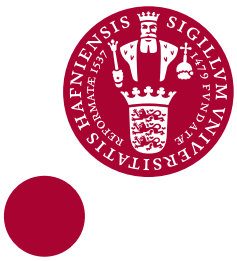

Functional genomic approaches and genome-wide transcript profiles for the investigation of plant responses towards powdery mildew infection

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Publication date:
2007

Document version
Publisher's PDF, also known as Version of record

FUNCTIONAL GENOMIC APPROACHES AND GENOME-WIDE TRANSCRIPT PROFILES FOR THE INVESTIGATION OF PLANT RESPONSES TOWARDS POWDERY MILDEW INFECTION

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PhD Thesis 2007
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I. Preface

The project I entered in 2004 was part of the coordinated framework program "Cell-specific analysis of host-plant responses to pathogens using a functional genomics approach". This project would soon confront me with the biological complexity of the interactions between living organisms. A complexity, not only in the physiology and molecular aspects of the interactors, but also at a higher 'meta' level. During my first six months on the project I had the opportunity to meet members of the international society of plant-microbe interactions at a conference in Norwich, and here I was impressed by the attempts by researchers to understand not only the behavior of host-plant responses, but also the calculations used by the pathogen in its attempt to attack and defend itself against counter attack during pathogenesis. Particularly, a Welsh scientist eagerly tried to make me express my working hypothesis on the interaction between barley and a grass powdery mildew fungus, as host- or pathogen-specific. Because, to his belief, only in the acknowledgement of the two separate motives one would be able to ask and address biologically meaningful questions related to the arms race of the interacting partners. His words have highlighted the crucial importance of experimental design in attempts to understand the biological processes I have investigated during my PhD studies. For the current project, the questions addressed and the data gathered have focused on the host response, though knowledge of the temporal and spatial changes during the pathogen development and attack was used. From my own point of view, I believe this study has made me a better biologist, in terms of exploiting both hypothesis-driven, and more importantly in the large-scale post-genomic era, data-driven research approaches.

Before continuing to read this thesis, a remark on the structure is needed. The thesis can be read from A-Z, starting from here and finishing with the conclusion. However, starting off with the introduction (Chapter I); three categorical sections encompassing the themes of my PhD studies are presented. The following chapters (2-4) serve as technical prescripts for the manuscripts enclosed in this thesis. Though termed prescripts, it is intended that these chapters are read concomitantly with the associated manuscripts, inferred at the beginning of each chapter (2-4). The three chapters provide insights to the methodological backgrounds of the papers presented. The conclusions and perspectives, serve best to be read at the end of introductory chapters 1-4 and the presented manuscripts I-IV.

II. Acknowledgements

The study presented in this thesis was carried out at (i) Section of Plant Pathology, Department of Plant Biology at Faculty of Life sciences, University of Copenhagen (KU-LIFE), and (ii) Department of Biosystems, Risoe National Laboratory, Technical University of Denmark. Funding was provided by a PhD scholarship from KU-LIFE (formerly The Royal Veterinary and Agricultural University) and research financed by a Danish Research Council grant for "Cell-specific analysis of host-plant responses to pathogens using a functional genomic approach" SJVF 23-03-0167.

I am grateful to my supervisors David B. Collinge and Michael F. Lyngkjaer for their patient guidance and confidence in my activities. Also, I would particularly like to thank my friend and colleague Jesper Henrik Rung for countless discussions on experimental concerns and the meaning of it all. Moreover, Karsten Bjerre, Anja Thoe Fuglsang, Michael Hansen, Torben Gjetting and Hans Lyngs Joergensen have all had great impact on the technical aspects of my studies. Especially, Qiyuan Li and Peter Hagedorn, have shown me the power and beauty of microarray data analyses and visualization for thorough biological in-sight. I sincerely thank you all for your contributions to my scientific development.

Additionally, I gratefully acknowledge Roger Wise of Iowa State University, USA, and Patrick Schweizer and Dimitar Douchkov from IPK, Gatersleben, Germany, for sharing unpublished data and for providing vector constructs of interest.

Furthermore, I thank Steen Malmmose, Anne Olsen and Margit Andersen for helping me taking care of my plants at KU-LIFE and Risoe National Laboratory, respectively. I also thank colleagues at KU-LIFE and Risoe National Laboratory; Nina Joehnk, Gitte Erbs, Thomas Sundelin, Mojtaba Mamarabadi, Lene Klem, Mari-Anne Newman, Mette Lübeck, Carl Erik Olsen, Jakob Skov, Pernille Olsen, and Kim Kristiansen. Thank you all for your help and attention. Additionally, I thank Karen Skriver and Charlotte O'Shea from Institute of Molecular Biology, University of Copenhagen, for reaching out a helping hand when needed.

Also, I am embedded to family and friends outside my scientific community, especially my mother, father and brother, to Allan Funder Kaas, and 'Hundene'; class of ´97.

Last but not least, I dedicate this thesis to my beloved wife, Sara; thank you for sacrificing your own interests and standing by my side through times when results were sparse and frustrations increasing.

III. Abstract

Living organisms respond to environmental stimuli by a range of regulatory mechanisms. In this respect plants, being sessile organisms, are particularly obliged to adapt to (as opposed to escape from) environmental stresses by the use of their 'at-hand' endogenous genetic apparatus. Besides undergoing physiological and structural changes, a predominant molecular adaptation to a given stimulus is conferred at the level of transcriptional activation and repression of specific genes. One approach for improved understanding of the molecular mechanisms affected by a given environmental stimulus is to look at the changes of gene transcript abundances. From the vast sequence abundances of transcripts expressed upon different environmental conditions, researchers subsequently face the challenges of characterizing the functional capacities encoded by such transcripts. From transcript sequence information, a range of reverse genetic approaches have become available for subsequent functional genomic studies, providing means to illuminate the molecular aspects of regulation conferred by the transcripts of interest.

In this thesis, I have studied the well-characterized interaction between the obligate biotrophic powdery mildew fungus *Blumeria graminis* f. sp. *hordei* (*Bgh*) and its natural host barley (*Hordeum vulgare*) as a model system for the functional characterization of genes expressed in barley upon powdery mildew infection. My studies have focused on members of the plant-specific NAC transcription family originally identified in our lab from powdery mildew infected barley leaves. Using a functional genomics approach including both transient gene silencing and over-expression assays of individual NAC members, I present the identification of a nuclear-localized transcription factor positively regulating penetration resistance, hereby providing an efficient and agro-economically important restriction of pathogen entry of host cells upon *Bgh* penetration attempts. The abundance of the NAC gene transcript accumulates in barley upon *Bgh* inoculation in both infected and healthy tissues. Both phenotype and transcript profiles were studied in the closest homologue of *Arabidopsis thaliana* upon *Bgh* inoculation. Interestingly, a loss-of-function mutant allele of the *Arabidopsis* homologue to the identified barley NAC transcription factor phenocopies the gene silencing effect on papillae formations in *Arabidopsis* towards *Bgh* penetration attempts, and the gene transcript accumulates in a similar temporal pattern. Genome-wide expression profiling and mutational analysis in *Arabidopsis* were assessed for the illumination of signalling cascades affected by the stable mutation of the NAC transcription factor upon *Bgh* inoculation. From these experimental approaches, it is concluded that changes in ABA biosynthesis and signalling in mutant plants may cause attenuation of the papilla-mediated resistance in *Arabidopsis*, and possibly in barley.

Overall, data from this study suggest that a nuclear-localized NAC transcription factor,

showing cross-species functional conservancy, positively regulates papilla-mediated penetration resistance upon *Bgh* inoculation. Regulatory targets of the NAC transcription factor are suggested, and indications of hormonal perturbations as a new and interesting component for effective papillae formations in plants upon fungal penetration attempts, are presented.

IV. Resumé

Levende organismer reagerer på miljøpåvirkninger ved hjælp af en række regulatoriske mekanismer. Da planter typisk ikke er mobile, er de i særlig grad nødsaget til at tilpasse sig (modsat flygte fra) det omkringliggende miljø, ved hjælp at deres forhåndenværende genetiske materiale. Udover at undergå en række fysiologiske og strukturelle forandringer for at imødegå miljøpåvirkninger, gør planter også brug af transkriptionel regulering ved aktivering og repressering af specifikke gener. For at forstå de molekylære mekanismer der påvirkes af bestemte miljøpåvirkninger, kan man derfor studere ændringer i geners transkript-mængder. Ud fra de enorme mængder af transkriberet sekvensmateriale der akkumuleres under forskellige miljømæssige påvirkninger, står forskere typisk overfor den udfordring det er, at funktionelt karakterisere disse transkripter. Ved hjælp af transkripternes sekvensinformation, kan der gøres brug af en række *reverse genetic*-tilgange til brug ved den funktionelle karakterisering, hvilket betyder at man har mulighed for at få et indblik i de molekylære aspekter af miljøafhængig regulering der bliver påvirket af de enkelte transkripter.

I denne afhandling har jeg taget udgangspunkt i den velstuderede interaktion mellem den biotrofe meldug svamp *Blumeria graminis* f.sp. *hordei* (*Bgh*) og dens værtsplante, byg (*Hordeum vulgare*), som model for funktionel karakterisering af gener der udtrykkes under meldug sygdommen. Mine studier har fokuseret på medlemmer af den plante-specifikke NAC transkriptionsfaktor familie, der oprindeligt blev identificeret i vores laboratorium fra meldug-inficerede byg planter. Ved brug af molekylærbiologiske metoder til slukning og over-udtrykkelse af individuelle NAC gener, præsenterer jeg herved identificeringen af en kernelokaliseret transkriptionsfaktor, der positivt regulerer dannelsen af et cellevægsassocieret forsvar, kaldet penetreringsresistens, for herved, under *Bgh* angreb, at udruste planten med et effektivt og økonomisk vigtigt forsvar mod meldug infektion. Transkript-mængden af det identificerede NAC gen ophobes under *Bgh* angreb i både inficeret og raskt væv. Både *Bgh*-associeret fænotype og transkript-mængde blev undersøgt i byg NAC genets nærmeste homolog i modelplanten *Arabidopsis thaliana*. Interessant nok, udviste *Arabidopsis* mutanten nedsat penetreringsresistens mod *Bgh*, identisk med fænotypen ved slukning af det homologe gen i byg planten. Derudover, var *Arabidopsis* genet også underlagt samme temporære transkript akkumulering under *Bgh* angreb, som det der blev fundet for byg homologen. Undersøgelse af samtlige *Arabidopsis* geners ekspressionsmønstre, samt uddybende eksperimentelle forsøg med vildtype og NAC mutant planter, blev udført for at belyse de signaleringsveje der bliver påvirket i mutanten under *Bgh* angreb. Ud fra disse tiltag, konkluderes det at mutant-specifikke ændringer i ABA biosyntese og signalering formentlig er medvirkende til at svække cellevægsassocieret forsvar mod *Bgh* i *Arabidopsis*, og muligvis byg.

Overordnet viser data indsamlet fra dette studie, at en funktionelt konserveret kernelokaliseret transkriptionsfaktor positivt regulerer penetreringsresistens som forsvar mod *Bgh*. Derudover foreslås en række regulatoriske mål for NAC transkriptionsfaktoren, og indikationer af hormonelle ændringer, som en ny og interessant komponent afgørende for effektivt cellevægsassocieret forsvar mod *Bgh*, bliver præsenteret.

V. Enclosed Manuscripts

This thesis is based on the following manuscripts, which will be referred to by their roman numerical (paper I-IV) throughout this thesis.

I Jensen, M.K., Rung, J.H., Gregersen, P.L., Fuglsang, A.T., Gjetting, T., Hansen, M., Fuglsang, A.T., Joehnk, N., Lyngkjaer, M.F. and Collinge, D.B.
The *Hv*NAC6 Transcription Factor: A Positive regulator of Penetration resistance in Barley and *Arabidopsis*.
Accepted for publication in *Plant Molecular Biology* on the 15th of June. See appendix VII[1]

II Jensen, M.K., Hagedorn,P., Rung, J.H., Collinge, D.B., and Lyngkjaer, M.F.
ATAF1 Negatively regulates Abscisic Acid Signaling for Efficient Penetration Resistance in *Arabidopsis* towards *Blumeria graminis* f.sp. *hordei*.
Manuscript in preparation

III Collinge, D.B., Jensen, M.K., Lyngkjaer, M.F., Rung, J.H.
How do we exploit functional genomics to understand the nature of plant defences? Barley as a case study.
Submitted for publication in *Eur. J. Plant Parthol*. Pending revision. See appendix VII

IV Moeller, A. B., Jakobsen, M. K., Jensen, M. K., Holm, P. B. and Palmgren, M. G.
The Barley Type V ATPase *Hv*P_5, a functional equivalent to *Spf1* and *MIA* secretory pathway pumps, is highly expressed during endosperm development.
Manuscript in preparation

1 The *Hv*NAC6 Transcription Factor: A Positive Regulator of Penetration Resistance in Barley and *Arabidopsis*.
DOI: 10.1007/s11103-007-9204-5

Contents

1

Introduction

1.1. Plants and Stress

Unlike animals, plants are sessile organisms, and therefore cannot move to escape environmental challenges. This obliges plants to perceive and respond to these challenges where they are, in order to successfully adapt and survive. The most important environmental constraints include abiotic stresses like drought, salt, extreme temperature and biotic stresses from attacking microbes. Such stresses impose a range of morphological, structural and molecular changes in the stressed plant, and most often the plant's response is a combination of induced adaptations, rather than a 'one-size-fits-all' approach. For instance, plants suffering from water deficiency readily shut down evaporation by closing their stomata to reduce transpiration rates, elicit processes related to seed maturation and reproduction, and systemic root-shoot signals effectuate the physiological adaptation necessary for increased tolerance in diverse tissues throughout the plant. Another example is the constant battle and evolutionary arms race between plants and pathogenic microbes. Besides using preformed 'hostile' structural barriers like cuticular waxes and glyco-proteinaceous polysaccharides, plants also rely on pathogen-induced morphological adaptations and biochemical constituents for its – usually successful – battle for resistance and survival. In all cases, plants, like animals, react to stress by consuming large quantities of energy. As a consequence, this energy can then no longer be used for vital physiological processes such as growth and carbon fixation in photosynthesis. This point is relevant in terms of plant production in an ever changing environment, and more information is needed to determine the nature of the components of adaptations towards environmental stresses.

