTTCATGCTACAATGGACGTACTACTGCT	CGCGAGGATTCAAAAGCGG
121084_sp_2200_R	*Rps8* recombination screen	GAAGGTCGGAGTCAACGGATTATGACTATGTCGGTCGGGTG	GAAGGTGACCAAGTTCATGCTATGACTATGTCGGTCGGGTT	CGATACCACTCTGGCGCC

3. A draft genome of *Ecdeiocolea monostachya* – a grass-like plant representing a closely related outgroup to the Poaceae

3.1 Chapter Summary

Ecdeiocolea monostachya is a wild perennial herb native to western Australia. It is one of three species within the Ecdeiocoleaceae; the family within the order Poales most closely related to the family Poaceae. *Ecdeiocolea monostachya* therefore represents an important outgroup for genomic studies of species within the Poaceae; a family which includes important crops such as wheat, rice, maize, barley, and millet. We used paired-end Illumina, long-read Nanopore sequencing, and the hybrid MaSuRCA genome assembler to assemble a draft genome of a wild accession of *Ecdeiocolea monostachya* designated EM_001. Gene annotations using RNAseq data from sheath, root and flower tissue predicted 84,7000 gene models with 95.2% complete BUSCO representation. The final assembly of the heterozygous, diploid *Ecdeiocolea monostachya* genome encompasses 1.3 Gbp over 3,605 scaffolds with N50=756 Kbp, and an estimated diploid genome coverage of 84%.

3.2 Introduction

3.2.1 The order Poales

The Poales are an order of monocotyledonous plants which can be traced back to the mid-Cretaceous period (approximately 115 Mya) in the Gondwanan region (present-day South America and Africa) (GPWG 2001; Bremer 2000). Poales are ecologically significant, being found on every continent, and are the defining feature of grasslands: the second largest biome (after oceans) on the planet. Especially notable are members of the family Poaceae;

which is known for species richness (Species Number >11,000) and includes all cereal crops, representing the primary source of calories utilised in human nutrition worldwide (Bouchenak-Khelladi, Muasya, and Linder 2014). Within the Poales, the Poaceae are a member of the Graminid clade, which also encompasses their nearest relatives the Ecdeiocoleaceae (3 species), Joinvilleaceae (4 species), and Flagellareaeceae (5 species). Although there have been many published genomes and transcriptomes within the Poaceae; the other families within the Poales are much less well-characterised (of the 481,639 SRA entries in NCBI classified under "Poales", 479,420 were from the Poaceae, at the time of writing).

3.2.2 Comparative genomics within the family Poaceae

Traditional approaches to understanding the evolution of the Poaceae and their close relatives using morphological and archaeological evidence (Simon 2007) are now supplemented by comparative genomic analyses. Evolutionary relationships can be interrogated by comparing nucleotide sequences of conserved genes such as those found in the BUSCO database (Seppey, Manni, and Zdobnov 2019), as well as structural indicators of ancestral genomic events such as chromosome re-arrangements, syntenic regions, and gene duplications (Hilu 2004; Salse et al. 2009; Eric Schranz, Mohammadin, and Edger 2012). Our understanding of these species is also greatly enhanced by volumes of high-quality genetic information derived from reference genomes, transcriptomes from a variety of tissue types and environmental conditions, and pan-genomes of major species (Bayer et al. 2020) such as *Hordeum vulgare* (Jayakodi et al. 2020) *Brachypodium distachyon* (Gordon et al. 2017), *Oryza spp.* (Huang et al. 2012; Zhao et al. 2018; Wang et al. 2018), and *Triticum aestivum* (Montenegro et al. 2017).

Comparative genomic studies within the Poaceae can identify agronomically relevant features which may not be found in wider monocots, or in dicot. However, the exact origin of these features can be difficult to determine due to the relative paucity of high-quality genomic information for their closest relatives in the graminid clade (Ware et al. 2002; Monat et al. 2019; Hochbach, Linder, and Röser 2018; Chase et al. 2006). Some well-studied features include expansions of MADS-box genes (Becker and Theißen 2003; Linder et al. 2018) such as the *FRUITFULL* genes *FUL1*/*FUL2*, and their loss in some lineages (Preston and Kellogg 2006;

Preston et al. 2009), C_4 photosynthesis, which has evolved independently 10 to 24 times in the PACMAD clade of the Poaceae (Grass Phylogeny Working 2012), the development of gene clusters such the *Bx* gene cluster of maize (Dutartre, Hilliou, and Feyereisen 2012), specialisation of other CYP450 families (Li and Wei 2020), and the duplication of genes controlling ADP-glucose synthesis from the plastid genome into the plant genome, permitting increased cytosolic starch production (Wu et al. 2008; Tang et al. 2010; McKain et al. 2016). Several features are directly linked to the *rho* whole genome duplication event which occurred in the common ancestor to the Poaceae, prior to the radiation of the Anomochlooideae (Paterson, Bowers, and Chapman 2004; McKain et al. 2016). The Anomochlooideae are themselves an outgroup within the Poaceae, being the most basal lineage composed of the genera Anomochloae and Streptochaetae, and lacking signatures of the true grasses such as the spikelet, a single exon *rpoC1* gene and a single bp deletion in the *matK* gene (Clark and Judziewicz 1996; Hilu, Alice, and Liang 1999; Morris and Duvall 2010) relative to other Poales. However, identifying the evolutionary origin of a trait found in a majority of Poaceae can be difficult, given the lack of high-quality reference data for their closest relatives in the Poales to provide a point of comparison.

3.2.3 The Ecdeiocoleaceae represent a critical outgroup to the Poaceae

Studies into the evolutionary history of the Poales have identified a number of metrics to evaluate their relationships. These include phylogenetic analysis of conserved single-copy gene families, identification of three signature inversions of the plastid genome, comparisons of chromosomal organisation and synteny across nuclear genomes, and genomic signatures associated with WGD events (Doyle et al. 1992; Duvall et al. 1993; Cummings, King, and Kellogg 1994; Bremer 2002; Paterson, Bowers, and Chapman 2004; Yu et al. 2005; McKain et al. 2016; Givnish et al. 2010; Givnish 2010; GPWG 2001; Michelangeli, Davis, and Stevenson 2003; Givnish et al. 2018; Hochbach, Linder, and Röser 2018). Studies report minor inconsistencies between a subset of these methods, such as between nuclear and plastid genome organisation, which can make the phylogeny difficult to resolve (McKain et al. 2016; Saarela et al. 2018; Givnish et al. 2018). However, the Ecdeiocoleaceae and Joinvilleaceae are consistently placed as the two closest relatives to the Poaceae, either linearly or in their own sister clade depending on the method of analysis (**Figure 3.1**) (Bremer 2002; Hilu 2004; McKain et al. 2016; Givnish et al. 2018; Hochbach, Linder, and Röser 2018). Most of these

methods are limited by the degree of available sequence data, and historically the resolution of the graminid clade has been limited to methods using plastid genes or individual nuclear genes (Marchant and Briggs 2007; Givnish et al. 2018; Hochbach, Linder, and Röser 2018), which may have limitations at fully representing the species in question, as well as preventing more robust cross-species analysis of entire gene families.

To contribute to the development of a series of high-quality genomes of species within the Poales for comparative genomics, we sequenced the genome of *Ecdeiocolea monostachya* – a wild species with limited publically available genomic data. *E. monostachya* represents one of the closest living outgroups to the Poaceae (**Figure 3.1**), and therefore an important species for comparative genomics (Bremer 2002; McKain et al. 2016). *E. monostachya* resists cultivation in laboratory or botanical settings and is a perennial herb native to western Australia (Linder, Briggs, and Johnson 1998) (**Figure Beta**). It is presumed to be an outcrossing species with conflicting reports indicating a basic chromosome number of either 11 or 12 (Hanson et al. 2005; Tang et al. 2010). Flow cytometry has previously indicated a haploid genome size of approximately 0.8 Gbp. We assembled a diploid genome of 1.5 Gbp, with an estimated haploid size of 0.77 Gbp, containing 3,605 scaffolds with an N50 of 756 kbp, that is 95% BUSCO complete with 84,700 gene models.

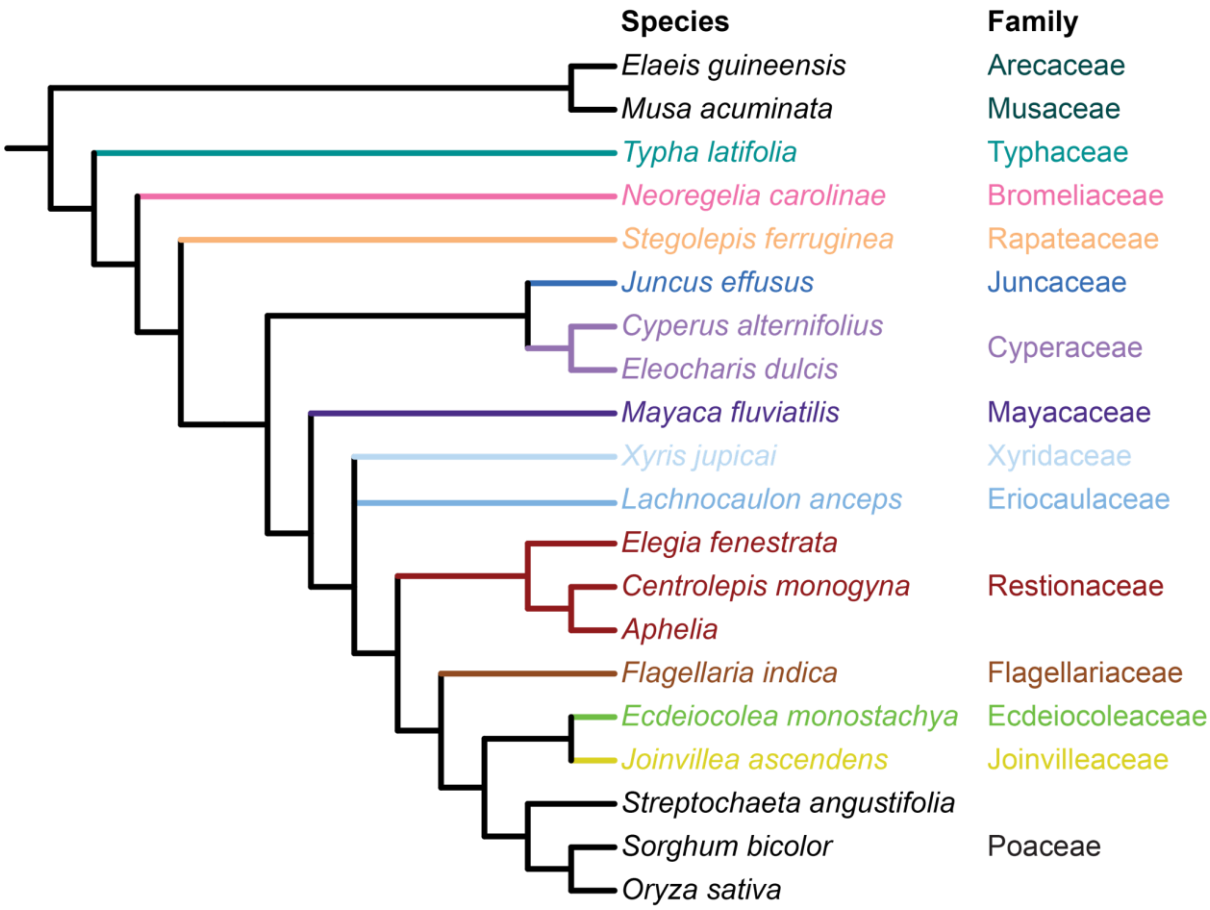

Figure 3.1 – Cladogram of selected commelinids based on (McKain et al. 2016). The commelinids are a clade of plants within the monocots, and represented here are *E. guineensis* (order Arecales), *M. acuminata* (order Zingiberales), and a member of each family within the order Poales. Of note are the Flagelliaraceae, Ecdeiocoleaceae and Joinvillaceae, which together form the graminid clade. The Ecdeiocoleaceae and Joinvilleaceae together form a sister clade to the Poaceae, an agronomically important and extremely large family. *E. monostachya* represents a critical outgroup for comparative genomics within the Poaceae. The data this figure is based upon represent 234 single-copy gene orthogroups conserved across these species.