This introduction presents approaches used for deciphering the complexity of molecular plant signalling events using functional genomic tools. Furthermore, it includes a detailed description of the biological models used in this thesis, and the regulatory genes of interest.

1.2. Functional Genomics in Plants

In my PhD studies, functional genomics approaches have been widely applied for the characterization of barley and *Arabidopsis* gene products of interest. This brief introduction to functional genomics tools in plants provides insight to the means by which researchers analyze gene functionality. Technologically, it encompasses many traditional molecular genetic and other biological approaches as well as the high-throughput approaches to whole genome or systems-oriented analyses. However, in this section special attention will be addressed transcriptomic approaches and modulation of expression by gene silencing and over-expression studies, as an introduction to the ditto applied experimental studies presented in this thesis.

1.2.1. How to infer functionality

Several plant genomes have been sequenced, including *Arabidopsis* (Tabata *et al.*, 2000), poplar (Kelleher *et al.*, 2007), and rice (Goff *et al.*, 2002; Yu *et al.*, 2002; Yuan *et al.*, 2005). Additionally, enormous amounts of expressed sequence tags (ESTs) and gene indices have been generated from more than 30 plant species including, soybean, sorghum and barley (see http://www.ncbi.nlm.nih.gov/dbEST/ and http://www.tigr.org). The challenge of the post-genome era of plant biology is to characterize the sequence data gathered in terms of function. In this respect, the term 'functional genomics' in its broadest sense refer to the discovery of the biological function of genes and how these genes and their products work together (Hieter and Boguski, 1997). For that purpose, parallel approaches need to be used for a thorough elucidation of the sometimes temporal and often redundant functions of individual genes in the complex sum of all gene activities in the plant cell (figure 1.1.) (Holtorf *et al.*, 2002).

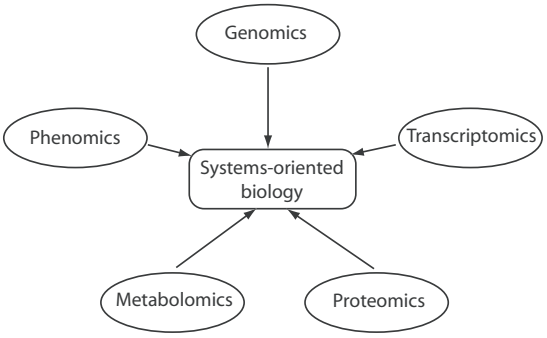


Figure 1.1. A diagrammatic presentation of functional genomics approaches.
Integration of several functional genomics technologies provides the opportunity for systems-oriented views of plant life. Figure adapted from Holtorf *et al.* (2002).

1.2.2. Transcriptomics – Gene Expression Profiling

Knowledge about a gene's function often relies on the analysis of spatial and temporal gene expression patterns (Zhang, 2003). The changes of individual mRNA steady state levels are mostly accomplished by changing the transcriptional rate of a gene, and are indicative of fluctuations in environmental and developmental conditions or responses to external and internal stimuli (Holtorf *et al.*, 2002). In order to understand gene function properly, it is of paramount importance to know when, where, and often to what extent genes are transcriptionally activated or repressed. Additionally, by putting the expression 'tag' of individual genes into the context of genome-wide changes in mRNA abundances, it is possible to infer co-regulations with the gene of interest, and over- and under-represented functional categories can be highlighted (Eisen *et al.*, 1998; Zhang, 1999). High-throughput and genome-wide analysis of differential gene expression using microarrays is a powerful tool for discovering new genes and for obtaining genome-scale information about biological processes in question (Lockhart *et al.*, 1996; Schena *et al.*, 1995). Hence, the primary goal of transcriptome analysis is to learn about how the large-scale changes in transcript abundances control growth and development of an organism and its response to the environment (Rhee et al., 2006).

Transcriptomics have greatly benefited from the development of microarrays. Microarray technology is generally based on one of two types of platforms. One contains a large number of relatively short (25-100-mer) probes synthesized directly on the surface of the arrays (for example Affymetrix GeneChip®, www.affymetrix.com), and other uses amplified PCR products or cloned cDNA fragments mechanically spotted directly on the array surface (Sreenivasulu *et al.*, 2002; Zierold *et al.*, 2005). By the use of such platforms, researchers have convincingly demonstrated how information from raw sequence data can be converted into a broad understanding of gene function by an unbiased data-driven approach (Delessert *et al.*, 2005; Leonhardt *et al.*, 2004). In this case, 'unbiased', refers to the fact, that working with large-scale data sets allows researchers unprecedented opportunities for the identification of over-represented elements or structures in collected data, even in the absence of working hypotheses. For instance, Leonhardt *et al.* (2004) applied single-cell microarray expression analyses of *Arabidopsis* guard cells for the identification and functional characterization of a ABA hypersensitive recessive protein phosphatase mutant, illustrating that the combination of guard cell expression data and functional genomic approaches can be used to identify gene function, without prior insight to the genes expressed in guard cells (Leonhardt *et al.*, 2004).

Though microarray analyses have proved their worth as a functional genomics tool, the simultaneous analysis of several thousand genes requires thorough planning of experiments, uniform and precise sampling or harvesting criteria, and high quality processing of extracted mRNA samples to ensure that biologically relevant information about the factor(s) in question are for obtained. For that reason, major efforts have been pursued for the easy comparison and interpretation of individual microarray experiments that can be verified

independently if needed (Brazma *et al.*, 2001; Spellman *et al.*, 2002), and standardized descriptions of experimental procedures in compliance with minimum information about microarray experiment (MIAME) are now mandatory prior to publication of research based on such data (Brazma *et al.*, 2006). Additionally, microarray analysis requires data processing to compensate for systematic biases (reviewed by Quackenbush, 2002), a topic covered thoroughly in Chapter 4.

1.2.3. Modulation of Gene Expression – Gene Silencing

A classical approach to elucidate gene functionality has been to knock-down gene expression for thereby identifying a phenotype indicative of gene functionality (Zhang, 2003). For this purpose, forward genetics have been performed by phenotypic screening of randomly mutagenized populations, perfectly exemplified from studies in search of components of plant defence and circadian rhythms (Collins *et al.*, 2003; Millar *et al.*, 1995). Once, an interesting phenotype is identified, relatively time-consuming positional cloning is needed to isolate the mutated gene (Jander *et al.*, 2002). Another forward approach includes insertional mutagenesis, which allow for a more rapid identification of insert positions, since the sequence of the inserted DNA is known (Sussman *et al.*, 2000). However, the large T-DNA insertion collections required to achieve good coverage of the genes in the genome constitutes a primary limitation to this procedure. Furthermore, the use of insertional mutagenesis is often limited by gene redundancy, lethal mutations, non-tagged mutants and the often "low-throughput" investigations to identify insert location in the genome (Matthew, 2004).

In contrast to insertional or chemically induced mutations, modulation of gene expression by RNA interference (RNAi) has the inherent advantage of enabling cost-effective targeted mutagenesis of genes of interest, circumventing screens for positional identification of mutational insertions (Chuang and Meyerowitz, 2000). RNAi is thought to be an ancestral defence mechanism against vira as well as a post-transcriptional endogenous regulating mechanism of gene expression, translation inhibition and RNA stability (figure 1.2.) (Brodersen and Voinnet, 2006; Waterhouse *et al.*, 2001). Applying RNAi for gene silencing involves the production of double-stranded RNA (dsRNA) homologues to the gene, or genes, of interest (Waterhouse *et al.*, 1998). This dsRNA can then endogenously be degraded into approx. 20-26-nucleotide small interfering RNA oligonucleotides (siRNAs), by the enzyme Dicer. The siRNAs will subsequently provide specificity to the endonuclease-containing, RNA-induced silencing complex (RISC), which targets homologues RNAs for degradation (recently reviewed in Brodersen and Voinnet, 2006). In addition to Dicer-derived siRNAs, plants and animals also encode small endogenous siRNA-like non-coding micro RNAs (miRNAs), inhibiting translation from complementing mRNA transcript sequences (Aukerman and Sakai, 2003).

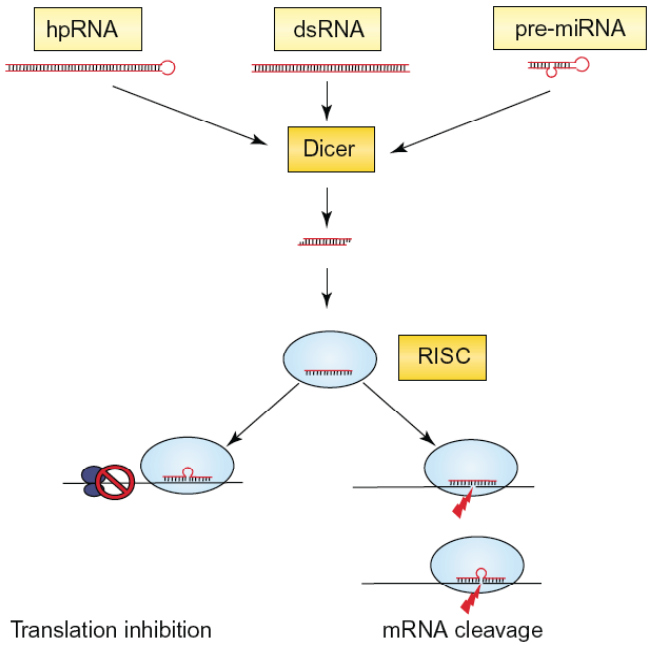


Figure 1.2. Schematic representation of part of the RNA interference cascade in plants.
Double stranded RNA (dsRNA) molecules are recognized in plants by a member of the RNase III family, Dicer, and digested into 20-26 nucleotide small interfering RNA fragments (siRNA). The siRNAs are unwound and one of the two strands is incorporated into the RNA-induced silencing complex (RISC). The antisense strand hybridizes to target mRNA and RISC cleaves the mRNA in close proximity of the centre of the siRNA. Furthermore, micro RNAs (miRNAs) can inhibit the translation by binding hybridizing to complementing mRNA sequences. Figure and text adapted from Kusaba (2004).

RNAi for mutational analyses in *Caenorhabditis elegans* has proved its worth as a precise high-throughput approach for investigating gene functions related to embryonic lethality, post-embryonic lethality, sterility, and morphological abnormality (Maeda *et al.*, 2001). Likewise, in plants, the use of dsRNA has been successfully applied for degradation of homologues RNAs with high specificity (Chuang and Meyerowitz, 2000; Di Serio *et al.*, 2001). Furthermore, it has proven ideal for silencing both individual and multiple members of large gene families, illustrated in the *Os*Rac gene family by Miki *et al.* (2005). In plants, this is of particular interest, as gene duplication events often account for the occurrence of large gene families with high sequence similarity and potential functional redundancy of individual family members (Itoh *et al.*, 2007).

Apart from the advantage to target predefined genomic locations, adequate design of vector constructs used for RNAi has added efficiency to this gene silencing approach (Miki and Shimamoto, 2004), even allowing the efficiency to be quantified at the single-cell level

(Panstruga *et al.*, 2003). Most notably, dsRNAs have been shown to be efficiently produced by intron-spliced hairpin transgenes (Smith *et al.*, 2000). Hence, high-throughput analysis of gene functions in barley have taken advantage of Invitrogenís Gateway cloning vectors (Hartley *et al.*, 2000) and cloning procedures for the easy production of gene silencing constructs harbouring inverted hairpin repeats (ihr) of individual clones of large cDNA libraries (Douchkov *et al.*, 2005). Subsequent delivery of dsRNA to plants can be achieved by (i) microprojectile-bombardment with silencing constructs coated onto particles for *in vivo* transcription, (ii) infiltration of plant tissue with an *Agrobacterium* strain carrying a T-DNA expressing an ihrRNA transgene, (iii) virus-induced gene silencing, and (iv) by stable transformation with ihrRNA expressing transgenes (Matthew, 2004).