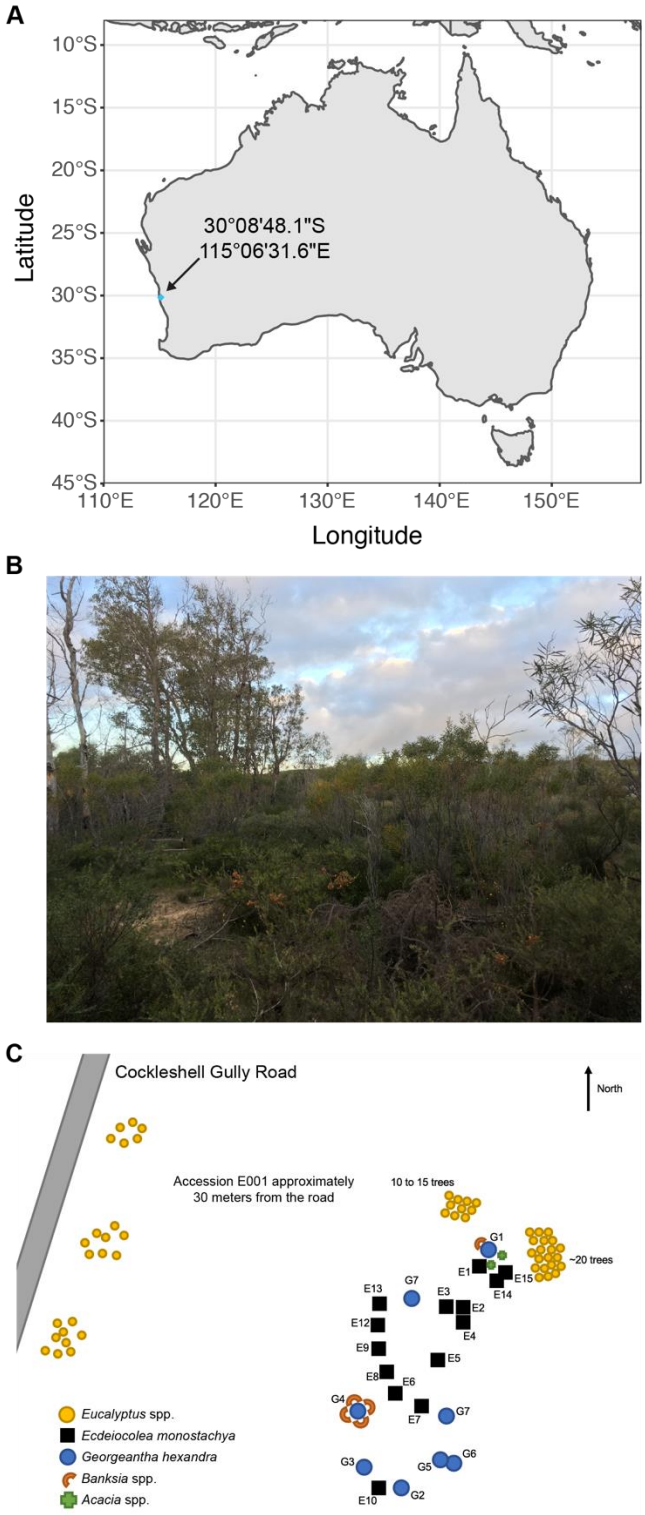

Figure 3.2. Field sampling of *E. monostachya*. A. The natural range of *E. monostachya* spans central and southern regions of western Australia. The sampling site for *E. monostachya* accession EM001 was 30°08'48.1"S 115°06'31.6"E (blue circle). **B.** Sampling site for *E. monostachya* accession EM001, facing east with *Euclyptus* groves (left). **C.** Map of sampling

site showing Cockleshell Gully Road, several *Euclyptus* groves, and the position of accessions of *E. monostachya*, *Georgeantha hexandra*, *Banksia* spp., and *Acacia* spp.

3.3 Results

3.3.1 Genome Assembly

E. monostachya is found throughout sandy regions of western Australia, it is a perennial grass-like shrub which resists cultivation in a laboratory and can grow to around 1 metre in height and width. An accession of *E. monostachya* was identified in a region that is suitable for future resampling (**Figure 3.2**). Plant tissue samples were taken of sheath, root tips, and fully developed flowers. Genomic DNA was extracted from sheath tissue of *E. monostachya* accession EM001 and sequenced using paired-end Illumina and single molecule Nanopore sequencing (Oxford Nanopore Technologies). RNAseq using paired-end Illumina sequencing was performed on RNA derived from sheath, root, and flower tissue. A k-mer based analysis of the raw Illumina gDNA data using Jellyfish and GenomeScope (k=27) predicted that accession E001 is diploid, with an estimated heterozygosity of 2.47% and estimated haploid genome size of 0.77 Gbp. This is a high degree of heterozygosity, suggesting substantial intraspecific variation. The hybrid assembly was performed with MaSuRCA (Maryland Super Read Cabog Assembler) using Illumina and Nanopore reads to assemble a draft diploid genome (Zimin et al. 2013). The total output size was 1.3 Gbp across 3,605 scaffolds, an amount predicted to cover approximately 84% of the diploid genome and with 50% of the genome spanning contigs of 756 kbp.

Table 5.1. Statistics summarising the *de novo E. monostachya* genome assembly. MaSuRCA was used to assemble a genome using Illumina reads and nanopore reads.

Assembly statistic	Result
Total length	1.3 Gbp
Number of scaffolds	3,605
N50	756 kbp
Shortest scaffold	1,673 bp
Longest scaffold	12.1 Mbp
Mean scaffold length	36 kbp
Median scaffold length	18 kbp
Total GC content	40.4%

Using KAT, we assessed the relative distribution of *k*-mers (k=27) in the Illumina data relative to the assembly genome (**Figure 3.3**). By comparing multiplicity of k-mers in each dataset, we assess the proportion of the genome that is present or absent in the assembly. The *k*-mer distribution has two local maxima at 80 and 160 multiplicity, representing *k*-mers found in a single haploid genome versus *k*-mers found in both haploid genomes. Generally, absence of *k*-mers in the assembly (shown in black) are associated with sequencing errors, as observed by the skewed distribution for low *k*-mer multiplicity or as *k*-mers lost due to the collapsing of allelic regions between haploid chromosomes (black region under first maxima). In this assembly 224 Mbp of sequence was not present in the final genome, likely due to collapsed regions between the haploid genomes. Given a predicted diploid genome size of 1.54 Gbp, this indicates the majority of the genome is present in the assembly. The genome is also clearly split across two haplotypes; with 1.3E+8 distinct *k*-mers appearing twice in the finished genome compared with 5.9E+8 of *k*-mers appearing once in the genome, indicating that approximately 18% of total genome sequence is duplicated across two contigs.

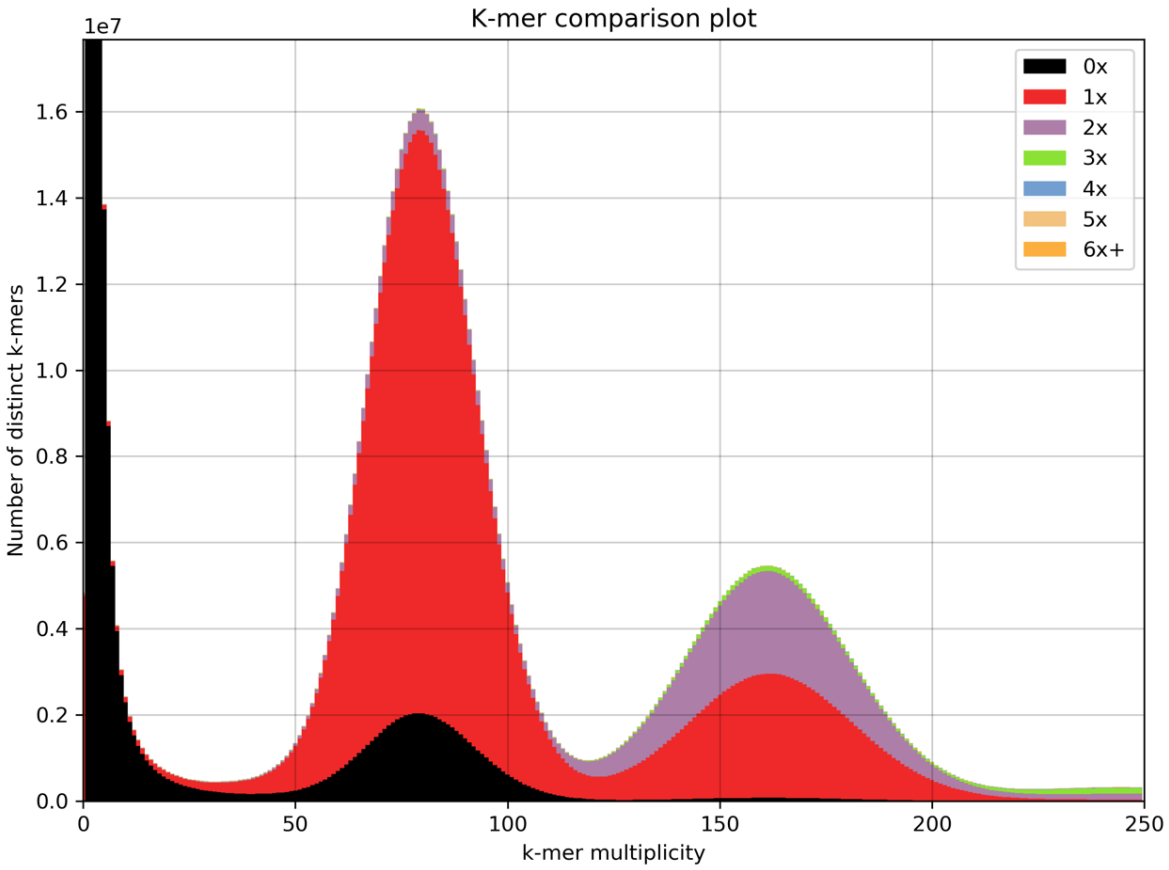

Figure 3.3: *E. monostachya* *k*-mer distribution from Illumina sequencing reads relative to the assembled genome. The *k*-mer analysis toolkit (KAT) was used to assess the multiplicity of *k*-mers in the unassembled illumina reads and the finished genome. The x-axis shows the number of times a given *k*-mer occurs in the Illumina data and the y-axis shows the occurrence of distinct *k*-mers. *k*-mers are displayed as a stacked distribution, shaded by the number of times that *k*-mer appears in the assembled genome.

3.3.2 Gene annotation

To annotate the genome, we used an RNAseq-based gene annotation strategy. RNA was extracted and sequenced *E. monostachya* using three tissues: sheath, root and flower. We aligned these data to the genome using hisat2 and identified gene models using Cufflinks. Open reading frames were identified with TransDecoder with and without Pfam-based prediction (**Table 3.2**). We evaluated the overall quality of these assemblies and annotations using the benchmark universal single copy ortholog (BUSCO) set of genes, which is a curated collection of species-appropriate, conserved, single-copy genes in different kingdoms. Using transcripts of the predicted gene models found the presence of 95.1% of BUSCO genes

predicted intact, 2.4% fragmented, and 2.5% missing. Evaluation of proteins based on TransDecoder prediction with or without Pfam-based prediction found a small reduction in BUSCO genes, indicating that a minority of predicted transcripts were rejected by transdecoder or Pfam and not passed to BUSCO. This indicates a high-quality genome assembly, with the majority of gene models assembled and correctly annotated. These predicted gene models of *E. monostachya* are suitable for further use in comparative genomics, without the risk of missing or ignoring valuable data. However, as shown in (**Table 3.2**), over half of genes which are considered single-copy in homozygous model genomes are multi-copy in all annotations the *E. monostachya* genome, reflecting the high level of heterozygosity (**Figure 3.3**).

Table 3.2: Gene representation of *E. monostachya* predicted transcripts and proteins based on a benchmark universal single copy ortholog (BUSCO) set of genes. BUSCO is a metric for describing the presence of conserved single-copy genes in a genome, used as a proxy for the overall quality of annotation. As the *Ecdeiocolea monostachya* EM001 genome is diploid with an overall heterozygosity of 2.5%, most single-copy BUSCO genes are expected to be detected with multiple copies, as each haplotype will be represented once.

	RNAseq alignment, Cufflinks	**RNAseq alignment, Cufflinks and Transdecoder**	**RNAseq alignment, Cufflinks, Transdecoder and Pfam**	**Maker pipeline**
Predicted open reading frames	84,700	62,132	90,736	539,444
BUSCO complete	95.1%	92.6%	93.5%	81.2%
BUSCO single-copy	33.0%	35.4%	35.0%	26.3%
BUSCO multiple copy	62.2%	57.2%	58.5%	54.9%
BUSCO fragment	2.4%	3.3%	3.3%	9.7%
BUSCO missing	2.5%	4.1%	3.2%	9.1%

3.3.3 Distribution of GC content in *E. monostachya* genome and exons

The relationship between genome size and overall GC content is complex, as GC base synthesis is more energy-intensive than AT synthesis, but also provides more stability and increased rates of recombination as well as allowing for more finely-tuned differential gene expression and epigenetic regulation (Singh, Ming, and Yu 2016; Šmarda et al. 2014). Unlike

the genomes of non-monocots the GC content of exons in Poaceae genomes outside the Anomochlooideae has been found to follow a bimodal distribution (Kuhl et al. 2004; Clement et al. 2015). The overall GC content of *Ecdeiocolea monostachya* is 40%, and the GC content of predicted exons follows a monomodal pattern with a peak at 45%.

3.4 Discussion

The genome presented here represents the first WGS available for a member of the Ecdeiocoleaceae, a close outgroup to the Poaceae. A hybrid assembly based on ONT long reads and Illumina reads, transcriptome-guided annotations providing high quality protein-encoding gene coverage, and large contigs which should be a useful resource for the scientific community. The high levels of heterozygosity in this outcrossing species present some unique challenges, and for any given gene of interest to a researcher there are likely to be two complimentary loci present in the full genome. Without chromosome sorting in advance of sequencing, or chromosome conformation capture data coupled with a long-read sequencing technology such as PacBio Hi-C, the ability to resolve the two haploid genomes of *E. monostachya* was limited by the degree of heterozygosity at a given locus. As can be inferred from **Figure 3.3**: some areas of the genome are represented by a single contig into which the differing sequences have been collapsed, and others by two contigs representing the two different chromosomes. Additionally, while multiple contigs containing areas of the plastid genome could be identified, no single contig appeared to capture the entire circular chromosome. The unequal fragmentation of the genome in this manner presumably also complicates the assembly of large scaffolds from their smaller components, as for increasing lengths of physical nucleotide sequence, the assembler will eventually alternate between being able to construct two separate phased contigs, or a single merged one, thus reducing the overall ability to create long, accurate scaffolds.

Attempts to phase the *E. monostachya* genome using Purge Haplotigs (Roach, Schmidt, and Borneman 2018) and redundans (Pryszcz and Gabaldón 2016) were made, however these resulted in a drop in BUSCO complete genes (to around 91%) without a significant reduction

in BUSCO duplicated genes (to around 40%). However, the level of heterozygosity in the genome also presents the opportunity to investigate multiple allelic variants of many genes of interest within *E. monostachya*, and only poses a minor obstacle to analyses which strictly require a single gene model.

The sequenced genome of *E. monostachya* has a predicted haploid size of 0.77 Gbp, and a predicted diploid size of 1.54 Gbp. The assembled diploid genome is 1.3 Gbp (84% Coverage) with an N50 of 756 kbp, 84,700 annotated genes and a BUSCO complete score of 95%. Compared to other reference genomes this is a high-quality genome, and we predict it will be an extremely useful resource for those working in the Poales, particularly as an outgroup for comparative genomic studies in the Poaceae, or for comparisons within the graminid clade. A phylogenetic analysis of the BUSCO genes of *E. monostachya*, as well as 15 other Poales places it as a sister to the *Joinvilleaceae,* in a subclade sister to the Poaceae. Sequencing of additional Ecdeiocoleaceae genomes, particularly of the other species in the family: *Ecdeiocolea rigens* and *Georganthia hexandra*, may further elucidate the relationships between the graminid species, as will the continued work of the Joint Genome Institute sequencing additional genomes from within understudied families of the Poales.
Assemblies of the *E. monostachya* genome were also performed using alternative assemblers – notably canu and miniasm, as well as illumina-only assemblers, but were of notably poorer quality than the hybrid assembly with MaSuRCA, or even of Trinity assemblies relying on mRNA rather than gDNA (https://github.com/matthewmoscou/Emo).

Future assemblies of *E. monostachya* or other wild outcrossing Poales species will undoubtedly benefit from the increasing prevalence of cheap and comparatively accurate long read sequence technologies such as Oxford Nanopore, but care must be taken to evaluate whether the additional complexities of these genomes, especially if they are expected to be large, heterozygous, or repetitive, will require additional investment in techniques such as CCS PacBio, chromosome flow sorting, HiC or even small breeding programmes to reduce heterozygosity in order to assemble the best possible genome.

3.5 Materials and Methods

3.5.1 Sampling and nucleic acid extraction

A number of *E. monostachya* accessions were identified at SITE A in western Australia. A single individual was selected (EM001) sampled for sheath, flower and root tissue. DNA was extracted from sheath tissue of sample EM001 using a method adapted from (Stewart and Via 1993). Tissue was harvested on site, frozen using liquid nitrogen, stored in dry ice during transport to a -80 C freezer. Frozen tissue was ground into a fine powder using mortar and pestle with grinding sand at -80 °C. Ground tissue was incubated in an extraction buffer (2% W/V CTAB, 100 mM Tris-HCL pH 8.0, 20 mM EDTA pH 8.0, 1.4 M NaCl, 1% W/V BME) at 55°C for 45 minutes. A ratio of 24:1 chloroform:isoamyl alcohol was added to the solution and mixed thoroughly to form an emulsion. The phases of the emulsion were separated by centrifugation and the lighter phase recovered. Nucleic acids were precipitated using 95% Ethanol. Nucleic acids were recovered and re-suspended in TE buffer. Resuspended DNA was incubated with RNase A for 2 hours at 37 °C. DNA was then re-precipitated using 24:1 chloroform:isoamyl alcohol and 95% ethanol as before. Finally, DNA was washed in 70% ethanol and re-suspended in TE buffer.

RNA was extracted from three tissue types: sheath, root and flower. Tissue was harvested on site, frozen in liquid nitrogen and transferred to the laboratory. Flower buds were excised from the petals before extraction to prevent tissue cross-contamination. For each tissue type, that tissue was ground to a fine powder using a mortar and pestle with grinding sand at -80 °C. Ground tissue was suspended in TRI reagent, allowed to incubate for 5 minutes at room temperature before centrifugation to pellet the lysate. The supernatant was recovered and mixed with chloroform. This mixture was incubated at room temperature for 15 minutes before the phases were separated by centrifugation and the lighter phase was recovered. Nucleic acids were precipitated from the lighter phase with isopropanol, pelleted via centrifugation, then washed with 75% ethanol and resuspended in water.

3.5.2 Whole genome sequencing

Short read sequencing of genomic DNA was performed using Illumina technology. Illumina paired-end libraries were generated using inserts of 250bp (DSW66921) and 350bp (DSW66909-V) and sequencing performed using a HiSeq 2500 platform

In total, 173.5 Gb of raw sequencing data was obtained. This data was cleaned using Trimmomatic v0.36, with a minimum final size requirement of 36 bp.

Sequencing was performed by Novogene

Long read sequencing of genomic DNA was performed using Oxford Nanopore technology. Genomic DNA was passed to a Nanopore PromethION platform, and sequenced to a depth of 103X.

Sequencing was performed by Novogene

Supplementary Table 5.1: Summary of Nanopore sequencing data. Assuming a haploid genome size of 0.78 Gbp, this translates to an average read coverage of 103X.

Number of reads	12835463
Maximum read length	849,007 bp
Median read length	4,652 bp
Total bp	7.96 Gbp

3.5.3 RNA sequencing

After extraction, RNA was purified, and QCd as described by (Dawson et al. 2016) RNA libraries were constructed using Illumina TruSeq RNA library preparation (Illumina; RS-122-2001). Barcoded libraries were sequenced using either 150 bp paired-end reads. Library preparation and sequencing was performed by Novogene.

Supplementary Table 5.2: Summary of Illumina RNAseq data. RNA was extracted from three tissue types, and sequenced by Novogene. Read length was 150 bp

Tissue	Total raw read length	Total trimmed read length
Sheath	85,068,573 bp	83,905,372 bp
Flower	87,661,519 bp	86,853,603 bp
Root	86,948,423 bp	86,071,563 bp

3.5.4 Genome assembly and analysis

The *E. monostachya* EM001 genome was assembled on Amazon AWS EC2 using SUSE Linux using MaSuRCA-3.3.0 with default configuration settings. Jellyfish was used to identify the distribution of k-mers using lengths of 17, 24, 27, and 31 and default parameters. Genome size was estimated using GenomeScope, which also provides estimates of heterozygosity and repetitive content. KAT 2.4.2 was used to assess the quality of the genome, and the relative distribution of k-mers in the raw and assembled genome. The output from KAT was visualised in R and analysed using custom Python scripts (https://github.com/matthewmoscou/QKkat). The full pipeline can be accessed at (https://github.com/matthewmoscou/Emo).

3.5.5 Gene annotation

To annotate the *E. monostachya* EM001 genome, RNAseq reads were aligned to the genome using hisat2 with a maximum intron length of 20 kb and no soft clipping. Samtools was used to convert .sam files into .bam files and sort reads with default parameters. Genes were then annotated and merged using the cufflinks pipeline, and passed through gffread.

For further assessment, putative protein models were generated using transdecoder, and interrogated for known protein domains using InterProScan (v3.20-59.0).

3.5.6 BUSCO assessment

To assess the overall quality of annotations, BUSCO, software which compares the presence of conserved single-copy genes over a variety of species was called on the total predicted transcripts using the lineage embryophyta_odb9, and default parameters.

3.5.7 Software used

NCBI Blast+ v2.2.31 (Altschul et al. 1990)

Trimmomatic v0.39 (Bolger, Lohse, and Usadel 2014)

Samtools v1.11 (Li et al. 2009)

Cufflinks v2.2.1 (Trapnell et al. 2010)

hisat2 v2.2.1 (Kim et al. 2019)

MaSuRCA v3.3.0 (Zimin et al. 2013)

Jellyfish v1.1.12 (Zimin et al. 2013)

GenomeScope (Vurture et al. 2017)

Transdecoder v5.5.0

KAT v2.4.2 (Mapleson et al. 2017)

BUSCO v3 (Seppey, Manni, and Zdobnov 2019)

Geneious v9.1.8

MUSCLE v3.8.31 (Edgar 2004)

RaxML v8.2.10 (Stamatakis 2014)

Python v2.7

Python v3.5.3

R v3.7.0 (Team 2013)

InterProScan v5.20-59.0 (Jones et al. 2014)

4. Expansion of the Exo70FX clade in the Poaceae

4.1 Chapter summary

Plants are endowed with several Exo70 families that exhibit substantial variation in copy number and sequence. A systematic gene family analysis by Cvrčková et al. (Cvrčková et al. 2012) demonstrated that Exo70 paralogues from eight gymnosperm species can be grouped into 10 distinct clades, the majority of which are conserved between monocots and dicots. However, among these clades, Exo70H, Exo70F, and Exo70FX exhibit substantial interspecific variation. Further studies have shown subfunctionalisation of Exo70 genes, based on observing tissue-specific expression and diverse biological functions including development, symbiosis, and immunity. To date, the majority of functional analysis of Exo70 in plants has been focused on dicot model systems, therefore we initiated a comprehensive analysis of interspecific and intraspecific variation between Exo70 genes in the monocot order Poales using a dataset encompassing RNAseq and genomic data from 37 Poaceae, 15 Poales species, and one Commelinid outgroup. While the majority of Exo70 are well-conserved between species, we show that the Exo70FX clade is originates within the graminid clade of the order Poales and exhibits remarkable intraspecific and interspecific variation in sequence and copy number – ranging from one copy in Streptochaeta angustifolia to 31 in the Triticum aestivum B subgenome. The rapid expansion and diversification of this gene family is analogous to the diversity observed in plant immune receptors (NLRs). Furthermore, two members of the clade are required for defence against biotrophic pathogens of barley, and no other roles have been associated with the clade. We hypothesise that the unusual diversity of this clade is due to a newly-acquired role in plant immunity, resulting in strong evolutionary pressures to maintain this function in tandem with the development of other components of plant immunity.