Though advantages are obvious, limitations are present. Both stable RNAi-based gene silencing and insertional mutagenesis, rely on efficient plant transformation techniques. An additional limitation of RNAi is the requirement of sequence information from the target gene(s). However, with the increasing number of genome and EST sequencing projects, sequence data *per se* is becoming much less of a limitation (Matthew, 2004). Additionally, the lack of transformation protocols for stable transformation of ihrRNA transgenes disqualifies RNAi in many plant species as a functional genomics tool for generation of stable loss-of-function mutations. However, RNAi has been used widely in *Arabidopsis* and rice, and additionally for transient transformation of many crop plants (reviewed in Kusaba, 2004; and Schweizer *et al.,* 1999<<<), and this tool has offered great potential for functional genomics in recent years (Douchkov *et al.*, 2005; Miki *et al.*, 2005).

Finally, though gene silencing is considered a major component for functional genomics, and several T-DNA and TILLING mutant populations exist (Panstruga, 2004), far from all of them have been reported to present informative phenotypes that provide direct clues for the function of individual genes (reviewed in Bouche and Bouchez, 2001). Functional redundancy may explain the lack of phenotypic alterations in some cases, and additionally, it appears that many mutations are conditional and/or do not alter plant morphology or physiology (Bouche and Bouchez, 2001). Particularly, aspects of functional redundancy are relevant in terms of functional genomics approaches to transcription factors, because they in general belong to large gene families, and often multiple transcription factor genes in a single gene family need to be knocked out to produce informative phenotypes (Kumaran *et al.*, 2002; Liljegren *et al.*, 2000). Of course, pinning down the exact function of uncharacterized genes depends upon how closely, and under what conditions, mutants are examined. However, researchers still often face the need for complementing gene silencing with over-expression studies for thorough analysis of gene functionality (Stevenson *et al.*, 2001).

1.2.4. Modulation of Gene Expression – Over-Expression

With the complete genome sequences and full-length cDNA clones of several organisms, it is possible to complement gene silencing studies with over-expression studies. Especially in the case in redundant functions, over-expression can provide more 'visible' phenotypes compared to gene silencing approaches (Fan and Dong, 2002; Yu *et al.*, 2005).

One important issue regarding strategies for over-expression is the choice of promoter. Apart from ectopically expressing genes under the control of their native promoter (Murray *et al.*, 2003; Xu *et al.*, 2006; Yi *et al.*, 2006), several studies use a strong constitutive promoter, like the cauliflower mosaic virus 35*s* (CaMV 35*s*) promoter for over-expression of genes of interest (Gu *et al.*, 2002; Park *et al.*, 2001). Using this and related constitutive promoters, a wealth of studies have deciphered gene functionalities by the identification of hypermorphic alleles (Gattolin *et al.*, 2006; Zabala *et al.*, 2005, and for a review see Zhang, 2003). However, it should be noted that irrespective of which regulatory sequences are used for over-expression studies, it is interesting that some of the most important quantitative trait loci (QTLs) implicated in the domestication of crops are linked to changes in the expression pattern of regulatory genes, rather than to changes in their coding sequences (Wang *et al.*, 1999). This could be particularly relevant when studying tissue- or developmental-stage specific regulatory gene products.

Another important aspect when applying over-expression for the functional characterization of a gene of interest, relates to considerations of how ectopic expression is manifested. If putative functional redundancy is believed to shatter the biological effect of expression modulation, knowledge of domain modularity may be used. This could imply the ectopic expression of gain-of function or loss-of function regulatory domains of, for instance, transcription factors or kinases, thereby making them constitutively active or dominant-negative, respectively (Leiva-Neto *et al.*, 2004; Ori *et al.*, 2007).

1.2.5. Additional Functional Genomics Approaches

Before completing the section on functional genomics approaches for characterization of gene products, a few lines need to be added regarding disciplines expanding in the post-genomic era for a detailed functional characterization of genes and their potential regulatory targets (figure 1.1.). This includes areas of proteomics, phenomics and metabolomics, However, these approaches are not applied in the present PhD study for inferring gene functionality, and therefore they will only be mentioned briefly to acknowledge their potentials for integrated functional genomics analyses.

The proteome is defined as the entire complement of proteins in the cell(s) or organisms in question (Pardanani *et al.*, 2002). Hence proteomics is the study of this complement at a given environmental condition. Likewise, metabolomics and phenomics in a functional genomics context, refer to the large-scale analyses of metabolome and plant diversity, respectively, to infer gene functionality (Holtorf *et al.*, 2002). The goal of proteomics is a comprehensive, quantitative description of protein expression and changes under the influence of biological perturbations (Anderson and Anderson, 1998). As most proteins exert their functions through transient or stable interactions with other proteins, proteomics not only rely on quantitative parameters, but also on parameters of protein-protein interactions and protein modifications (Angers *et al.*, 2006; Benschop *et al.*, 2007). For that purpose, classical yeast-two hybrid, and the methods derived thereof, have been used to identify, mostly, binary interactions (Pandey and Mann, 2000; Uetz *et al.*, 2000), and mass spectrometry-based (MS) methods have been used for identifying members of entire protein complexes (Peltier *et al.*, 2001; Yamaguchi and Subramanian, 2003). Furthermore, high-scale bioimaging approaches have successfully elucidated the importance of the timely subcellular co-localization of proteins for enabling a perfectly orchestrated response to environmental stimuli (Shen *et al.*, 2007).

No matter what means are used for the identification of protein interacting components, the identification of proteins and their interactors form a central bridge between genes and metabolites in living organisms, and subsequent potential higher-level understanding of phenotypic measures and advances towards integrated functional genomics (Tian *et al.*, 2007). For this purpose, it should be mentioned that high-throughput metabolic profiling and protein characterizations using MS-based platforms, offers the advantage of analyzing functional entities, rather than mere transmitters of gene expression as mRNA. To this end, the potential to fully understand gene functionality includes information gathered from several of the mentioned global-scale analysis platforms serving as a basis for system-oriented biological understanding of relationship between mutant genotype and its respective phenotype.

1.3. Plant Defence towards Powdery Mildew Infection

Though in constant contact with potential pathogenic microbes, plants are resistant to the majority of them. This kind of plant disease resistance is called basal resistance or non-host resistance. The latter term reflects the notion that incompatibility is most often provoked by specialization of a pathogen species, *i.e.* pathogenicity factors, to a narrow host range (Heath, 1981).

Results gathered during my PhD studies show that basal resistance in barley (*Hordeum vulgare*) and *Arabidopsis* towards the grass powdery mildew fungus *Blumeria graminis* f.sp. *hordei* (*Bgh*), is regulated by a NAC transcription factor (section 1.4.). Therefore this section summarizes the physiological and molecular events underlying elements of basal and non-host resistance upon of *Bgh* attack. However, to bring basal resistance into context of plant resistance, race-specific resistance will also be covered in this section when relevant.

1.3.1. The Morphological Characteristics of the Barley-*Bgh* Interaction

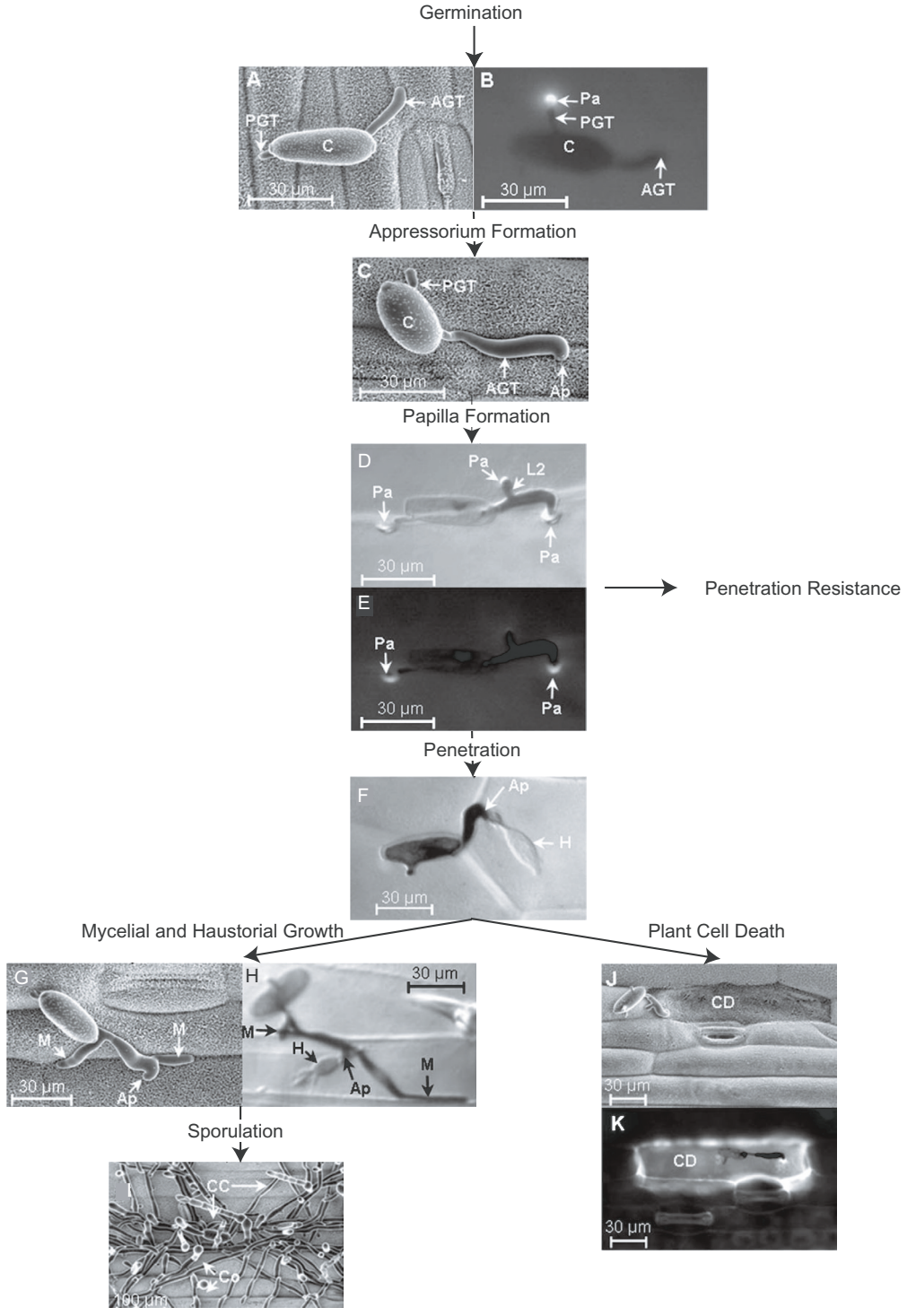
Bgh is the causal agent of powdery mildew; one of the most important and devastating diseases of barley worldwide. Thus, the barley-*Bgh* interaction has been widely investigated for understanding the bases of plant responses towards pathogen attack.

Before continuing with the outline of *Bgh* development a few general points regarding *Bgh*'s life strategy deserve to be addressed. Firstly, *Bgh* spores are wind-dispersed and, being obligate biotrophs, fungal development relies on uptake of nutrients from living host plants. This implies the need for *Bgh* germlings to breach the cell wall for successful reproduction. Secondly, upon successful ingress, key features of the pathogen is that its growth is restricted to the plant epidermis and *Bgh* germling development follows an ordered morphogenetic sequence (Green *et al.*, 2002; Prats *et al.*, 2006; Zimmerli *et al.*, 2004).

Within minutes after contact with barley leaves, *Bgh* conidia releases an extracellular matrix presumably involved in the directional growth of germ tubes (Carver *et al.*, 1999). The first germ tube to be established is the primary germ tube (PGT, figures 1.3A. and 1.3B.) emerging from the conidia approx. one hour after inoculation (hai), which is rapidly followed by formation of a short peg that only penetrates the cuticle (Edwards, 2002). Soon after a second germ tube, the appressorial germ tube (AGT; figures 1.3A. and 1.3B.), emerges. This elongates and differentiates a lobed, apical appresorium by ~ 10 hai (figure 1.3C.). A penetration peg then emerges from beneath the appressorial lobe (~ 10-12 hai) and attempts to breach the cuticle and epidermal cell wall to establish infection. At this point,

subsequent developmental outcome depends on the efficiency of the host response. In the barley-*Bgh* interaction, basal resistance is conferred by cell wall modifications, including papillae formations that effectively accumulate in the apoplast, thereby preventing *Bgh* germlings from entering the epidermal cells. Papillae are chemically complex appositions deposited in the apoplasm between the cell wall and plasma membrane underneath the attempted penetration site of attacked epidermal cells (Schulze-Lefert, 2004). Their formation involves the early generation of nitric oxide (NO) (Prats *et al.*, 2005) and hydrogen peroxide (H_2O_2) (Thordal-Christensen *et al.*, 1997), and they are comprised of callose, H_2O_2, and autofluorogenic phenolics (figures 1.3B. and 1.3E.)(Collins *et al.*, 2003; Hückelhoven *et al.*, 1999; Thordal-Christensen *et al.*, 1997). Furthermore, effective papilla-mediated resistance evokes cytoskeletal rearrangement (Kobayashi *et al.*, 1997; Opalski *et al.*, 2005) and redirected cytoplasmic streaming and aggregation (Zeyen *et al.*, 2002). Both the reorganization of cytoskeleton and cytoplasm are believed to be important events directing vesicles containing papillae components to the site of attempted penetration (An *et al.*, 2006; Collins *et al.*, 2003). The cell wall changes involved in basal resistance are induced and active against both virulent and avirulent *Bgh* genotypes. Effective papillae arrest the protruding penetration pegs of *Bgh* germlings, and thereby subsequent invasive growth, and are often referred to as the first line of defence (Heitefuss, 2001; Schulze-Lefert, 2004). However, the efficiency of basal resistance varies, and some *Bgh* penetration attempts will always succeed. In such cases the ineffective papilla becomes a 'collar' for the neck of the haustorium (figure 1.3F.). Though both the nature of origin and composition of papillae is multi-faceted, an unusual case of papilla-mediated resistance arises from recessive alleles of *MLO* (*mlo*) (*mildew resistance locus O*) conferring effective papilla-mediated penetration resistance against virtually all tested *Bgh* isolates (section 1.3.5.) (Jørgensen, 1992).

Figure 1.3. *Bgh* development and barley responses.
Cryo-SEM, transmitted light, and fluorescence (blue light excitation) micrographs of key stages in *Bgh* development and barley host cell responses. (**A-C**) During the first ~ 10 hours after infection (hai) no changes in the fungal development are observed when comparing virulent and avirulent *Bgh* attacks. Germling morphological development starts by emergence of a primary germ tube (PGT) from the conidia (C), followed by the protrusion of an appressorial germ tube (AGT). Autofluorogenic material accumulates within a small papilla (Pa) deposited beneath the site of PGT contact (**A**). (**C**) By ~ 10 hai the appressorium (Ap) has differentiated into a hooked apical lobe. (**D-E**) Papillae formations comprise the first line of defence towards both virulent and avirulent *Bgh*. (**D**) Transmitted light and (**E**) fluorescence images of a germling failing penetration from its first formed appressorial lobe, leading to formation of a secondary lobe (L2). Successful penetrations from secondary lobes are rarely observed. (**F**) In contrast to effective papilla-mediated penetration resistance 'locking' progression of the penetration peg, a penetration peg penetrates the cell wall beneath the appressorial peg (Ap) and develops into a haustorium (H). (**J-K**) The second line of defence, the race-specific resistance responses, as of 30 hai. In the avirulent attack, epidermal cell death (CD) occurs as a result of single gene-controlled hypersensitivity (HR) preventing further *Bgh* growth. This occurs at a much lower frequency in virulent *Bgh*-barley interactions. (**J**) SEM and fluorescence microscopy (**K**) showing the collapsed and autofluorogenic dead cells, respectively. (**G-I**) SEM (**G**) and (**H**) light microscopy images of developing colonies 30 hai. By this time mycelium (M) growth is emerging from AGT as H develops a digitate process from each end of its central body inside the cell. (**I**) Ninety-six hai, M growth is extensive and repeated penetration from appressoria results in more haustoria being developed. Finally, conidiophores (Co) generate chains of next-generation conidia (CC) for wind dispersal. Figure and text adapted from Prats *et al.* (2006).


Germination
A
AGT
PGT
C
30 µm
B
Pa
PGT
C
30 µm
AGT
Appressorium Formation
C
PGT
C
30 µm
AGT
Ap
Papilla Formation
D
Pa
L2
Pa
Pa
30 µm
E
Pa
Pa
30 µm
Penetration Resistance
Penetration
F
Ap
H
30 µm
Mycelial and Haustorial Growth
Plant Cell Death
G
M
M
30 µm
Ap
H
30 µm
M
H
M
Ap
J
CD
30 µm
K
CD
30 µm
Sporulation
CC
Co
100 µm

In 50-70% of the interaction attempts on susceptible host plants, penetration resistance fails to arrest the fungus breaching the cell wall and the penetration peg swells within the penetrated epidermal host cell (12-15 hai). The swelled structure differentiates into a haustorium (figure 1.3H.) that develops into numerous digitate processes over the next 4-5 days. The haustorium absorbs nutrients from the penetrated cell, and thereby enables ectophytic mycelia development (figures 1.3H. and 1.3I.) from which subsequent generations of haustoria are formed and conidia (figure 1.3I.) are produced (from 4 days after inoculation).

The second line of single-cell defence responses is associated with enhanced epidermal cell death, and this occurs much more frequently in resistant compared with susceptible barley genotypes (Trujillo *et al.*, 2004b). In the avirulent scenario, cell death results from a race-specific, single gene-controlled hypersensitive response (HR) preventing nutrient flow to the fungus (figures 1.3J.-K.). Among the genes controlling this second line of defence are the alleles at the *Mla* locus. In *Mla1* barley, a localized HR is elicited soon after a haustorium forms within epidermal cells attacked by an avirulent *Bgh* isolate. The first signs of HR are H^+ and Ca^{2+} effluxes from the apoplast (12-24 hai, (Felle *et al.*, 2004)) and, within the attacked cell, generation of NO (Prats *et al.*, 2005) and H_2O_2 (Hückelhoven and Kogel, 2003; Thordal-Christensen *et al.*, 1997). Subsequently, the whole cell becomes autofluorogenic (figure 1.3K., Vanacker *et al.*, 2000) as phenolic compounds accumulate. Following HR of the successfully penetrated epidermal cell, further development of the *Bgh* germling comes to a complete arrest. Hence, HR can be referred to as a back-up defence which comes into play when the fungus has accomplished breaching of both cell wall and papilla (Heitefuss, 2001; Thordal-Christensen, 2003).

To sum-up, host-cell entry represents a critical step during pathogenesis of both virulent and avirulent *Bgh* on barley (Lipka *et al.*, 2005). Hence, even in compatible barley-*Bgh* interactions, some epidermal leaf cells resist penetration by papillae formations. However, upon successful penetration, post-invasive race-specific resistance is decisive to avirulent fungal progression (Schulze-Lefert, 2004).

1.3.2. The *Arabidopsis-Bgh* Interaction

Arabidopsis is a non-host for *Bgh*. Non-host refers to the incompatibility between an entire plant species and all isolates of a microbe species, rendering attacking microbes disabled in regeneration upon attempting infection of a non-host plant (Thordal-Christensen, 2003).

In the *Arabidopsis-Bgh* interaction (figure 1.4.), the conidia will germinate and produce both PGT and AGT (figure 1.4A.). The fungus will try to breach the cell wall to gain entry to epidermal cell nutrients as in the compatible barley-*Bgh* interaction. However, the majority

(approx. 90-95%) of the *Bgh* germlings fails to overcome basal penetration resistance and fungal growth is therefore terminated during penetration attempts (figure 1.4A.). Thus, a block at the penetration step, during the transition from surface to invasive growth, is the most prevalent form of resistance towards *Bgh* in *Arabidopsis* (Zimmerli *et al.*, 2004). Following the rare events of successful penetration, *Bgh* spores may establish a haustorium inside the epidermal cells (~ 24 hai)(figure 1.4B.). Additionally, *Bgh* germlings overcoming penetration resistance can exhibit a limited secondary hyphal growth on the leaf surface by 48 hai, indicating a functional feeding structure. In all cases, however, successfully penetrated cells will accumulate fluorescent compounds and undergo HR (figures 1.4C. and 1.4D.) (Lipka *et al.*, 2005; Zimmerli *et al.*, 2004).

1.3.3. Morphological Overlap in Host and Non-host Responses

Taken together, fungal infection and morphological plant defensive manifestations observed in *Bgh* interactions with *Arabidopsis* overlap those observed with interactions in barley. Hence, basal defensive reactions such as cell wall appositions, HR, and accumulation of phenolic compounds in the attacked cell, also referred to as race non-specific resistance, is present in both host and non-host interactions (Carver *et al.*, 1992; Zimmerli *et al.*, 2004). However, though overlapping, quantitative differences when comparing the morphological outcome of the two outlined host and non-host responses are evident. In barley, virulent *Bgh* penetrates epidermal cells and produce a haustorium in approximately 50-70% of the cases, whereas *Bgh* development on *Arabidopsis* will be arrested at the step of penetration in approx. 90% of the cases. Furthermore, *Bgh* spores never penetrate more than one *Arabidopsis* epidermal cell, as observed in the host interaction and, finally, asexual reproduction is never observed on *Arabidopsis* (Zimmerli *et al.*, 2004).

Finally, non-host resistance is believed to be broad spectrum and durable under field conditions (Heath, 2000), and understanding the regulations conferring non-host resistance is therefore of potential agro-economical interest (Thordal-Christensen, 2003; Trujillo *et al.*, 2004a). Indeed, acknowledging the overlap of basal and non-host resistance has stimulated research aiming to understand this important line of defence. Particularly, the incorporation of the *Arabidopsis-Bgh* interaction as a competent reference patho-system has accelerated our understanding of the molecular aspects of basal resistance (sections 1.3.4. and 1.3.5.)

1.3.4. Molecular Events Underlying Penetration Resistance

In plants, much research has focused on the gene-for-gene resistance which render a plant cultivar carrying a specific resistance gene, resistant to a specific race of pathogen carrying a

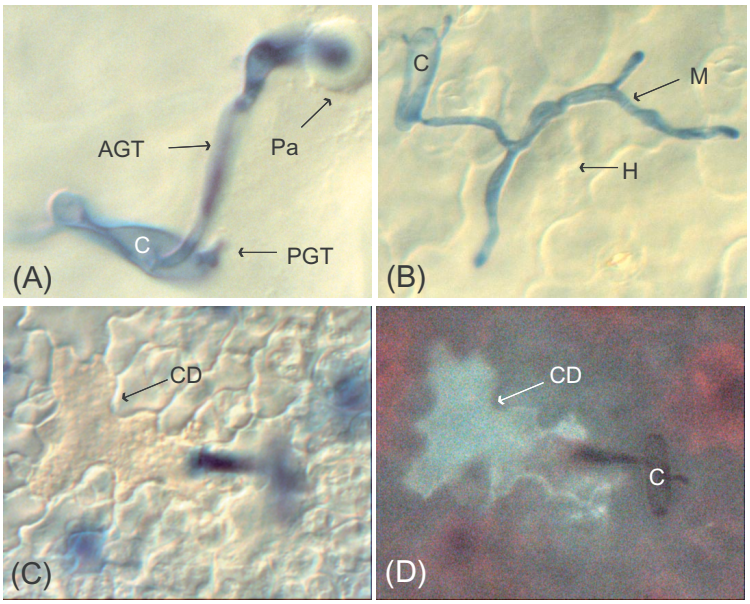


Figure 1.4. The *Arabidopsis-Bgh* interaction.
(**A**) Upon contact with the *Arabidopsis* leaf surface, *Bgh* conidia (C) will germinate within ~ 10 hours after inoculation (hai) and produce a primary germ tube (PGT), followed by an appressorial germ tube (AGT). The most frequent outcome of *Bgh* conidia attempts to infect *Arabidopsis* epidermal cells, is the formation of an effective papilla (Pa) beneath the site of attempted penetration. (**B**) Successful penetration attempts (5-10%) will enable *Bgh* germlings to produce a haustorium (H) inside the epidermal cell, and even sometimes subsequent ectophytic mycelial (M) growth. (**C**, **D**) In all cases, the *Bgh* germling development will be arrested by a post-invasive hypersensitive response leading to cell death (CD). This figure is also presented in paper I.

specific avirulence gene (Ellis *et al.*, 2000; Flor, 1971). This specific recognition observed in plants is a parallel to the adaptive immune system in animals, which generates specialized antibodies and T-cell receptors upon antigen perception (Gomez-Gomez and Boller, 2002). In both animals and plants this usually leads to immunity; in plants termed the HR (Hammond-Kosack and Jones, 1996). Apart from the adaptive immunity, plants have the capability to perceive potentially pathogenic microbes in a more general way through so-called pathogen-recognition receptors (PRRs) that recognize general pathogen-associated molecular patterns (PAMPs), like lipopolysaccharides, flagellins, glucans and chitins, hereby evoking basal resistance (Nurnberger and Kemmerling, 2006). This kind of perception of 'non-self' (Boller, 1995) is similar to what has been termed 'innate immunity' in the animal field (Kimbrell and Beutler, 2001). This section will cover some of the molecular mechanisms underlying penetration resistance in barley and *Arabidopsis*, an often very efficient resistance 'product' of plant basal resistance or innate immunity.