4.2. Introduction

4.2.1 Exo70 proteins and the Exocyst complex

Exo70 is one of eight proteins which comprise the Exocyst complex along with Sec3, Sec5, Sec6, Sec8, Sec10, Sec15, and Exo84 (Novick, Field, and Schekman 1980; TerBush et al. 1996; Hsu et al. 2004). The primary role of the Exocyst complex is tethering secretory vesicles to the plasma membrane in concert with soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins (Heider and Munson 2012). Unlike SNARE proteins, the Exocyst complex is specific to the plasma membrane, and separate multisubunit tethering complexes (MTCs) exist which localise to additional membranes such as the endoplasmic reticulum and the membranes of organelles (Ravikumar, Steiner, and Assaad 2017). The Exocyst complex is essential to polarised secretion, cell division, and cell growth (He, Xi, et al. 2007; He and Guo 2009). It has been implicated in roles as diverse as motility, cilliogenesis, autophagy, and defence against pathogens (Wu and Guo 2015). All eight subunits are essential for proper exocytosis and yeast cells deficient in any component either die, or accumulate a surfeit of un-secreted vesicles (Zhang et al. 2016)

Mei et. al. recently described a structure of the yeast Exocyst complex at 4.4 Å resolution by combining Cryo-EM data with established crystal structures and established crosslinking data (Mei et al. 2018). In this model, the eight subunits form pairs of heterodimers which are themselves joined into two complexes of four subunits. This model also describes the structure of the Exo70 protein, illustrated in (**Figure 4.1**), which is divided into five subdomains. In this model, the N terminal CorEx domain is intertwined with the CorEx domain of the Exo84 protein to create the first-level heterodimer. These domains are themselves part of a four-helix bundle with the CorEx domains of Sec10 and Sec15. The CAT-C and CAT-D domains of Exo70 also interface with the CAT-B and CAT-C domains of Sec5. Other work indicates that a conserved motif within the CAT-D domain is responsible for binding the phospholipid bilayer, specifically phosphatidylinositol 4,5-bisphosphate (PIP_2) (He, Xi, et al. 2007; Liu et al. 2007). This motif is generally conserved across eukaryotes, although its role in phospholipid binding has not been experimentally verified in plants, and not all plant Exo70 copies exhibit this motif.

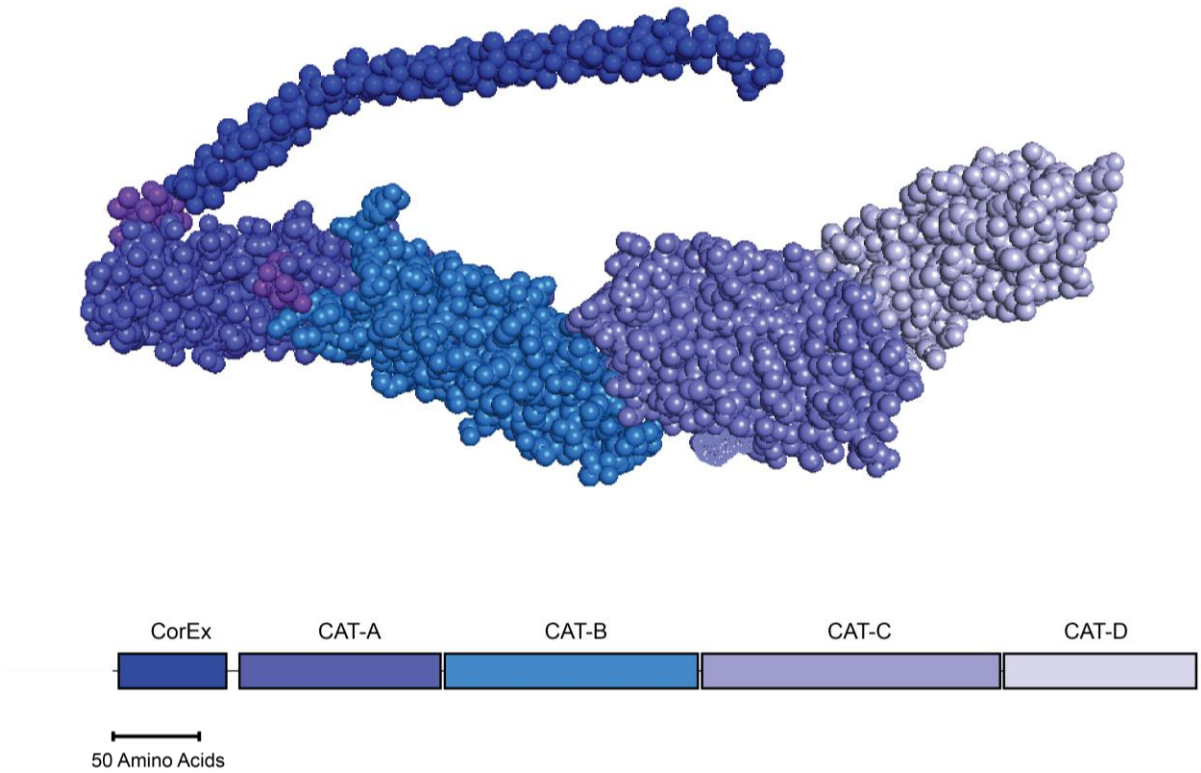

Figure 4.1. Physical structure of Exo70. Proposed crystal structure of ScExo70 with atoms represented as balls, shaded according to their domain. Exo70 domains are shown to scale in cartoon form beneath. This figure is a visualisation of PDB entry 5YFP **(Mei et al. 2018)** using PyMol.

Mechanistic differences have been observed between the Exo70 proteins of fungi, animals, and plants. A Rho3 interacting domain has been identified within the N-terminus in mammals, the C terminus in yeast, and is absent in plants (He, Xi, et al. 2007; Inoue et al. 2003). In plants, Y2H (Pečenková et al. 2011) assays have shown that the Exo70B2 and Exo70H1 proteins of *Arabidopsis thaliana* are capable of forming homomeric and heteromeric complexes outside of the Exocyst complex, an activity which is unique to plant isoforms of Exo70. Furthermore, in plants it has been observed that some Exo70 isoforms may localise to unique membrane domains other than PIP_2, an observation supported by the variation in the conserved PIP_2 binding motif in these proteins (Sekereš et al. 2017; Žárský et al. 2009; Kubátová et al. 2019).

4.2.2 Exo70 genes in plants have proliferated and diversified

The multi-copy Exo70 genes of land plants can be grouped into Clades: Exo70A, Exo70B, Exo70C, Exo70D, Exo70E, Exo70F, Exo70G, Exo70H, Exo70I, Exo70FX, and Exo70BNG

(Cvrčková et al. 2012; Synek et al. 2006). Clades Exo70A, Exo70B, Exo70C, Exo70D, Exo70E, and Exo70G are highly conserved between monocot and dicot plants, clade Exo70F is expanded in monocots, clade Exo70H is expanded in dicots, and clade Exo70FX is the most divergent in terms of sequence (as represented by branch lengths) and is unique to monocots. Clade Exo70I is specific to plants which form mycorrhizal symbioses (Zhang et al. 2015). Clade Exo70BNG is specific to basal non-angiosperms (ie, mosses), which only exhibit Exo70s in clades Exo70BNG, Exo70A, Exo70G, and Exo70I. On a wider scale, the Exo70 clades can be grouped into three major families: Exo70.1 consists of the Exo70A clade, and is most closely related to the Exo70s of animals and fungi (Synek et al. 2006). Exo70s in this group are observed to have multiple exons, while the remaining families are usually single-exon, or single-intron. Exo70.2 includes the Exo70B, Exo70C, Exo70D, Exo70E, Exo70F, Exo70FX, Exo70H, and Exo70BNG clades. Exo70.3 includes the Exo70G and Exo70I clades (Synek et al. 2006).

Within these clades, individual Exo70 genes are known to exhibit tissue and stimulus-specific expression patterns (Li et al. 2010a; Žárský et al. 2009). It has been found that the proliferation of Exo70 isoforms and expression patterns has allowed for regulatory fine-tuning of exocytosis in plants, and that certain Exo70 isoforms have acquired novel roles, and do not participate in the same functions as the ancestral isoforms (Synek et al. 2006). These explanations are not mutually exclusive, and a large body of work exists elucidating functions particular to certain Exo70 isoforms and species, some of which is summarised in (**Table 1.1**)

Table 1.1: Selected Exo70s associated with particular roles. A brief overview of Exo70 genes in for which a role has been ascertained. These roles are not exclusive, as in some cases multiple Exo70s may provide redundancy to a role, and many Exo70 proteins appear to have multiple functions. For more complete overviews see (Žárský et al. 2013; Žárský et al. 2019; Wu and Guo 2015)

Gene	Species	Role	Reference
EXO70A1	Arabidopsis thaliana	Cytokinesis	(Fendrych et al. 2010)
EXO70A1	Arabidopsis thaliana	Xylem differentiation	(Li et al. 2013)
EXO70A1	Arabidopsis thaliana	Pollen compatibility	(Samuel et al. 2009)
EXO70B1	Arabidopsis thaliana	Immunity	(Stegmann et al. 2013)
EXO70B2	Arabidopsis thaliana	Immunity	(Pečenková et al. 2011)
EXO70E	Arabidopsis thaliana	Non-Exocyst secretion	(Ding et al. 2013)
EXO70H1	Arabidopsis thaliana	Immunity	(Pečenková et al. 2011)
EXO70H4	Arabidopsis thaliana	Trichome development	(Kulich et al. 2015) (Kubátová et al. 2019)
EXO70I1	Medicago truncatula	Arbuscule development	(Zhang et al. 2015)
Exo70FX8	Hordeum vulgare	Immunity	(Ostertag et al. 2013)
Exo70E1	Oryza sativa	Immunity	(Zhao et al. 2016)
Exo70F2	Oryza sativa	Immunity	(Fujisaki et al. 2015)
Exo70D3	Oryza sativa	Immunity	(Fujisaki et al. 2015)
Exo70	Homo sapiens	Immunity	(Nichols and Casanova 2010)

To identify the evolutionary origin of the Exo70FX clade and assess the extent of diversity within the Exo70FX clade, we characterised Exo70 in eight genomes and 29 transcriptomes from species within the Poaceae, a family which includes all the major cereal crop plants but

has not been the focus of work characterising the Exocyst complex or its components. We also included the genomes of *Joinvillea ascendans* (order poales, graminid clade) *Ananas comosus* (order Poales), *Elaeis guineensis* (order Arecales), and *Musa acuminata* (Zingiberales), as well as transcriptomes from 14 species representing the major families within the Poales. To more conclusively place the emergence of the Exo70FX subclade, we sequenced the genome of *Ecdeiocolea monostachya*: a species in the Ecdeiocoleaceae; a sister order to the Poaceae. We found that the Exo70FX clade is present in *E. monostachya*, but dramatically expanded after the radiation of *Streptochaeta angustifolia* in the Poaceae. We found high levels of interspecific and intraspecific diversity in the Exo70FX subfamily compared to other subfamilies for both nucleotide and amino-acid sequence and presence/absence variation within individual subclades. We find that extreme examples of subclade expansion in the Exo70FX clade are driven predominantly by tandem repeats at the locus of the ancestral subclade members. These results collectively indicate that the Exo70FX clade first evolved within the Poales, and subsequently diversified within the Poaceae.

4.3 Results

4.3.1 The Exo70 gene at the *Rps8* locus is a member of the Exo70FX clade, which exhibits species-specific expansions in the Poaceae

The genomes of land plants encode multiple copies of the Exo70 gene, which can be grouped into distinct clades. The Exo70FX clade has only been observed in Poaceae and exhibits unusual variation in copy-number and amino acid sequence (Cvrčková et al. 2012). We aimed to catalogue this diversity, as well as identify the evolutionary origin of this unusual clade. We mined the genomes of nine species within the Poaceae, *Joinvillea ascendans* (order poales) *Ananas comosus* (order Poales), *Elaeis guineensis* (order Arecales), and *Musa acuminata* (Zingiberales). We also investigated the transcriptomes of 29 species within the Poaceae and of 14 species representing the major families within the Poales. Our initial work focused on the species *Hordeum vulgare, Triticum aestivum, Brachypodium distachyon, Oryza sativa, Setaria italica, Sorghum bicolor, Oropetium thomaeum*, and *Zea mays*. These species each have at least one high-quality reference genome as well as transcriptomic data available to support gene annotations. After obtaining genome annotations and performing *de novo* transcriptome assembly, we interrogated all open reading frames for the Exo70 Pfam motif

(PF03081). Once identified, Exo70 genes were extracted manually from each dataset and curated to a single reference transcript per gene. Predicted protein sequences were then aligned, trimmed to relevant sites, and used to develop a maximum likelihood phylogenetic tree (**Figure 4.2**). We annotated these proteins based on the previously annotated proteins of *Oryza sativa* and *Brachypodium distachyon* (Cvrčková et al. 2012). For all clades but Exo70FX, the original nomenclature was preserved and proteins from other species were annotated based on their relationships to existing clades and subclades. In the Exo70FX subclade, we discarded the existing protein subclades (eg, Exo70FX1, Exo70FX2…) as we could not reconcile them with the orthology we identified between species.

The putative Exo70FX subclades identified through sequence similarity and phylogenetic tree of the gene family are shown in (**Figure 4.2**). Putative subclades were not well supported by bootstraps due to higher levels of sequence divergence between species than in other clades. However, when we inspected the genomes of these species, we discovered that members of putative Exo70FX subclades were usually found at syntenic loci across the majority of species, and in tandem repeats if they are multi-copy. We therefore adopted synteny as a criterion for delineating Exo70FX subclades. The Exo70 at the *Rps8* locus (**Chapter 2**) was designated as Exo70FX12a, a single-copy subclade exhibiting strong sequence similarity with subclade Exo70FX11 but existing as a *trans*-species presence/absence polymorphism on Triticeae chromosome 4 rather than at the Exo70FX11 locus on Triticeae chromosome 2. A partial copy (409 bp vs 509 bp in barley) was identified in the *Triticum aestivum* B genome, but not the other genomes of wheat. (In total, we identify 14 subclades within the Exo70FX clade.

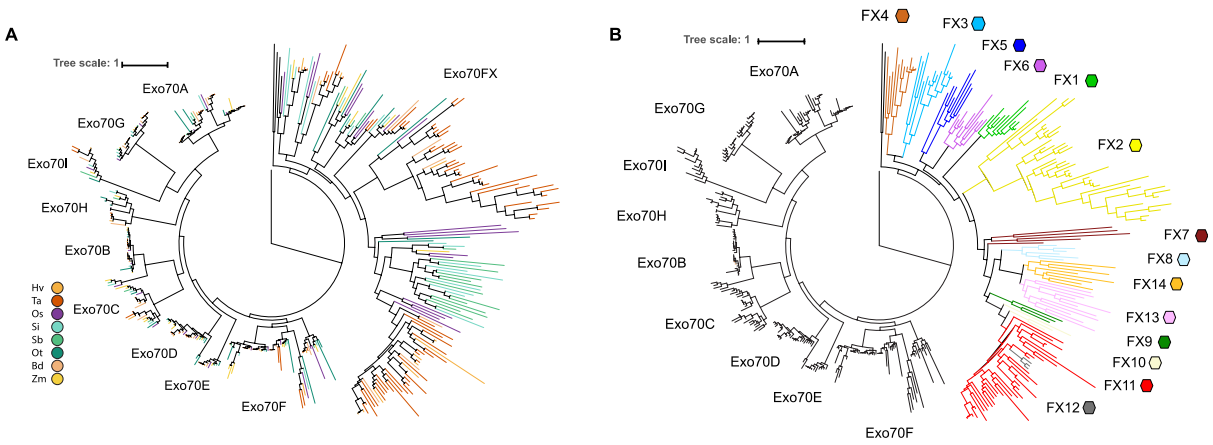

Figure 4.2. The Exo70FX clade exhibits species-specific subclade diversity. Maximum likelihood tree of the Exo70 proteins of *Hordeum vulgare, Triticum aestivum, Brachypodium distachyon, Oryza sativa, Setaria italica, Sorghum bicolor, Oropetium thomaeum*, and *Zea mays* with **(A)** species-based or **(B)** Exo70FX subclade-based colour-coding (see respective legends). The phylogenetic tree was generated using MUSCLE-based multiple sequence alignment of full-length protein sequence and RAxML using polymorphic amino acid sites with 40% coverage and 1000 bootstraps. Visualisation was performed using EMBL-iTOL. The outgroup is S*accharomyces cerevisiae* Exo70.

4.3.2 The Exo70FX clade emerged shortly before the radiation of the Poaceae

To determine whether the Exo70FX clade is an innovation of the Poaceae, we sequenced and annotated the genome of *Ecdeiocolea monostachya*: a member of the closest extant sister family to the Poaceae. We then interrogated the genomes of *Streptochaeta angustifolia, E. monostachya, M. acuminata, E. guineensis*, *Ananas comosus* and the transcriptomes of 31 Poaceae and 15 Poales for Exo70 genes as previously described and used our existing Poaceae annotations as a guide for annotating these proteins.

Within the Poaceae, all species had Exo70FX genes distributed between the subclades previously identified, although we were only able to identify a single Exo70FX (Exo70FX5) in the genome of *S. angustifolia*, which is the most basal member of the Poaceae. In *E. monostachya*, three Exo70FX genes were identified, and all three were located at a single locus. These genes grouped into the Exo70FX4 and Exo70FX5 families and exhibited clear heterozygosity between the two haplotypes of the locus present in the sequenced individual.

One gene (Exo70FX4-1) was located immediately adjacent to an NLR gene, however no single ORF encoding an integrated NLR/Exo70FX4-1 protein could be identified based on RNAseq read mapping. Exo70FX genes are absent in the genomes of *Musa acuminata*, *Elaeis guineensis*, and *Ananas comosus*. No Exo70FX transcripts were identified in the remaining Poales transcriptomes, however we cannot exclude the possibility that genes are present but not expressed under the sampling conditions, or low expression level and/or insufficient sequencing depth was used. These results are summarised in **Figure 4.3**.

Exo70FX12a (**Chapter 2**) was additionally identified in *Agrostis stolonifera* (**Figure 4.3**)., however it was not expressed in this dataset. Exo70FX12a is known to exist in an accession-dependent manner in *H. vulgare*, and analysis of additional accessions within the Pooideae may identify additional members of this, or other subclades.

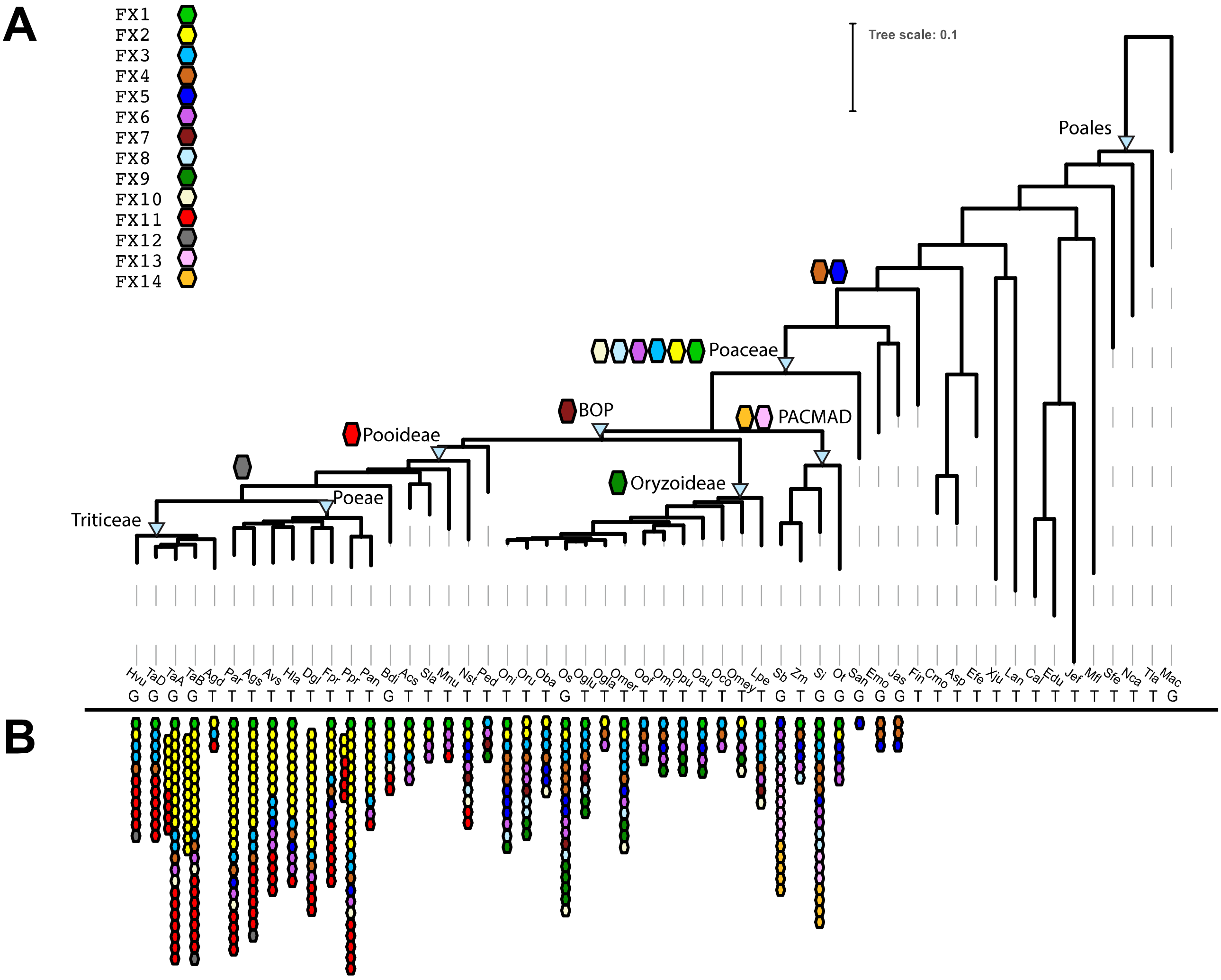

Figure 4.3. The Exo70FX subfamily emerged in the Poales and is expanded in the Poaceae. (A) Phylogenetic relationship between these species using codon-based alignment of BUSCO genes with 40% coverage, concatenation of alignments, and maximum likelihood using RAxML. Three letter species abbreviations from **Table Beta** are shown at the base of the tree. Whether sequence was derived from genomes and transcriptomes (G), or only transcriptomes (T) is shown below the species abbreviation. Major taxonomic groups are labelled at the corresponding node, along with hexagons indicating the hypothesised emergence of Exo70FX subclades, coloured according to the key in the top left **(B)** Coloured

hexagons represent presence of a gene encoding a member of that Exo70FX subclade in the corresponding species. For *Hordeum vulgare*, the reference accession Morex was used.

4.3.3 Variation in conserved Exo70 domains is clade and dependant

It has previously been observed that the greatest amount of variation in Exo70 sequences in plants occurs within the first 300 bases (100 residues) of the N-terminus (Cvrčková et al. 2012). This region includes the CorEx domain and the beginning of the CAT-A domain (**Figure 4.1**). The model for Exo70 integration into the Exocyst complex proposed by Mei et al. (Mei et al. 2018) features the CorEx domains of Exo70 and Exo84 interacting to form a pair of intertwined coiled coils (Mei et al. 2018). It is unclear how variation in the N-terminal region, especially in the form of large deletions, impacts the integration of Exo70 into the Exocyst complex. To assess variation in subdomain composition among Exo70, we constructed an alignment of Exo70 proteins from *B. distachyon, H. vulgare*, and *O. sativa* with ScExo70. Mei et al define the Exo70 domains in ScExo70 by the following residues: CorEx: 5-67, CAT-A: 74-190, CAT-B: 194-340, CAT-C: 341-513, CAT-D: 514-623. Gaps are due to disordered regions. A domain was considered present in the plant Exo70 if at least 30 total residues were present over the alignment region corresponding to that domain in ScExo70. (**Table 4.2**). We observe that among Non Exo70FX clades, the CAT-A and CorEx domains were almost always present, with the exception of the Exo70D and Exo70E clades, where the CorEx domain was only present in in 17% (1/6) and 0% (0/3) of proteins respectively. In the Exo70FX clade, however, the reverse is true, and CorEx domains were only found in 17% (5/30) of Exo70FX proteins. Primarily those of the Exo70FX4 and Exo70FX5 clades, which appear to be the most ancestral (**Figure 4.3**). Similarly, the CAT-A domain, which is almost always present in non-Exo70FX clades is only present in 12/30 (40%) of proteins.

Table 4.2. Presence/absence variation in Exo70 CAT-A and CorEx subdomains in three Pooideae species. A domain was considered present in the plant Exo70 if at least 30 total residues were present corresponding to that domain. Numbers given indicate the percent of proteins with that domain present, as well as the number of proteins examined.
Other domains were always present, with one exception: OsExo70FX7 has neither an intact CAT-C or CAT-D domain.