A recent series of publications have significantly improved our understanding of the molecular aspects of penetration resistance to mildews (Collins *et al.*, 2003; Consonni *et al.*, 2006; Gjetting *et al.*, 2004; Lipka *et al.*, 2005; Stein *et al.*, 2006). With respect to penetration resistance, the *Arabidopsis-Bgh* interaction is particularly well suited because of its level of penetration resistant cell (~ 90%) (Zimmerli *et al.*, 2004). Moreover, at least two species of powdery mildew that cause disease on *Arabidopsis* are known; *Golovinomyces orontii* and *Golovinomyces cichoracearum* (Consonni *et al.*, 2006; Vogel *et al.*, 2002), allowing for easy comparisons between host and non-host responses (Zimmerli *et al.*, 2004). Moreover, the identification of barley and *Arabidopsis MLO* genes and their mutant alleles have greatly accelerated our understanding of the molecular mechanisms of penetration resistance (section 1.3.5.) (Collins *et al.*, 2003; Consonni *et al.*, 2006).

1.3.5. The Genes

Being a biotrophic fungus, the survival and reproduction of *Bgh* relies on nutrient uptake from host plants. However, to succeed in this, a transition from extra-cellular to invasive growth is needed, which is accommodated by penetration of the plant cell wall (Schulze-Lefert, 2004). Molecular insights to the mechanisms conferring penetration resistance have greatly benefited from the barley *MLO* gene isolated by Büschges *et al.* (1997). The gene encodes a protein with 7 transmembrane domains reminiscent of a G-coupled receptor (Buschges *et al.*, 1997). MLO functions in susceptibility to *Bgh* via binding of calmodulin (Bhat *et al.*, 2005; Kim *et al.*, 2002). Though detailed molecular evidences for MLO functionality upon *Bgh* attack are scarce, it is generally believed that MLO is a susceptibility factor (ab)used by *Bgh* to gain entry to the host epidermal cell (Schulze-Lefert and Panstruga, 2003). In barley, recessive loss-of-function alleles of the *MLO* locus are known to mediate durable and broad-spectrum resistance towards *Bgh* by effective penetration resistance (Jørgensen, 1992), whereas over-expression of *MLO* confers super-susceptibility, rendering essentially all attacked barley epidermal cells deficient in effective penetration resistance (Kim *et al.*, 2002). All tested *mlo* alleles analyzed stop fungal growth at the same developmental stage within the papilla directly beneath the site of abortive fungal penetration (Wolter *et al.*, 1993). The resistance mediated by *mlo* appears to be expressed in a single-cell autonomous manner, as concluded from single-cell transient *MLO* over-expression studies in *mlo*-resistant barley leaves (Shirasu *et al.*, 1999) and transient single-cell *MLO* gene silencing experiments in susceptible barley leaves carrying wild-type *MLO* (Schweizer *et al.*, 2000). In *Arabidopsis,* non-host resistance to *Bgh* is reminiscent of *mlo*-mediated resistance; almost all penetration attempts fail (section 1.3.2.). Interestingly, the barley *mlo*-phenotype upon *Bgh* inoculation is mimicked in *Arabidopsis AtMLO2*-mutants (Consonni *et al.*, 2006), demonstrating that MLO is a *Bgh* susceptibility factor both in monocots and dicots. In barley, *mlo*-mediated penetration resistance is 100% effective (Piffanelli *et al.*, 2004), whereas in *Arabidopsis* triple knock-out lines of the three closest barley *MLO* homologues; *AtMLO2*, *AtMLO6* and

AtMLO12, was required for complete penetration resistance to the virulent host fungus *G. orontii* (Consonni *et al.*, 2006).

The *mlo* alleles have controlled mildew resistance in the majority of cultivated European spring barley elite varieties for approximately three decades (Consonni *et al.*, 2006; Piffanelli *et al.*, 2004). Though *mlo* barley and *Arabidopsis* plants show developmentally controlled pleiotropic effects like spontaneous callose deposition and mesophyll cell death (Consonni *et al.*, 2006; Wolter *et al.*, 1993), Consonni *et al.* demonstrated that the *mlo* disease resistance phenotype can be uncoupled from the unwanted pleitropic effects by mutations in the salicylic acid signalling pathway (Consonni *et al.*, 2006). In barley, papilla-mediated resistance towards *Bgh* is believed to be SA-independent (Hückelhoven *et al.*, 1999). However, in *Arabidopsis*, SA signalling mutants have enhanced susceptibility to other biotrophic pathogens (Glazebrook, 2005). Additionally, in barley, *mlo* mutant alleles mediate super-susceptibility against the hemibiotrophic rice blast fungus *Magnaporthe grisea* and the necrotrophic fungus *Bipolaris sorokiniana* (Jarosch *et al.*, 1999; Jørgensen, 1977; Kumar *et al.*, 2001), calling for improved understanding of the molecular aspects underlying this durable and broad-spectrum type of resistance to enable agronomically beneficial traits.

The identification of the *mlo* phenotype has proved important for thorough understanding of basal and non-host resistance. So how does *MLO* modulate defence responses to *Bgh*? In barley, *mlo*-mediated penetration resistance to *Bgh* is dependent on *ROR1* and *ROR2* (*required for mlo-specified resistance 1,2*) (Collins *et al.*, 2003; Freialdenhoven *et al.*, 1996). Though not inferred from their full names, *ROR1* and *ROR2* are additionally required for basal penetration resistance and non-host resistance (see below) (Collins *et al.*, 2003). Mutation of *ROR* genes suppresses *mlo*-mediated penetration resistance and *MLO* suppresses *ROR2* dependent penetration resistance (Collins *et al.*, 2003; Freialdenhoven *et al.*, 1996). Hence, as null-*mlo ror* genotypes compromises penetration resistance in barley towards *Bgh* compared to *mlo* single mutant genotypes, it is suggested that MLO is not simply a susceptibility factor needed by *Bgh* to recognize its host, and possibly non-host, but rather a negative regulator of basal resistance responses triggered by *Bgh* (Collins *et al.*, 2003; Freialdenhoven *et al.*, 1996). *ROR2* and *ROR1* on the other hand, are believed to encode resistance factors required not only for *mlo*-mediated resistance and basal resistance to *Bgh*, but also non-host resistance (see below) (Collins *et al.*, 2003; Freialdenhoven *et al.*, 1996; Trujillo *et al.*, 2004b). The *ROR* genes are not required for race-specific resistance (Peterhansel *et al.*, 1997). Interestingly, the *Arabidopsis* orthologue of *ROR2*, *PEN1* (*PENETRATION1*), isolated by map-based cloning, is required for limiting non-host pathogen *Bgh* and host pathogen *G. cichoracearum* entry to the *Arabidopsis* plant epidermal cells (Collins *et al.*, 2003; Consonni *et al.*, 2006), highlighting the conservancy of additional signalling components of immunity for limiting this important step of fungi development and subsequent pathogenicity. *ROR2* and *PEN1* encode functional homologues syntaxins, members of the SNARE superfamily (Collins *et al.*, 2003). SNARE (soluble N-ethylmaleimide-sensitive-factor association protein receptor) proteins are conserved in eukaryotic cells and necessary for most membrane-fusion events

(Battey *et al.*, 1999; Schulze-Lefert, 2004). Both ROR2 and PEN1 become concentrated in plasma membrane microdomains at attempted sites of fungal penetration (Assaad *et al.*, 2004; Bhat *et al.*, 2005). Because SNARE proteins play a key role in vesicle trafficking in eukaryotic cells (Bonifacino and Glick, 2004), these findings provoke the assumption of the existence of a vesicle-associated resistance mechanism preventing *Bgh* ingress reminiscent of lipid rafts and/or exosomes induced upon attempted entry of pathogenic bacteria in animal cells (An *et al.*, 2006; Bhat *et al.*, 2005). It is also speculated that these vesicles allow the transport of building blocks to be readily assembled into papillae and antifungal compounds to be discharged against fungal penetration (An *et al.*, 2006). In this respect, it is interesting that actin filaments and microtubules are polarized and direct cytoplasmic aggregates towards the fungal pentration site (Opalski *et al.*, 2005; Shimada *et al.*, 2006). Actin filaments play a central role in trafficking of vesicles and endosomes in the plant cell (Battey *et al.*, 1999).

Knowledge of typical SNARE interactors upon membrane fusion events (Jahn *et al.*, 2003), prompted Collins *et al.* to identify other factors required for basal penetration resistance towards *Bgh* in barley, possibly related to membrane fusion events (Collins *et al.*, 2003). In addition to PEN1 and ROR2, they isolated a SNAP-25 (SNARE-assocoaited protein of 25 kDa) protein barley homologue required for full resistance, named *Hv*SNAP34 (Collins *et al.*, 2003). *Hv*SNAP34 interacts with ROR2, and this interaction is enhanced when a 31 amino acid deletion of a central cytosolic a-helix was deleted (ROR2Δ31) (Collins *et al.*, 2003). Additionally, over-expression of *ROR2Δ31* in *mlo* plants carrying a wild-type *ROR2* allele acted as a potent inhibitor of penetration resistance (Collins *et al.*, 2003). Overall, it is hypothesized that ROR2 and PEN1 mediate penetration resistance by their participation in SNARE complexes acquired for a vesicle-mediated secretory pathway (Collins *et al.*, 2003; Shimada *et al.*, 2006). Hence, ROR2 interaction with *Hv*SNAP34 constitutes a transient binary intermediate in the assembly of ternary SNARE complexes, acquired through the interaction with a vesicle-associated R-SNARE component (Collins *et al.*, 2003). The ROR2Δ31-enhanced binding to *Hv*SNAP34 is then supposed to hijack ROR2 interacting partners into non-functional complexes. Furthermore, MLO is hypothesized to sequester ROR2/PEN1 syntaxins with *Hv*SNAP34 in inactive complexes, thereby inhibiting ternary SNARE complex formation (Panstruga, unpublished). In this case, the stronger interaction between *Hv*SNAP34 and the truncated ROR2 version (ROR2Δ31) compared to wild-type ROR2, could explain the observed super-susceptibility phenotype associated with *ror2* mutants in wild-type *MLO* backgrounds (Collins *et al.*, 2003; Schulze-Lefert and Panstruga, 2003). Future studies on the cargo of the vesicles and the last component of ternary SNARE complexes required for exocytosis of vesicle cargo await further characterization for additional insights to the means by which *ROR2* and *PEN1* restrict *Bgh* entry to epidermal cells.

Lastly, underscoring the topic on penetration resistance, it should be mentioned that further *Bgh* growth is abolished in both *Arabidopsis* wild-type and *pen* mutants, coincident with cell death of attacked and penetrated epidermal cells (Collins *et al.*, 2003).

In addition to *PEN1*, the *PEN2* and *PEN3* genes limit fungal ingress in *Arabidopsis* towards non-host powdery mildew fungi (Lipka *et al.*, 2005; Stein *et al.*, 2006). *PEN2* and *PEN3* encode a glycosyl hydrolase and an ABC transporter, respectively (Lipka *et al.*, 2005; Stein *et al.*, 2006), and as *Atmlo2 pen2* and *Atmlo2 pen3* double mutants allow conidiation of host pathogen *G. cichoracearum* at a higher frequency compared to *Atmlo2* plants, it is believed that these two players of penetration resistance may have additional roles in post-invasive host defences (Consonni *et al.*, 2006). Post-invasive defence was not affected in the *Atmlo2 pen1* double mutant compared to *Atmlo2*, and the fact that *pen3* is epistatic to *pen2* in at least one assay (Stein *et al.*, 2006), indicates that PEN2 and PEN3 may act together in a defensive signalling pathway distinct from PEN1 (Consonni *et al.*, 2006; Ellis, 2006), possibly through active export of toxic material to locations of both pre- and post-invasive defences (Consonni *et al.*, 2006; Stein *et al.*, 2006).

Though not much is known about the regulatory aspects of penetration resistance, Shen *et al.* (2007) recently discovered that the barley *Hv*WRKY1 and *Hv*WRKY2 transcription factors are sequestered to an MLA immune receptor, thereby relieving repression of penetration resistance, upon virulent *Bgh* inoculation (Shen *et al.*, 2007). Mutant lines of the closest homologues in *Arabidopsis* agreed with the *HvWRKY1/2* gene silencing phenotypes, producing less successful penetration attempts compared to wild-type (Shen *et al.*, 2007). Another *Hv*WRKY transcription factor have also been shown to repress penetration resistance (Eckey *et al.*, 2004). However, the super-susceptible phenotype is believed to be WRKY-domain-independent as the *Hv*WRKY transcription factor SUSIBA2 do not alter *Bgh* infection type (Shen *et al.*, 2007).