	Non-Exo70FX proteins		Exo70FX proteins	
Species	CAT-A domain	CorEx domain	CAT-A domain	CorEx domain
B. distachyon	100% (19/19)	79% (15/19)	67% (4/6)	17% (1/6)
H. vulgare	100% (20/20)	70% (14/20)	20% (2/10)	10% (1/10)
O. sativa	90% (19/21)	66% (14/21)	42% (6/14)	21% (3/14)

We also investigated the canonical PiP_2 (K-X-X-K) and Arp2/3 (K-E-X(53)-K-N-P) binding sites identified in Exo70 (He, Xi, et al. 2007; Zuo et al. 2006). Zarsky et al. (Žárský et al. 2009) previously observed clade-specific variation in these domains within *A. thaliana* Exo70 proteins, implying a diversity of membrane localisation specificities. Using the aligned Exo70 proteins of *B. distachyon, H. vulgare*, and *O. sativa* with ScExo70, we interrogated that position. The PIP_2 binding site was described as intact if both amino acids were of the same polarity as those in yeast, and the Arp2/3 binding site was described as intact if only one or fewer of the five amino acids were different to those in yeast (**Table 4.3**). Overall, amongst non-Exo70FX proteins Arp2/3 binding sites were frequently disrupted in clades Exo70B, Exo70G, Exo70H, and Exo70I, and PIP_2 binding sites were frequently disrupted in clades Exo70B, Exo70D, Exo70H and Exo70I. In the Exo70FX clade, no intact Arp2/3 binding sites were identified, and only 36% (11/30) of proteins exhibited an intact PIP_2 site. These results, and those in (**Table 4.2**) strongly suggests that Exo70FX clade members have unusual localisation and binding specificities compared with the ancestral Exo70A clade.

Table 4.3. Sequence variation in Exo70 Arp2/3 and PIP_2 binding sites in three Pooideae species. Binding sites were aligned with the ScExo70 protein sequence, and examined for polarity. A binding site was considered present if each amino acid agreed with the polarity of its matching amino acid in ScExo70 for PIP_2 binding, and if up to one amino acid did not match the polarity of ScExo70 for Arp2/3 binding. Numbers given indicate the percent of proteins with that domain present, as well as the number of proteins examined.

	Non-Exo70FX protenis		Exo70FX proteins	
Species	Arp2/3 site	PIP_2 site	Arp2/3 site	PIP_2 site
B. distachyon	63% (12/19)	68% (13/19)	0% (0/6)	17% (1/6)
H. vulgare	55% (11/20)	65% (13/20)	0% (0/10)	60% (6/10)
O. sativa	61% (13/21)	61% (13/21)	0% (0/14)	29% (4/14)

4.4 Discussion

In this chapter we describe the origin and expansion of the Exo70FX clade in monocots, specifically their origin in the Poales, either at or before the radiation of the Ecdeiocoleaceae and their subsequent diversification in the Poaceae after the radiation of *Streptochaeta angustifolia*. When present, this unusual gene family is often the largest of all Exo70 families. Furthermore, it exhibits the highest levels of diversity of all Exo70 families in the species we studied, by the metrics of overall sequence diversity (branch length), N-terminal domain presence/absence, subclade copy number and presence/absence variation between species and intra-specific sequence (branch length), and presence/absence variation in *Hordeum vulgare* transcriptomes. These results are congruent with previous analyses using alternative datasets within the Poaceae (Cvrčková et al. 2012; Zhao et al. 2019).

The origin of the Exo70FX clade appears to be in the graminid clade, being found in the genomes of *Ecdeiocolea monostachya* (**Chapter 3**) and *Joinvillea ascendans*. However, the dramatic expansion of the subclade after the radiation of *Streptochaeta monostachya* is a source of interest, as it coincides with the speciation and expansion of the "true grasses", which make up the remainder of the Poaceae, which are extremely diverse comprising over

11,000 species (Soltis et al. 2009). Other traits of the Poaceae include bimodal GC distribution, the spikelet, cytoplasmic ADP synthesis, and an expanded LRR-XII receptor kinase family (Lehti-Shiu, Zou, and Shiu 2012; Preston et al. 2009; McKain et al. 2016)

Given that conserved clade or isoform-specific roles have been identified for many Exo70s, we find the expansion of the Exo70F and Exo70FX clades in Poaceae particularly intriguing. Where present, the Exo70FX clade often includes over half of all Exo70s within a plant genome, and this dramatic increase in gene content is not associated with expansions of the other clades, or with increased diversity in those clades. To our knowledge only two genes in the Exo70FX clade have been characterised, and both are involved in plant immunity to fungal pathogens *Rps8* / Exo70FX12a (Chapter 2) and *Exo70F-like* / *Exo70FX11-3*) (Ostertag et al. 2013). Furthermore, the Exo70 proteins which have formed chimeric fusions with nucleotide-binding, leucine-rich repeat proteins (NLRs) in the Poaceae all originate within these clades (Brabham et al. 2018).

Based on the separation of biological roles for the other established clades of Exo70 in plants, we hypothesise that members of the Exo70FX clade also share a role in the species where they are present. We propose that this role is likely one which places the clade under a selective pressure to expand and diversify, leading to the high number of recent (<70 Mya) species-specific expansions and contractions of the Exo70FX clade in the Poaceae (Cvrčková et al. 2012; Zhao et al. 2019; Kellogg 2001), we speculate that this is congruent with a Clade-wide role in plant immunity.

4.5 Materials and Methods

4.5.1 Identification of Exo70 genes and transcriptome assembly

De novo assemblies of transcriptomes were performed using Trinity (v2.4.0). Open reading frame prediction and translations were performed using TransDecoder (v2.0.1), putative Exo70-encoding genes identified using HMMer (v3.2.1) with the Pfam motif (PF03081), and

all other domains identified using InterProScan (v5.20-59.0). Genes containing putative Exo70 domains (Pfam PF03081 or Superfamily SSF74788) were manually inspected for length and the presence of other domains. When a fragment was identified, it was retained and recorded as a fragment. NCBI BLAST v.2.2.31+ was used with default parameters. For genomes with annotations, Exo70s were identified using InterProScan and compared to any relevant transcripts for additional verification. When the sequence of an Exo70 gene was in question and both genomic and transcriptomic data were available, RNAseq data was aligned to the appropriate region of the genome using Bowtie2 or Hisat2. The aligned reads were sorted and converted to bam format using samtools, and visualised in Geneious to curate the gene model.

4.5.2 Phylogenetic tree construction

Alignments were performed using MUSCLE v3.8.31 using default parameters. The QKphylogeny set of scripts (https://github.com/matthewmoscou/QKphylogeny) was used to process alignments of DNA or protein sequences to remove entries which do not meet a length criterion of 40% the total alignment length and alignment positions which do not meet a depth criterion of 40% coverage. Maximum likelihood trees were constructed from these processed alignments using RAxML (v8.2.10) using the PROTGAMMAJTT AA substitution model, the rapid bootstrap analysis with either 100 or 1000 bootstraps, and search for best-scoring ML tree.

4.5.3 Genome-based Exo70 annotation and curation

Full length Exo70 protein sequences from *O. sativa, B. distachyon, H. vulgare*, and *Arabidopsis thaliana* were aligned and parsed to remove sequences with a total length of less than 100 amino acids and alignment positions with a coverage of less than 40%. The curated alignment was used to construct a maximum likelihood tree in RaxML. Proteins comprising each clade (as defined by the previously annotated previously annotated Exo70s of *O. sativa, B. distachyon* and *A. thaliana* proteins) were partitioned and this approach was repeated to produce clade-specific trees and alignments. Alignments and trees were manually inspected to identify (1) proteins with multiple predicted gene models and (2) proteins with significant deviation from others in their clade. In the latter case, this includes areas of complete

sequence mismatch or large insertions/deletions, which may indicate a poorly annotated protein, a pseudogene, or a true deviation in protein sequence compared to other Exo70. Gene models were validated using transcriptomic data and manual inspection of genomic sequence where available. When several gene models existed and manual curation did not resolve the issue, the version which most closely matched a verified homologue in an appropriate species was retained. Curated Exo70 proteins were used to construct a maximum likelihood phylogenetic tree using RAxML as described previously, and reclassified based on their phylogenetic relationship.

The following naming strategy was adopted:
[Xy]Exo70[Z][α]-[β].[γ]
[Xy] is the two letter abbreviation for the species (as seen in table Beta)
[Z] is the letter-based clade identifier. Clades include 10 distinct groups formed by the first order branches of a maximum likelihood tree of Exo70s (eg figure 4.2).
[α] is a number-based subclade identifier. Members of a subclade from different species are more closely related to one another than other members of the same clade from the same species.
[β] is a number referring to variants. When there are multiple members of a subclade from the same species these are assigned an arbitrary number.
[γ] is a letter referring to alleles, where sequence from multiple accessions of a species indicates that there is allelic variation for a given Exo70.

For non-Exo70FX clades, the designations by Cvrčková et al. (Cvrčková et al. 2012) were retained and used as a guide to name the previously undescribed *H. vulgare* Exo70s. For example, an *H. vulgare* Exo70 protein more similar to OsExo70A1 than to any other protein in *H. vulgare* would be classified as HvExo70A1. A hypothetical novel *H. vulgare* protein which was in the Exo70A clade but not orthologous to any of the existing four subgroups would be named HvExo70A5. A hypothetical novel *H. vulgare* protein which was more similar to OsExo70A1 than to any *H. vulgare* protein except HvExo70A1 would prompt a renaming of these proteins HvExo70A1-1 and HvExo70A1-2.

For the Exo70FX clade, subclades were named by manually inspecting the groups of proteins with above 70% bootstrap support for protein identity and synteny between their respective genomic positions, as well as by performing reciprocal blasts to identify closely related sequences. These additional parameters were required due to the overall lack of conservation within the Exo70FX clade, where far more subclades are present with multiple variants and greater intra- and inter-specific diversity than non-Exo70FX clades. The original designations were not retained for the Exo70FX family, as the inclusion of additional species generated subclades which could not be reconciled with the existing designations.

The approach was repeated using the annotated genomes of *S. italica, O. thomaeum, S. bicolor, Z. mays*, and the three subgenomes of *T. aestivum* (A, B and D). The curated Exo70s of *H. vulgare, O. sativa* and *B. distachyon* were included in the alignment and used to provide a guide for classifying the Exo70 complements of these species.

For the genomes of species outside the Poaceae in **Supplementary Table 4.1**, the same approach was used, but this time with all curated Poaceae Exo70 proteins included in the alignment and used as a guide for classifying the Exo70 complements of these species.

4.5.4 Transcriptome-based Exo70 annotation and curation

For the remaining species in **Supplementary Table 4.1**, Exo70s were identified from *de novo* assembled transcriptomes using the same method as before. An alignment was constructed using the genome-derived Exo70s of the Poaceae and the Exo70s of that species and used to construct a phylogenetic tree as before. For each transcriptome, individual protein sequences were compared to Exo70s with genomic support using Blast+. The Blast+ results, alignment and phylogenetic tree were cross-referenced in order to place the Exo70FX proteins within an existing clade and subclade, or into a new subclade. Proteins which could not be conclusively placed into a subclade or a new subclade, were placed into “subclade 0”, to indicate a lack of significant homology with any other Exo70FX protein. This was commonly observed for truncated transcripts from species which are evolutionarily distant from the Poaceae. As many transcripts were not full length, multiple transcripts from the same species were often placed in the same subclade and may represent partial reads from the same full-

length transcript. When possible, these were merged into a single transcript. The implementation of this was as follows:

(1) partial transcripts aligning to the same reference transcript, which overlapped with no polymorphisms between them were candidates for merging into a single transcript

(2) partial transcripts aligning to a transcript does not exhibit multiple variants in related species, and with no polymorphisms between them were candidates for merging

(3) if transcripts overlapped but exhibited polymorphisms then they were never merged

(4) if the subclade in question exhibited multiple variants in related species then transcripts were not merged without overlap between their sequences.

This approach refined the alignments by removing several truncated proteins, which contain limited information and are disruptive to the overall alignment, as well as preventing an over-estimation of the diversity in Exo70s by falsely presenting multiple transcripts of a single full-length protein as multiple proteins.

4.5.5 Physical structure of Exo70s

Predicted protein sequences of *Saccharomyces cerevisiae* Exo70 and plant Exo70s with genomic support (as described above) were aligned in MUSCLE, and the protein domains of ScExo70 were used as a scaffold to annotate the protein domains of the plant Exo70s

Mei et al define the Exo70 domains in ScExo70 by the following residues: CorEx: 5-67, CAT-A: 74-190, CAT-B: 194-340, CAT-C: 341-513, CAT-D: 514-623. Gaps are due to disordered regions. A domain was considered present in the plant Exo70 if at least 30 total residues were present over the alignment region corresponding to that domain in ScExo70. The annotated ARP2/3 binding site and PIP_2 binding site in ScExo70 were also used to identify those same sites in plant Exo70 proteins. A binding site was considered present if each amino acid agreed with the polarity of its matching amino acid in ScExo70 for PIP_2 binding, and if up to one amino acid did not match the polarity of ScExo70 for ARP2/3 binding.

4.5.6 Software used

NCBI Blast+ v2.2.31 (Altschul et al. 1990)

Bowtie2 v2.1.0 (Langmead et al. 2009)

Trimmomatic v0.39 (Bolger, Lohse, and Usadel 2014)

Samtools v1.11 (Li et al. 2009)

Cufflinks v2.2.1 (Trapnell et al. 2010)

Transdecoder v5.5.0

hisat2 v2.2.1 (Kim et al. 2019)

Geneious v9.1.8

MUSCLE v3.8.31 (Edgar 2004)

RaxML v8.2.10 (Stamatakis 2014)

Python v2.7

Python v3.5.3

R v3.7.0 (Team 2013)

R v3.7.0 (Team 2013)

InterProScan v5.20-59.0 (Jones et al. 2014)

4.6 Appendix

Supplemental Table 4.1: Species and accessions from which sequencing data was obtained in this chapter.

Species	Acronym	Accession	Identifier	Type
Achnatherum splendens	Acs		v3	Transcriptome
Agropyron desertorum	Agd		v3	Transcriptome
Agrostis stolonifera	Ags	Penncross	v3	Transcriptome
Aphelia sp.	Asp		v3	Transcriptome
Arabidopsis thaliana	At	Col-0	v10	Genome
Avena sativa	Avs	Victoria	GGDX01000000	Transcriptome
Brachypodium distachyon	Bdi	Bd21	v3.1	Genome
Centrolepis monogyna	Cmo		v3	Transcriptome
Cyperus alternifolius	Cal		v3	Transcriptome
Dactylis glomerata	Dgl	Sparta	v3	Transcriptome
Ecdeiocolea monostachya	Emo		v3	Transcriptome
Elegia fenestrata	Efe		v3	Transcriptome
Eleocharis dulcis	Edu		v3	Transcriptome
Festuca pratensis	Fpr	Westa	v3	Transcriptome
Flagellaria indica	Fin		v3	Transcriptome
Holcus lanatus	Hla		v3	Transcriptome
Hordeum vulgare	Hvu	Abed Binder 12	GGCM01000000	Transcriptome
Hordeum vulgare	Hvu	Aramir	GGCO01000000	Transcriptome
Hordeum vulgare	Hvu	Barke	GGCN01000000	Transcriptome
Hordeum vulgare	Hvu	Baronesse	GGCP01000000	Transcriptome
Hordeum vulgare	Hvu	BCD12	GGCQ01000000	Transcriptome
Hordeum vulgare	Hvu	BCD47	GGCR01000000	Transcriptome
Hordeum vulgare	Hvu	Betzes	GGCS01000000	Transcriptome
Hordeum vulgare	Hvu	Bowman	GGCT01000000	Transcriptome
Hordeum vulgare	Hvu	CI 16139	GGCU01000000	Transcriptome
Hordeum vulgare	Hvu	CI 16147	GFJN01000000	Transcriptome
Hordeum vulgare	Hvu	CI 16153	GFJL01000000	Transcriptome

Hordeum vulgare	Hvu	CIho 4196	GFJK01000000	Transcriptome
Hordeum vulgare	Hvu	Commander	GGCV01000000	Transcriptome
Hordeum vulgare	Hvu	Duplex	GGCW01000000	Transcriptome
Hordeum vulgare	Hvu	Emir	GGCX01000000	Transcriptome
Hordeum vulgare	Hvu	Finniss	GGCY01000000	Transcriptome
Hordeum vulgare	Hvu	Fong Tien	GGCZ01000000	Transcriptome
Hordeum vulgare	Hvu	Golden Promise	GGDA01000000	Transcriptome
Hordeum vulgare	Hvu	G.Z.	GGDB01000000	Transcriptome
Hordeum vulgare	Hvu	Haruna Nijo	GFJJ01000000	Transcriptome
Hordeum vulgare	Hvu	Heils Franken	GGDC01000000	Transcriptome
Hordeum vulgare	Hvu	Hindmarsh	GGDD01000000	Transcriptome
Hordeum vulgare	Hvu	HOR 1428	GGDE01000000	Transcriptome
Hordeum vulgare	Hvu	I 5	GGDF01000000	Transcriptome
Hordeum vulgare	Hvu	Igri	GGDG01000000	Transcriptome
Hordeum vulgare	Hvu	Manchuria	GFJO01000000	Transcriptome
Hordeum vulgare	Hvu	Maritime	GGDH01000000	Transcriptome
Hordeum vulgare	Hvu	Morex	2017v1	Genome
Hordeum vulgare	Hvu	Pallas	GGDI01000000	Transcriptome
Hordeum vulgare	Hvu	Q21861	GGDJ01000000	Transcriptome
Hordeum vulgare	Hvu	Russell	GGDK01000000	Transcriptome
Hordeum vulgare	Hvu	Sultan 5	GGDL01000000	Transcriptome
Hordeum vulgare	Hvu	SusPtrit	GGDM01000000	Transcriptome
Hordeum vulgare	Hvu	WBDC 008	GGDN01000000	Transcriptome
Hordeum vulgare	Hvu	WBDC 013	GGDO01000000	Transcriptome
Hordeum vulgare	Hvu	WBDC 085	GGDP01000000	Transcriptome
Hordeum vulgare	Hvu	WBDC 109	GGDQ01000000	Transcriptome
Hordeum vulgare	Hvu	WBDC 110	GGDR01000000	Transcriptome
Hordeum vulgare	Hvu	WBDC 172	GGDS01000000	Transcriptome
Hordeum vulgare	Hvu	WBDC 259	GGDT01000000	Transcriptome
Joinvillea ascendens	Jas		v1.1	Genome
Juncus effusus	Jef		v3	Transcriptome

Lachnocaulon anceps	Lan		v3	Transcriptome
Leersia perrieri	Lpe		v3	Transcriptome
Mayaca fluviatilis	Mfl		v3	Transcriptome
Melica nutans	Mnu		v3	Transcriptome
Musa acuminata	Mac		v1	Genome
Nardus stricta	Nst		v3	Transcriptome
Neoregelia carolinae	Nca		v3	Transcriptome
Oropetium thomaeum	Ot		v1.0	Genome
Oryza australiensis	Oau		v3	Transcriptome
Oryza barthii	Oba		v3	Transcriptome
Oryza coarctata	Oco		v3	Transcriptome
Oryza glaberrima	Ogla		v3	Transcriptome
Oryza glumipatula	Oglu		v3	Transcriptome
Oryza meridionalis	Omer		v3	Transcriptome
Oryza meyeriana	Omey		v3	Transcriptome
Oryza minuta	Omi		v3	Transcriptome
Oryza nivara	Oni		v3	Transcriptome
Oryza officinalis	Oof		v3	Transcriptome
Oryza punctata	Opu		v3	Transcriptome
Oryza rufipogon	Oru		v3	Transcriptome
Oryza sativa	Os	Nipponbare	7.0	Genome
Phalaris arundinacea	Par		v3	Transcriptome
Phyllostachys edulis	Ped		v3	Transcriptome
Poa annua	Pan		v3	Transcriptome
Poa pratensis	Ppr		v3	Transcriptome
Setaria italica	Si		2.2	Genome
Sorghum bicolor	Sb		3.1.1	Genome
Stegolepis ferruginea	Sfe		v3	Transcriptome
Stipa lagascae	Sla		v3	Transcriptome
Streptochaeta angustifolia	San		V1	Genome

Triticum aestivum	TaA	Chinese Spring A	v1.0	Genome
Triticum aestivum	TaB	Chinese Spring B	v1.0	Genome
Triticum aestivum	TaD	Chinese Spring D	v1.0	Genome
Typha latifolia	Tla		v3	Transcriptome
Xyris jupicai	Xju		v3	Transcriptome
Zea mays	Zm	B73	2010-01	Genome

5. General discussion

5.1 Summary

In this thesis we present evidence that *Rps8,* a locus providing resistance to wheat stripe rust in barley requires two genes: *Exo70FX12a* and *LRR-RK*, which are inherited together in a large In/Del polymorphism on chromosome 4H of barley. We sequence a genome of *Ecdeiocolea monostachya*, a close outgroup of the Poaceae and assemble a diploid genome of 1.3 Gpb, with 84,700 gene models that is 95% BUSCO complete, and use it to analyse the evolutionary history of the Exo70FX clade – a clade of Exo70 genes unique to the monocots, and find that it originated in the Graminid clade, but drastically expanded in Poaceae after the radiation of the Anomochlooideae. We further catalogue extensive species-specific variation in Exo70 genes amongst the Poaceae and identify the origins of the Exo70FX12 subclade in the Pooideae.

5.2 Evolution of a genetic module providing resistance to *Pst* in barley

5.2.1 Background of resistance to *Pst* in barley

The resistance of barley to the non-adapted pathogen *Puccinia striiformis* f. sp *tritici* is primarily derived from a Natural Stack of three R-genes, each capable of providing resistance to the pathogen, although minor effect QTLs have also been reported (Dawson 2015; Dawson et al. 2016). In the field, distinguishing between the *formae speciales* of stripe rusts can only be accomplished with molecular evidence, however to our knowledge there are no reports of wheat stripe rust infecting cultivated barley and none of *Rps6, Rps7* or *Rps8* has been identified as providing resistance to *Puccinia striiformis* f. sp *hordei*. The strongest evidence to support the durability of these genes is in Australia, where geographic isolation has prevented *Puccinia striiformis* f.sp *hordei* from taking hold. Wheat and barley have been grown in the same parts of the country for over 60 years, and while wheat stripe rust is a perennial issue, the Australian Department of Agriculture, Water and the Environment reports that barley grown in the country does not suffer from stripe rust.

5.2.2 Similarities between *Rps8* and other well characterised R-genes

Both *Rps6* and *Rps7* encode NLR proteins, which are a common class of protein involved in intracellular recognition of pathogen-specific effectors. This accounts for the strength of resistance provided by these genes, as well as their specificity towards a particular *formae speciales* rather than to a broad-spectrum of *Puccinia striiformis* lineages. In **Chapter 2** we show that *Rps8* requires two genes: an LRR-RK and Exo70, suggesting that it recognises an extracellular ligand. LRR-RK is a member of the LRR-XII family, and combines traits identified in other members with certain unique properties. Similarly to Xa21, *LRR-RK* does not provide resistance to as broad an array of pathogens as FLS2 or EFR, which recognise epitopes widely conserved across bacteria. Similarly to FLS2, *LRR-RK* requires an Exo70 gene in order to function. However this gene is from the Poales-specific Exo70FX clade, rather than the widely-conserved Exo70B clade. Proper FLS2 function is associated with both EXO70B1 and EXO70B2, and EXO70B1 is required for proper membrane localisation of FLS2. No Exo70 genes have been associated with the proper functioning of EFR or Xa21, however there is still a possibility that an uncharacterised Exo70 or set of Exo70s are required for their proper localisation and function. Exo70FX12a shares some features with EXO70B2; both have a disrupted ARP2/3 and PIP_2 binding site, although EXO70B2 has a complete N-terminal region including a CorEx domain, and Exo70FX12a does not.

The fact that *Rps8* requires an LRR-RK indicates that recognition is provided by an extracellular ligand; the ligand is likely to be a peptide as LRR-domains preferentially recognise peptides. *Rps8* does not limit mycelial growth within the leaf as strongly as *Rps6* and *Rps7* (Dawson 2015) which could indicate either a weaker overall defence response or that the ligand is not detected until a later stage of infection. The downstream signalling components of LRR-RKs in barley have not been well characterised, however they are likely to be similar to those in Rice and so LRR-RK is predicted to have a co-receptor, within the SERK family. In rice, OsCERK2 is known to be required for Xa21 mediated resistance, and in barley HvSERK2 is expressed in response to *Blumeria graminis* f. sp. *hordei* (Li et al. 2018). Investigation of LRR-RK co-receptors should therefore focus on whether HvSERK family mutants are compromised in *Rps8*-mediated resistance, or whether LRR-RK colocalises with or phosphorylates these proteins *in planta*. Downstream signalling components involved in *Rps8* mediated defence

could also be investigated by a phosphoprotemic approach, comparing the overall status of phosphorylated proteins in the cell, before and after stimulation by *Pst*, as well as between mutant alleles of LRR-RK (Reinders and Sickmann 2005; Thingholm, Jensen, and Larsen 2009). The mutant allele derived from accession TM98, which has an early stop codon inside the kinase domain will be an excellent resource for this kind of investigation.