Finally, to sum up the content of this section (1.3.5.), several genes, of both host and non-host plants, have been identified to be associated with basal penetration resistance towards *Bgh*. Most importantly, loss-of-function *mlo* mutant alleles (Buschges *et al.*, 1997), have provided a breaking tool for the identification of additional components of the multi-layered basal and non-host resistance (Collins *et al.*, 2003; Consonni *et al.*, 2006; Freialdenhoven *et al.*, 1996). Apart from the negative regulation of basal penetration resistance mediated by MLO and members of the WRKY transcription factor family (Buschges *et al.*, 1997; Consonni *et al.*, 2006; Shen *et al.*, 2007), crucial components encaged in vesicle trafficking for effective papilla formations, like ROR2/PEN1, PEN2, PEN3 and the barley SNAP-25 homologue *Hv*SNAP34, have been identified (Collins *et al.*, 2003; Consonni *et al.*, 2006; Douchkov *et al.*, 2005; Freialdenhoven *et al.*, 1996; Lipka *et al.*, 2005; Stein *et al.*, 2006).

1.3.6. The Transcripts

At the transcript level, basal resistance is characterized by the accumulation of defence-related transcripts and accumulation of phenolic compounds (Röpenack et al., 1998; Shirasu & Shultze-lefert 2000; Piffanelli et al., 2002, Gregersen et al., 1997). Futhermore, Zierold *et al.* (2005), have performed wild-type and *mlo* epidermis-specific gene expression profiling of barley attacked by powdery mildew for identification of candidate genes for mediators and effectors of *mlo*-mediated resistance in barley (Zierold *et al.*, 2005). Based on the transcript profiles of 293 significantly differentially regulated genes between *mlo* and wildtype *Bgh* inoculated plants (http://pgrc.ipk-gatersleben.de/epidermis_mlo), it was evident that by far the most prominent changes observed between the two genotypes were of quantitative nature (Zierold *et al.*, 2005). Hence, both the overall amplitude of gene induction and repression was higher in *mlo* plants compared to wildtype plants undergoing basal resistance (Zierold *et al.*, 2005). Additionally, Caldo *et al.* (2004) have described the interaction-dependent temporal gene expression of 22.798 host genes towards virulent and avirulent *Bgh* inoculation (Caldo *et al.*, 2004). Here they found that no significant changes in the transcriptome between compatible and incompatible interactions were observed until 16 hai. However, from 16 to 32 hai, the majority of significantly differentially expressed genes in the compatible interaction were suppressed (Caldo *et al.*, 2004). Therefore, it is speculated that general elicitors (PAMPs) evoke identical immediate host responses at the transcript level, irrespective of *Bgh* virulence, and that race-specific resistance evolved from the recognition and prevention of pathogen-induced suppression of host genes required for basal resistance (Caldo *et al.*, 2004; Zierold *et al.*, 2005). In contrast, virulent *Bgh* is believed to escape the plant's defence system by avoid recognition and/or suppress pre- and post-invasive defences (Eichmann *et al.*, 2006; Schulze-Lefert and Panstruga, 2003). Hence, basal resistance in appropriate compatible interactions and non-host interactions require common genetic and mechanistic elements of plant defence – even the transcriptome only undergoes slight quantitative changes at early stages of pathogen attack (Caldo *et al.*, 2004; Eichmann *et al.*, 2006; Zierold *et al.*, 2005). This supports other observations on resistance being regulated by shifts in the balance among defence mechanisms, and by quantitative and/or kinetic enhancements that make the defence response more effective (Katagiri, 2004).

Although several studies have addressed the question of how penetration resistance is regulated in both *mlo* and wild-type plants at the transcript level, it is only recently that data have provided the first evidence for gene regulation associated specifically with effective papilla-mediated resistance expressed in susceptible wild-type barley leaves (Gjetting *et al.*, 2007). As stated in section 1.3.1., even compatible interactions display a certain degree of papilla-mediated resistance. Therefore, it is difficult to relate 'total leaf-' or even epidermis-specific transcript profiles of *MLO*-based barley-*Bgh* interactions to resistance or susceptibility scenarios. By capillary extraction of single-cell mRNA, Gjetting *et al.* (2007) provide insights to the transcriptome of papilla-mediated *Bgh* resistant cells compared to both susceptible (infected) and non-attacked cells of the same inoculated leaf (Gjetting *et*

al., 2007). Most notably, the authors confirmed assumptions on the cytoplasmic vesicle transport underlying papilla-mediated penetration resistance, by highlighting the specific up-regulation of a member of exocyst complexes in papilla resistant cells, and additionally identified genes encoding peroxidases and germin-like proteins to be up-regulated, providing further proof on the notion of H_2O_2 production (section 1.3.2.) at attempted attack sites, possibly hampering *Bgh* virulence (Gjetting *et al.*, 2007).

1.3.7. The Hormones

Briefly, though it has been inferred that penetration resistance is largely salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) independent (Consonni *et al.*, 2006; Hückelhoven and Kogel, 2003), phytohormone levels influence the outcome of many plant-pathogen interactions (Glazebrook, 2005). Apart from these three former mentioned phytohormones, increasing evidence associates abscisic acid (ABA) in plant defence modulation (Mauch-Mani and Mauch, 2005). Most studies have shown that enhanced ABA levels correlates with increased susceptibility (Audenaert *et al.*, 2002; Edwards, 1983; Mohr and Cahill, 2003; Torres-Zabala *et al.*, 2007). Specifically a few points argue for ABA involvement in penetration resistance. Firstly, ABA treatment has been shown to inhibit the activity and transcript accumulation of the phenylalanine ammonium lyase (PAL) encoding gene (Ward *et al.*, 1989). PAL is the entry-point enzyme to phenylpropanoid biosynthesis (Shadle *et al.*, 2003), and much physiological evidence indicates that phenylpropanoids related to lignin biosynthesis are associated with effective papillae (Zeyen *et al.*, 2002). Interestingly, PAL mRNA levels have been shown to be specifically up-regulated in papilla-mediated penetration resistant cells (Gjetting *et al.*, 2004; Gjetting *et al.*, 2007). Secondly, exogenously application of ABA prior to pathogen inoculation has been shown to increase susceptibility of host plants (Audenaert *et al.*, 2002; Mohr and Cahill, 2003; Ward *et al.*, 1989), most notably compromising penetration resistance of barley towards *Bgh* (Edwards, 1983). Thirdly, and in contradiction to the two previous points, ABA has been shown to confer a positive effect on callose accumulation restricting necrotrophic and viral disease progression (Adie *et al.*, 2007; Beffa *et al.*, 1996; Kaliff *et al.*, 2007; Ton and Mauch-Mani, 2004). As papillae are associated with callose depositions for effective penetration resistance towards *Bgh* (section 1.3.1.) (Aist, 1976), seemingly contrasting observations on outcomes of ABA treatment are inferred. Additionally, the stimuli-dependent observations on ABA mediated susceptibility and resistance potentates the often-stated truism on the antagonistic relationship between signalling responses which confer resistance towards necrotrophs and biotrophs, respectively (Glazebrook, 2005).

In conclusion, though the implications of ABA on abiotic stress interactions has been studied widely (Verslues and Zhu, 2005), much less is known about the influence of this hormone in basal resistance towards pathogenic microbes. In recent years, increasing evidence implicates

ABA as an interesting component in plant-pathogen interactions, although its exact role in susceptibility or basal resistance of plants against different pathogens, and the different means by which these signals are transmitted, remains elusive (reviewed by Mauch-Mani and Mauch, 2005).

1.3.8. Non-host for the Future

Overall, apart from the common mechanistic and genetic elements of basal resistance and non-host resistance, possible regulatory overlaps of broad spectrum immunity are also conserved between mono- and dicots (Shen *et al.*, 2007). As the common monocot and dicot angiosperm progenitor dates back 200 million years (Wolfe *et al.*, 1989), the durability of common elements of basal resistance and non-host resistance to restrict host cell entry of powdery mildew fungi is unusual given the assumed gains and losses of pathogenicity during evolution of the pathogenic fungi (Consonni *et al.*, 2006). Clearly, this highlights the potential of pre-invasive defences to pathogenic fungi as an agronomical beneficial trait. However, basal and non-host resistance is presumed to be a complex, multi-component form of resistance, including both constitutive and inducible defences (Thordal-Christensen, 2003), and understanding how *Bgh* cause disease on barley but not on *Arabidopsis* and subsequently benefiting from this, is the future challenge for breeders (Ellis, 2006).

1.4. NAC Transcription factors

Regulation of gene expression at the level of transcription influences or controls many of the biological processes in the plant cell, including cell cycle progression, metabolic and physiological balance, and responses to the environment (Riechmann and Ratcliffe, 2000). In order for this to be achieved, a battery of nuclear proteins modulates chromatin structure and contributes to the transcription machinery (AGI, 2000). Among these proteins, transcription factors are crucial components for regulation and recognition of target gene promoters (Guo and Gan, 2006). The largest plant transcription factor families are NAC, WRKY, C2H2 type zinc finger, AP2/EREBP, and MYB proteins (Buchanan-Wollaston *et al.*, 2005; Chen *et al.*, 2002; Guo *et al.*, 2004; Lin and Wu, 2004), and most of these families have been further divided into subfamilies according to sequence homology (Ooka *et al.*, 2003; Qu and Zhu, 2006). Recent progress has been made on the cloning of open reading frames (ORFs), sequence analysis and expression profiling of members of different transcription factor families (Buchanan-Wollaston *et al.*, 2005; Chen *et al.*, 2002; Kusano *et al.*, 2005). From such studies, major progress has been made within the field of functional characterization of plant transcription factors (Guo and Gan, 2006; Hu *et al.*, 2006). However, much more information on the regulatory targets and signalling pathways by which transcription factors operate await in the future, as only ~ 10% of the transcription factors have been molecularly or genetically characterized in *Arabidopsis* and rice (Qu and Zhu, 2006).

To enable a perspective view of the results obtained during my studies I will briefly cover the progress made within research of members of the NAC transcription factor family, highlighting overall structural similarities, expression profiles and functionalities.

1.4.1. The Domain Structure of Characterized NAC Transcription Factors

The NAC genes belong to a plant-specific gene family playing important roles in many aspects of growth, development and environmental stresses (Olsen *et al.*, 2005a). The NAC family is large, with predicted 111, 142 and 167 members in *Arabidopsis*, rice and poplar, respectively (Riano-Pachon *et al.*, 2007). The N-terminal region of the encoded gene products contains a highly conserved NAC (for NAM, ATAF1,2, and CUC2) domain (Aida *et al.*, 1997; Aida *et al.*, 1999; Souer *et al.*, 1996) (figure 1.5.). Structurally, the NAC domain consists of a twisted antiparallel β-sheet aligned by helical elements (Ernst *et al.*, 2004; Olsen *et al.*, 2004). Functionally, it encompasses a homo- and heterodimerization region and most often a nuclear localization signal (NLS) (Olsen *et al.*, 2005b). Furthermore, as site-directed mutagenesis of residues needed for dimerization abolishes DNA-binding, it is believed that the NAC domain encodes the DNA-binding region (Olsen *et al.*, 2005b; Xue *et al.*, 2006), though the exact DNA-binding motif is yet to be characterized.

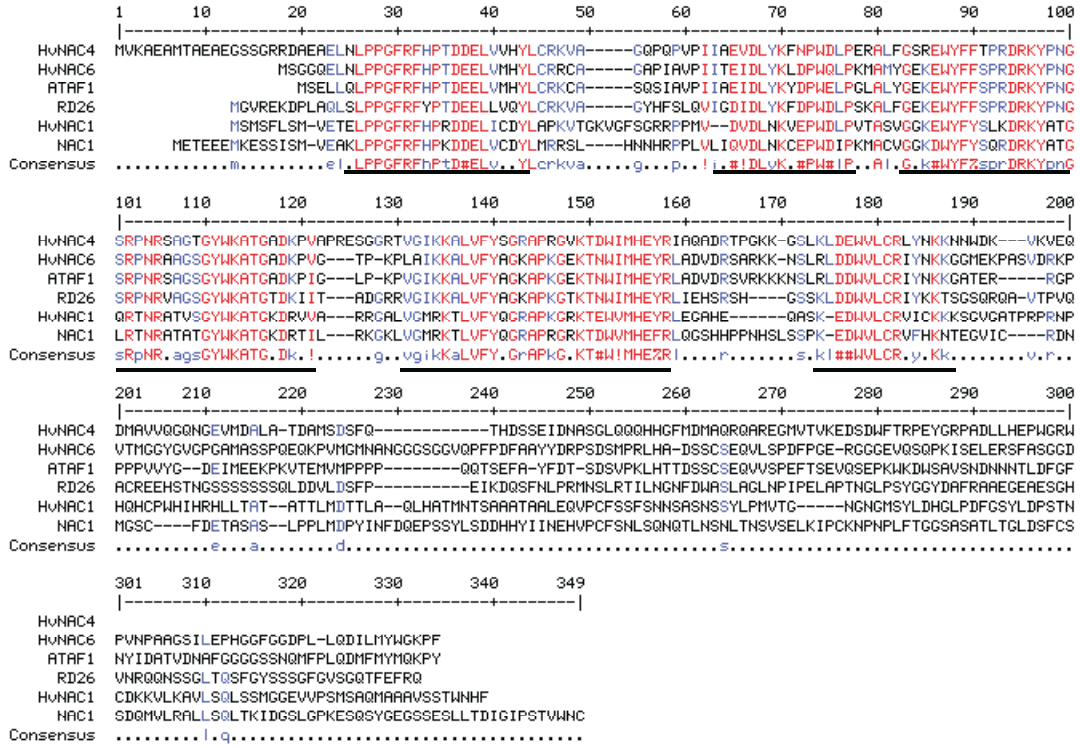


Figure 1.5. Multiple alignment of three full-length barley NAC members isolated in our lab (see paper I), and three additional characterized NAC members from *Arabidopsis*.
Alignment was made in CLUSTALW (Thompson *et al.*, 1997). Five motifs comprising the conserved NAC domain are underscored. Accession numbers are as follows; *HvNAC1* (AM500855), *HvNAC6* (AM500853), *HvNAC1* (AM500854), *ATAF1* (NM_100054), *RD26* (NM_118875) and *NAC1* (NM_104479).