5.2.3 Hypotheses regarding the mechanism of *Rps8*

In order to further understand the interactions between *Exo70FX12a* and *LRR-RK* it will be important to characterise their behaviour *in planta*. Little is currently known about the behaviours of Exo70FX12a inside the cell, but some predictions can be made based on the established behaviours of other Exo70 proteins.

There are three hypotheses as to how these genes might be co-operate:

1. Exo70FX12a localises LRR-RK to an appropriate domain of the plasma membrane with the involvement of other members of the Exocyst complex. In this case we would expect mutants deficient in Exo70FX12a but not *LRR-RK* (eg, m3535 and Heils Franken haplotypes) to express *LRR-RK* but for the protein to accumulate in the cytoplasm, and not be delivered to the cell membrane. This could be tested by transient expression of tagged variants of the protein in a heterologous system, or by stable expression of tagged variants in barley. We would also expect that Exo70FX12a can localise to the plasma membrane, and interact with other members of the Exocyst complex, especially Sec3 and Exo84, which could be tested using yeast-2-hybrid assays, or by co-immune precipitation of candidate subunits. We would also expect that mutations in other *Sec* genes would also inhibit *Rps8* function, and that this might be identified in the uncharacterised mutants in **Chapter 2**.
2. Exo70FX12a localises LRR-RK to an appropriate domain of the plasma membrane in an unconventional manner. Again, we would expect not to see localisation of the LRR-RK protein to the plasma membrane in plants without Exo70FX12a. However in this case we would not expect to see Exo70FX12a interact with other members of the exocyst complex. We would expect that induced inhibition of the Exocyst complex has no effect on *Rps8* localisation or function, which could be tested by disruption of Exocyst assembly as in (Vukašinović et al. 2017) or (Huang et al. 2019).
3. Exo70FX12a is not involved with localisation of LRR-RK. In this hypothesis Exo70FX12a may be involved somehow in signal transmission, or in elicitor binding rather than trafficking. Exo70FX12a could also be involved in post-recognition autophagic processes, either directly interacting with LRR-RK or with cytoplasmic proteins. Exo70 genes have been implicated in a number of diverse roles. Although the mechanisms for these roles is largely unknown, there is currently no information to exclude the possibility Exo70FX12a has a role outside of polarised

exocytosis. In this case we would expect that Exo70FX12a is not required for LRR-RK to localise at the membrane, which would be detectable either in a heterologous system or through expression of tagged LRR-RK in barely. We would also expect Exo70FX12a to associate with downstream components of defence, or a pathogen ligand.

In all of these cases, the two identified mutant alleles of Exo70FX12a (**Chapter 2**) as well as the *Triticum aestivum* B genome Exo70FX12 gene (**Chapter 4**) may provide crucial insights into the role of Exo70FX12a. Mutant TM3535 exhibits an L130F polymorphism in the CAT-A domain and Heils Franken exhibits an E33K polymorphism in the CAT-C domain. The TaBExo70FX12 gene has an N-terminal truncation; reducing its size to 409 amino acids compared to 508 in barley, as well as 36 amino acid polymorphisms (91% protein identity). When investigating the localisation and interactors of Exo70FX12a, as well as its effect on the localisation and function of LRR-RK, mutant variants of Exo70FX12a which are known to be expressed but non-functional will aid in establishing its mechanism. Furthermore, the presence of an orthologous Exo70FX12 gene, as well as an orthologous LRR-RK (84% protein identity) in the B genome of bread wheat, raises the questions of whether these genes provide resistance to any lineage of stripe rust in wheat, whether these genes interact in the same manner *in planta* as those at the *Rps8* locus, and whether expression of the barley *Rps8* genes in wheat can provide resistance to *Pst.*

5.3 Evolution of the Exo70FX12 subclade

In order to better understand *Rps8*, we decided to investigate the evolutionary history of the Exo70 gene at the locus; Exo70FX12a, and the Exo70FX clade more generally. Historically the Exo70FX clade has not been the focus of much research, and it was only identified in the Poaceae (Cvrčková et al. 2012; Žárský et al. 2019; Zhao et al. 2019). In order to accomplish this we assessed the Exo70 gene complement of genomes belonging to eight Poaceae, three Poales, *Elaeis guineensis* (order Arecales), and *Musa acuminata* (Zingiberales) as well as 29 transcriptomes within the Poaceae and 14 from within the Poales (**Chapter 4**). We identify the emergence of the Exo70FX clade within the graminid clade, and a subsequent expansion and innovation within the Poaceae, transitioning from two subclades in *E. monostachya* and

J. ascendans to 14 across the Poaceae as a whole, and up to 10 in a single species (*O. sativa*). The Exo70FX subclade exhibits extremely long branch lengths compared to other Exo70 clades, and a rich species-specific diversity. Certain families, notably Exo70FX2, Exo70FX11, Exo70FX13, and Exo70FX14 can be extremely large, with up to 20 members of the Exo70FX2 clade in the B subgenome of wheat (**Chapter 4)**. Where this kind of species-specific subclade expansion occurs, it is almost always in the form of tandem repeats at a single locus.

Exo70 genes in plants are subfunctionalised, relative to the animal and fungal Exo70 gene, with individual Exo70 proteins associated with particular roles ((Žárský et al. 2009; Žárský et al. 2019; Li et al. 2010b). The diversity of the Exo70FX clade may indicate that additional subfunctionalisation has taken place in the Graminids. Given that only two Exo70FX proteins have been characterised (**Chapter 4**) (Ostertag et al. 2013), and both are involved in defence it seems likely that this expansion is connected to an overall role in immunity. Plant immune genes such as receptor kinases and NLRs are also under pressure to expand and diversify in order to counteract the rapid evolution of pathogens in a phenomenon sometimes described as a "Molecular arms race" (Stahl and Bishop 2000) or "the Red Queen hypothesis" (Pearson 2001). The mechanisms of Exo70FX clade members are still unclear, but it is possible that each Exo70FX has evolved to localise a particular cargo, or to do so under a particular condition and that this gene expansion provides additional control over PM localisation for defence, or additional resilience to disruption by pathogens.

Exo70FX12 is a comparatively recent innovation in the Exo70FX clade. We were able to identify an Exo70FX12 subclade member in Barley, Wheat, and *Agrostis stolonifera,* and therefore place Exo70FX evolution within the Pooideae, after the radiation of the Brachypodieae. Notably, in **Chapter 2**, we identified that Exo70FX12a exhibits presence/absence polymorphism across a panel of 40 barley accessions. It is therefore possible that Exo70FX12 exists as a pan-species presence-absence polymorphism within a wider set of Pooideae, and was simply undetectable in the accessions chosen for study in **Chapter 4**.

Exo70FX12 was also placed within the Exo70FX11 subclade in the phylogenetic tree (**Figure 4.2**) based on amino acid sequence. However due to the difficulties resolving subclades with little bootstrap support, we adopted synteny as a secondary metric of subclade placement.

Given that Exo70FX11 is a subclade containing up to 12 members at a single locus, the unique translocation of Exo70FX12 to chromosome 4H, rather than 1H warranted its placement in a unique subclade. There is little doubt, however, that Exo70FX12 is derived from Exo70FX11 given its sequence similarities and appearance subsequent to the emergence of Exo70FX11 at the base of the Pooideae.

5.5 Recommendations for future work

- **Validate the role of LRR-RK in *Rps8***. A transgenic construct encoding *LRR-RK* under the control of its native promoter and terminator has already been designed and assembled, along with a construct encoding both *Exo70FX12a* and *LRR-RK*. Both constructs have been used to generate primary transgenics in the *rps8* accession SxGP DH-47, and testing of transgenic plants for resistance to *Pst* should be completed at the soonest possible time. This will validate the requirement for both genes, acting together to provide *Rps8*-mediated resistance against wheat stripe rust in barley

- **Investigate the physical effects of *Rps8.*** Previous work (Dawson 2015) found that while *Rps6* and *Rps7* have a strong effect on colonisation of the leaf (ie, hyphal growth) and pustule formation, *Rps8* only prevents the formation of pustules and has little to no effect on leaf colonisation. Preliminary results indicate that *Rps8* does, in fact, slow the development of hyphae between the mesophyll through an unknown mechanism. As *Rps6* and *Rps7* encode NLR proteins, it is likely that defence is provided through the hypersensitive response at an early stage of infection (Dalio et al. 2020). Investigating whether *Rps8* provides a hypersensitive response, and what stage of infection defence is stimulated at will assist in determining how to evaluate the mechanism of LRR-RK *in planta.*

- **Investigate the signalling partners of *Rps8*.** LRR-RK is likely to signal through a co-receptor from the SERK family of receptor kinases (Chinchilla et al. 2009; Gust and Felix 2014; Chen, Zuo, et al. 2014; Li et al. 2018). To our knowledge, identifying a co-receptor will provide the first evidence for this kind of interaction in barley. An initial step towards identifying this partner, or additional signalling partners will be to characterise the remaining 6 *Rrs* mutants obtained in **Chapter 2**. This can be achieved through an F_2 recombination screen to rough-map a mutant gene of

interest coupled with RNAseq analysis to identify SNPs or other polymorphisms in the candidate regions, as well as crosses to determine additional complementation groups. Special attention should be paid to orthologues of genes identified as being required for other LRR-XII family genes such as SERK family members, chaperonins such as PSL1, PSL2, STT3A, SDF2, ERdj3B, and BiP (Saijo et al. 2009; Nekrasov et al. 2009)., and downstream signalling components such as MAPK family members and BIK1 (Lin et al. 2014).

- **Investigate the interaction partners of Exo70FX12a.** The mechanism of Exo70FX12a is still unknown. Determining where it localises within the plant cell, whether it interacts directly with LRR-RK, and which members of the Exocyst complex and other secretion machinery it interacts with *in planta* as discussed in **5.2.3** will be greatly beneficial towards understanding the role of the Exo70FX family as a whole, as well as the mode of action of LRR-XII RK family members in monocots.

5.6 Lessons Learned

No PhD project goes entirely according to plan. Many experiments only succeeded after numerous tries, others were never successful at all. However an experiment that doesn't produce a positive result is not necessarily a total failure – it is important to learn from mistakes, and to take into account negative data when considering hypotheses and experimental design for future experiments.

The most important lesson I learned over this project was to *value your time*. It is very simple to think "I will keep trying until this goes perfectly". Tenacity can be a great virtue, but as touched on in **Section 2.3.5** I was unable to clone the *LRR-RK-Rps8* gene and surrounding promoter and terminator for use in subsequent experiments. In fact I attempted to clone the gene for almost 9 months, before ordering a synthetic copy of the locus from a commercial provider. In the end, I was unable to include the results from experiments using the *LRR-RK-Rps8* gene in this thesis as they were conducted after the conclusion of my PhD project! It is important not to get so caught up in a task that you lose sight of your long-term goal, and not to treat your own time as disposable simply because it is *yours*.

Another important lesson was to *take multiple approaches to solving a problem*. Often there exist a number of specialist tools for a particular analysis, and deciding which one to use can be daunting. It's usually simplest to go with a tool you, or a colleague is familiar with, and once you have a result to simply move on. However, during the processes of assembling the

E. monostachya genome, and especially during Exo70FX annotation I often discovered that it wasn't until after I had done an analysis that the limitations of the approach I had chosen became clear. Should I base my alignments on nucleotide or protein sequence? How much noise is it appropriate to filter out? Which software produces the highest quality annotations? Eventually it became clear that a more comprehensive approach: trialling multiple methods and comparing them was often best in the long run, and that planning this from the start rather than retroactively "bolting on" additional analyses helped me to plan better experiments, and also to really think about what data I had, and what I would need to get the most out of it.

Finally *Actively Listen*. Many times over the course of my project I came across an idea for an experiment or analysis and only discovered after beginning that somebody I knew could have given me useful advice, if I had asked. Sometimes it's not enough just to take in what someone tells you, but instead you need to ask them for more – more information, more explanation, more advice, sometimes even just to explain it one more time! I am incredibly grateful for all of the assistance given to me by my friends and colleagues at TSL and further afield, but if I could go back in time and tell myself one thing at the start of my PhD it would be to ask more questions of all of them.

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