Apart from identification of individual residues important for NAC domain DNA-binding and dimerization (Olsen *et al.*, 2005b; Xue *et al.*, 2006), several reports have in recent years shed light on the NAC core DNA-binding site (Fujita *et al.*, 2004; Olsen *et al.*, 2005b; Tran *et al.*, 2004; Xue *et al.*, 2006).

Table 1.1. summarizes the results obtained from characterized NAC proteins, illustrating the core recognition motif -CGT-, and its reverse complement -ACG-, to have been identified for members of a broad range of NAC transcription factors in eudicots. Furthermore, the absolute requirement of a palindromic binding site for *Ta*NAC69 and *At*NAP implies that *Ta*NAC69 and *At*NAP may bind DNA in a dimeric form (Xue *et al.*, 2006). Additionally, ANAC019 was shown to be able to bind to both single and palindromic NAC recognition sites (Olsen *et al.*, 2005b). This suggests that both single and palindromic NAC recognition sites may have relevance *in vivo*, possibly determined by interactions with other components of the transcription apparatus (Weir *et al.*, 2004). Overall, the consensus binding site [TA]

Table 1.1. NAC Recognition Sites

Protein name §	Consensus NAC Recognition Sites **	Organism	NAC subfamily*	Reference
ANAC019 ANAC055 ANAC072	CACGCATGT	*Arabidopsis*	ATAF	Tran *et al.*, 2004
ANAC019	[TA][TG]NCGT[GA]	*Arabidopsis*	ATAF	Olsen *et al.*, 2005b
ANAC092	TTGCGTGT	*Arabidopsis*	NAM	Olsen *et al.*, 2005b
TaNAC69	CGT[AG]NNNNN[CT]ACG	Wheat	NAP	Xue *et al.*, 2006
AtNAP	CGT[AG]NNNNN[CT]ACG	*Arabidopsis*	NAP	Xue *et al.*, 2006

* Classification according to Ooka *et al.* (2003).
** N = A, C, T or G
§ NAC nomenclature is as follows: *At, Arabidopsis thaliana; Ta, Triticum aestivum.*

[TG][TACG]CGT[GA] has been suggested from studies of the ANAC019 member (table 1.1.) (Olsen *et al.*, 2005b).

The C-terminal regions of NAC proteins are highly diverse (figure 1.5.). However, all NAC proteins to date have been shown to encode a transcriptional activation domain in the C-terminal part (Fujita *et al.*, 2004; Robertson, 2004; Xie *et al.*, 2000). Consistent with these observations, C-terminal regions of NAC proteins frequently include characteristics of plant transactivation domains, often regions rich in serine, threonine, proline and glutamine, or acidic residues (Kikuchi *et al.*, 2000; Liu, 1999). Interestingly, another characteristic of a subgroup of *Arabidopsis* NAC transcription factors is, that the C-terminal region of at least 13 members of the NAC family in *Arabidopsis* contain strong α-helical transmembrane motifs (TMs), and Kim *et al.* (2007) recently confirmed the predicted membrane-association of one *Arabidopsis* NAC transcription factor by transient expression in *N. benthimiana* leaves.

1.4.2. Expression Characteristics

Altering the expression of NAC transcription factors have been shown to influence plant stress tolerance and development (Delessert *et al.*, 2005; Hu *et al.*, 2006; Zhong *et al.*, 2007). Hence, knowledge about spatio-temporal changes in the – often transient – transcript accumulation of individual transcription factor genes has made way for data-driven functional analysis of such genes.

Several methods have been used to elucidate transcript accumulations of NAC transcription factors (Collinge and Boller, 2001; Delessert *et al.*, 2005; Gregersen and Collinge, 2001; Hegedus *et al.*, 2003; Schenk *et al.*, 2003). However, by the introduction of quantitative hybridization- and PCR-based methods for transcript profiling, robust hypotheses-

generation for down-stream analyses of genes of interest has been boosted. From such expression profiles, researchers have targeted functional studies for the elucidation of the regulatory potential of genes encoding transcription factors upon induction or repression (Lorenzo *et al.*, 2004; Lu *et al.*, 2007; McGrath *et al.*, 2005).

One quantitative method is microarraying (see section 1.2.2.). Microarray is an important post-genomic tool for generating large amounts of data for parallel expression analysis (Kilian *et al.*, 2007), and has accommodated new genome-wide insights to the stimuli-dependent activation of regulatory proteins, like transcription factors (Chen *et al.*, 2002). Figure 1.6. shows an expression profile matrix of 97 *Arabidopsis* NAC genes identified on the Affymetrix ATH1 GeneChip®, upon biotic, abiotic and hormone stimuli. The matrix is presented as a heatmap[2]. From this figure it is clear that, though NAC members only comprise one gene family, expression profiles of individual NAC members deviate largely, both quantitatively and qualitatively. Hence, some members are coordinately induced rapidly upon cold and salt treatments (cluster III in figure 1.6.), but not affected by hormones, and others are late induced by osmotic stress (cluster II). Another interesting observation gained from such matrices, is that the six members of the ATAF subfamily of NAC transcription factors identified on the ATH1 GeneChip® co-express upon biotic stress and ABA treatment (cluster I). These data confirm reports on transcript profiles of individual ATAF subfamily members from *Arabidopsis* (Fujita *et al.*, 2004; Greve *et al.*, 2003; Lu *et al.*, 2007), as well as from rice (*OsNAC6*) and potato (*StNAC*) ATAF subfamily members (Collinge and Boller, 2001; Ohnishi *et al.*, 2005). Additionally, a wheat *OsNAC6* homologue was recently reported to be induced by senescence (Gregersen and Holm, 2007). Though the members in this cluster are well separated by the dissimilarity matrix underlying the hierarchical clustered heatmap (*i.e.* many treatments with no regulation, or large quantitative differences in transcript levels between common inducing and repressing stimuli), the NAC members of the ATAF subfamily are not only evolutionary related in terms of primary structure, but also show a certain degree of co-expression, indicating additional evolutionary conservancy of key regulatory sequences (Fujita *et al.*, 2004). Hence, from expression matrices it can be inferred that plants co-ordinately express multiple repressor- and activator-type transcription factors to modulate perception of environmental stimuli (McGrath *et al.*, 2005).

Another high-quality approach for transcript analysis is quantitative real-time PCR (QRT-PCR) (Bustin, 2000). With the arise of large-scale hybridization-based techniques, QRT-PCR transcript analysis is often limited to validation of microarray data (Schenk *et al.*, 2003). In contrast to hybridization-based techniques like micro- and macroarrays, QRT-PCR, however, is acknowledged as a more sensitive approach for characterization of low-abundance transcripts (Czechowski *et al.*, 2004; Horak and Snyder, 2002). As genes encoding transcription factors often show low-abundant transcript levels, a QRT-PCR-

2 Heatmaps are two-dimensional colored grids applied to microarray data by Eisen *et al.* (1998) and have become a standard visualization method for expression data. The color of each rectangle is determined by the value of the corresponding entry in the matrix, in this case log2-fold changes in the treated samples compared to control samples. Both columns and rows can be ordered so that similar rows are placed next to each other, and similar for the columns. Expression profile matrices are often depicted using hierarchical clustering, with dendrograms added (Huber *et al.*, 2005).

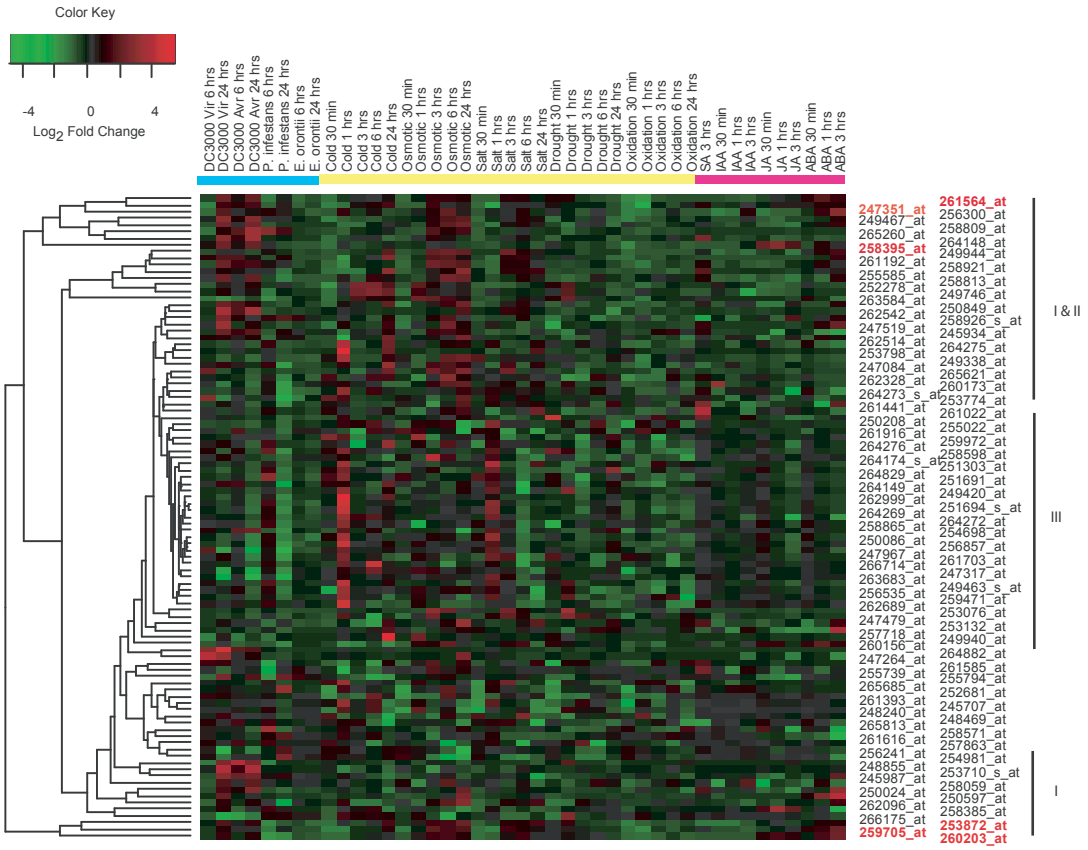


Figure 1.6. Hierarchical clustering of NAC gene expression patterns upon biotic, abiotic and hormone treatments of *Arabidopsis*.

Ninety-seven ATH1 probe-sets matching *Arabidopsis* NAC encoding genes, were clustered using Affymetrix GeneChip® expression data from the AtGenExpress Consortium (http://www.weigelworld.org/resources/microarray/AtGenExpress/) and Integrated Microarray Database System (IMDS, http://ausubellab.mgh.harvard.edu/imds/). Biotic stress (blue colour bar) treatments include those of virulent and avirulent bacteria *P. syringae DC3000*, hemibiotrophic *P. infestans* and biotrophic *E. orontii*, all samples harvested at 6 and 24 hrs after inoculation. Abiotic stress (yellow colour bar) treatments include cold, osmotic, salt (NaCl), drought and oxidation (mannitol), all samples harvested at 0.5, 1, 3, 6, and 24 hrs after treatment. Hormone treatment (pink colour bar) include SA, IAA, JA, and ABA, all sampled at 0.5, 1, 3 hrs after treatment, except for SA. All raw data sets were normalized and interpreted using the GCRMA function (Wu *et al*., 2004) of the Bioconductor microarray analysis package (http://www.bioconductor.org/) (Gentleman *et al*., 2004). Mean values of two-three biological replicates from each sample group were used. Hierarchical clustering was performed using a scaled correlation (*i.e.* each row was scaled to have a mean of zero and standard deviation one) and complete linkage clustering. Genes induced relative to their control are coloured red, those suppressed coloured green, whereas genes unchanged in their expression are coloured black. Colour key displays correlation between colour and $\log_2$ fold changes. Probe-set names are given to the right. Members of the ATAF subfamily are highlighted in bold red. Lines to the right, labelled I (hormone and biotic stress responsive), II (biotic and osmotic stress responsive), and III (early cold and salt responsive) highlights co-expression clusters.

based resource for genome-wide quantitative measurements of transcripts of transcription factor genes has been developed (Czechowski *et al.*, 2004). Using this resource, large-scale efforts have recently been made to uncover small but biologically meaningful changes in tissue-specific and nitrogen responsive transcripts (Czechowski *et al.*, 2004; Scheible *et al.*, 2004). Furthermore the resource has illustrated the significant hormonal and biotic stress responsive perturbations of 12 NAC genes (McGrath *et al.*, 2005).

Furthermore, apart from transcriptional regulation, NAC gene products are under post-transcriptional and post-translational control. In *Arabidopsis* and pumpkin, post-transcriptional regulation by miRNA-mediated repression and phloem transport of NAC transcripts, respectively, have been reported (Laufs *et al.*, 2004; Mallory *et al.*, 2004; Ruiz-Medrano *et al.*, 1999). Post-translational regulation by ubiquitin-mediated protein degradation have been reported in *Arabidopsis*, and a possible post-translational modification of a barley NAC gene product interacting with an *N*-acetylglucosaminyltransferase have been speculated (Robertson, 2004; Xie *et al.*, 2002). Additionally, the membrane-associated NAC members (see section 1.7.2.) constitute yet another level of regulation by the rapid "activation" of such gene products by regulated intramembrane proteolysis (RIP) or regulated ubiquitin-proteasome dependent processing (RUP)(Hoppe *et al.*, 2001).

Finally, an important aspect of the current research interest using functional genomic tools is the identification of key regulators based on gene expressions studies related to abiotic and biotic stress (Chen and Zhu, 2004). The expression patterns of the studied transcription factors are complex and suggest that stress tolerance and disease resistance are controlled at the transcriptional level by an intricate regulatory network (figure 1.6.). Accordingly, apart from the NAC family, virtually every major transcription factor family includes members both induced and functionally implicated in biotic stress perception and adaptation (for review, see Singh *et al.*,(2002). However, with respect to the NAC transcription factors, functional characterizations have until recently remained scarce (section 1.4.3.).

1.4.3. NAC Functionality

The first NAC genes to be identified, were originally observed from developmental mutant phenotypes (Aida *et al.*, 1997; Souer *et al.*, 1996). In this section, however, a brief overview will be attributed NAC genes with biological functions in defence and abiotic stress signalling.

Though high-throughput screens of genome-wide expression profiles of plants upon biotic and biotic stress provides large amounts of candidate genes encoding transcription factors, only a small fraction of these have been functionally characterized (McGrath *et al.*, 2005; Qu and Zhu, 2006; Schenk *et al.*, 2003). In many instances, however, genes

encoding transcription factors involved in plant defence and abiotic stress signalling are also transcriptionally regulated by biotic and abiotic stresses (Delessert *et al.*, 2005; Fujita *et al.*, 2004; Hu *et al.*, 2006). This suggests that a possible strategy to identify transcription factors with roles in stress perception and/or adaptation may be to first identify transcription factor genes showing altered transcript levels during responses to biotic and abiotic stresses, and then to follow up by a functional characterization of these candidate genes.

Indeed, Hu *et al.* (2006), identified the SNAC1 transcription factor enhancing drought resistance and salt tolerance from a cDNA microarray screen of rice drought responsive genes. Another key regulator of abiotic stress perception and adaptation identified from large-scale expression profiling is *At*NAC2 (He *et al.*, 2005). *AtNAC2* is induced by salt, and over-expression lines show increased lateral root formation, suggested to represent an adaptive response to of plants to salt stress (He *et al.*, 2005). Furthermore, Fujita *et al.* (2004) report unpublished data on ABA, dehydration and salt-responsiveness of all seven *Arabidopsis* ATAF subfamily members, and isolate a cDNA clone of the ATAF member *RD26* from a cDNA library of dehydrated *Arabidopsis* plants. *RD26* over-expressing plants were furthermore shown to be highly sensitive to ABA, while repressed plants were insensitive (Fujita *et al.*, 2004). Other NAC genes characterized from transcript data, include the SA- and JA-activated *ATAF2* and the tomato leaf curl virus-induced *SlNAC1* from *Arabidopsis* and tomato, respectively (Delessert *et al.*, 2005; Selth *et al.*, 2005). Both of these genes were shown to be positive regulators of disease progression from necrotrophic *Fusarium oxysporum* and geminiviral infections, respectively. Hence, over-expressing *ATAF2*, was shown to repress pathogenesis-related transcript accumulation and increase susceptibility to *F. oxysporum* (Delessert *et al.*, 2005), whereas *SlNAC1* expression enhanced the accumulation of TLCV ssDNA, possibly by interacting with the viral replication enhancer protein, REn (Selth *et al.*, 2005).

Furthermore, in an attempt to understand the genetics underlying senescence in wheat, Uauy *et al.* (2006) identified a NAC gene by map-based cloning, believed to accelerate senescence, characterized by massive programmed cell death and increased nutrient remobilization from leaves to developing grains (Uauy *et al.*, 2006). Furthermore, leaf senescence has been shown to be delayed in an *At*NAP knock-out plant (Guo and Gan, 2006), originally identified from microarray analysis of plant senescence (Buchanan-Wollaston *et al.*, 2005). Hence, several aspects of the complex regulations underlying plant responses to biotic and abiotic stress and senescence-associated cell death include NAC transcription factors identified and functionally characterized from expression profiling.

Apart from transcript profiles, NAC proteins have also been characterized by their physical interaction with DNA and proteins, using yeast one- and two-hybrid screens (Greve *et al.*, 2003; Tran *et al.*, 2004; Tran *et al.*, 2007). Interestingly, the *Arabidopsis* NAC protein TIP, was found to be required for resistance towards turnip crinkle virus, by its interaction with the viral capsid protein (Ren *et al.*, 2000), and the wheat NAC members GRAB1 and GRAB2

have been shown to directly or indirectly inhibit wheat dwarf geminiviral DNA replication, possibly by its interaction with the viral RepA protein (Xie *et al.*, 1999). However the case, it is peculiar that these three NAC proteins, in addition to SlNAC1 (Selth *et al.*, 2005), have all been characterized from their interaction with viral components. Moreover, the individual interactions impinge differently on the outcome of the viral infection. Additionally, no two-hybrid screens have revealed NAC-interacting proteins when using NACs as baits.

Finally, apart from the drought tolerance-conferring SNAC1 (Hu *et al.*, 2006), over-expression of three homologues *Arabidopsis* NAC transcription factors isolated from one-hybrid screens using the *early responsive to dehydration stress 1* promoter as a bait (Tran *et al.*, 2004), have been shown to increase drought tolerance. Interestingly, the close homologue ATAF1, is believed to be a negative regulator of drought tolerance, presumably by repressing the expression of stress responsive genes (Lu *et al.*, 2007). Once again this illustrates the complex – sometimes redundant, sometimes contrasting – functionalities of members of this large gene family.

1.5. Recap and Aims of Research

In this introduction I have covered aspects on functional genomics in plants, highlighting transcriptomics (1.2.2.) and modulation of gene expression (1.2.3. and 1.2.4.). Moreover, a detailed description of the morphological and molecular aspects of barley and *Arabidopsis* penetration resistance towards *Bgh* have been presented (1.3.), and finally, a status on the knowledge of NAC transcription factors and their regulatory potentials (1.4.). All three isolated sections (1.2., 1.3. and 1.4.) provide background knowledge for the presented chapters and papers.

The work performed during my PhD studies has aimed at providing novel insights to the plant-pathogen interactions between the biotrophic fungal host and non-host pathogen *Blumeria graminis* f.sp. *hordei*, and barley and *Arabidopsis*, respectively. This included both studies of plant defence responses in the two reference pathosystems and the plant genes involved in early responses to the pathogen.

Following topics motivated for the presented research:

– Does modulation of isolated barley NAC genes interfere with *Bgh* development and/or pathogenicity?

– What are the molecular mechanisms or regulatory targets of the NAC transcription factors upon *Bgh* inoculation?

– Can modulation of the identified NAC regulated mechanisms proportionally mimic the gene modulation approaches of *HvNAC1* and *ATAF1*?

2

Experimental Modulation of Barley NAC Genes Expression

This chapter is based on the following manuscripts:

Jensen, M.K., Rung, J.H., Gregersen, P.L., Fuglsang, A.T., Gjetting, T., Hansen, M., Fuglsang, A.T., Joehnk, N., Lyngkjaer, M.F. and Collinge, D.B.
The *Hv*NAC6 Transcription Factor: A Positive Regulator of Penetration Resistance in Barley and *Arabidopsis*. Accepted for publication in ***Plant Mol. Biol.*** Appendix VII. Paper I.

Collinge, D.B., Jensen, M.K., Lyngkjaer, M.F., Rung, J.H.
How can we exploit functional genomics to understand the nature of plant defences? Barley as a case study. Submitted for publication in *Eur. J. Plant Parthol.* Pending revision. See appendix VII.

2.1. Abstract

Transient expression systems enable reverse genetic approaches to study promoter, gene and protein functionality in native or near-native environments. Furthermore, transient expression systems have proved useful for high-throughput scoring of gene functions in plants upon powdery mildew infection. In this chapter, background considerations of the transient assay and subsequent data analyses will be provided for a thorough insight to the technical aspects underlying the studies and results presented in paper I.

2.2. Background

The detailed time-course studies performed by Dr. P. L. Gregersen, while working at the Section of Plant Pathology from 1986-2001, provided the material for starting this project. The overall aim of the research performed in the late nineties was the identification of novel transcripts expressed in barley epidermis and underlying mesophyll tissues during infection with virulent and avirulent *Bgh* races. Using differential display and RACE techniques, Gregersen and co-workers extracted sequence material to be used for further characterization (Gregersen *et al.*, 1997;Gregersen and Collinge, 2001). Among the differentially displayed clones extracted from barley inoculated with *Bgh* conidia, a putative NAC transcription factor member was identified. Probing a *Bgh*-infected barley cDNA library with the 5' end of the differentially displayed cDNA clone, additional NAC sequences were isolated (figure 1.5.). In my PhD study, I have attempted functional characterization of the isolated full-length NAC cDNA clones.

2.3. The Single-Cell Transient Expression System

Despite considerable technical advances in plant transformation approaches in recent years (Lombari *et al.*, 2003;Yan and Rommens, 2007), stable genetic manipulation of large

cereal genomes with long generation times is not yet widely established on a routine basis (Panstruga, 2004;Travella *et al.*, 2005). This has been believed to be one of the primary limitations to discovery of genes involved in plant-microbe interaction of cereal crops (Birch, 1997). This still holds true a decade later. However, the technical and biological limitations restricting cereal gene discovery and subsequent functional characterization have been partially overcome by the use of transient transformation techniques (Finer *et al.*, 1999;Zierold *et al.*, 2005). Specifically, delivery of target DNA by particle bombardment for *in vivo* transcription has proved useful for studying the impact of RNAi and over-expression of candidate genes involved in plant-microbe interactions (figure 2.1.) (reviewed in Panstruga, 2004). The biolistic delivery of target DNA for *in vivo* transcription is particularly well suited for studying the outcome of papilla formation in barley-*Bgh* interactions due to the high degree of cell-autonomy exhibited by barley cells responding to *Bgh* attack (Nielsen *et al.*, 1999;Panstruga, 2004). Additionally, *Bgh* only attacks epidermal cells, which are the primary targets of the biolistic approach used. Furthermore, *Bgh* spores are easily generated and barley epidermal cells are comparatively large. This ensures simple optimization of inoculum density compared to number of transformed cells, ensuring good coverage of transformed cells attacked by *Bgh* for subsequent easy inspection by light microscopy.

Technically, in the approach used in my studies, the β-Glucuronidase (GUS) reporter gene (*uidA*) was used as transformation control (Schweizer *et al.*, 1999). Applying this reporter gene allows researchers to obtain detailed biological information from conserved/fixed material. Especially when looking into interactions with a spatio-temporal development, as the fungal progression on a host plant, transient approaches suitable for fixed material are preferred, since the time to evaluate the interaction at desired time-points after contact is adequate when very large numbers of individual interactions shave to be scored. In our hands, GUS staining of pUbi-GUS transformed cells was carried out in a time span from 30 to 48 hrs after bombardment without any decrease in β-Glucuronidase activity. In addition, as fungal inoculations have been shown to be applied in varying time-spans after bombardment (Douchkov *et al.*, 2005), this system allows for easy and widespread applications for use of studies between transformed epidermal cells and conidia progression.

2.4. The Limitations of the System

Though widely applied (Christensen *et al.*, 2004;Kristensen *et al.*, 2001;Schultheiss *et al.*, 2002;Schweizer *et al.*, 1999), the transient system of RNAi has some limitations compared to stable knock-down transformants. Meaningful answers to questions related to possible

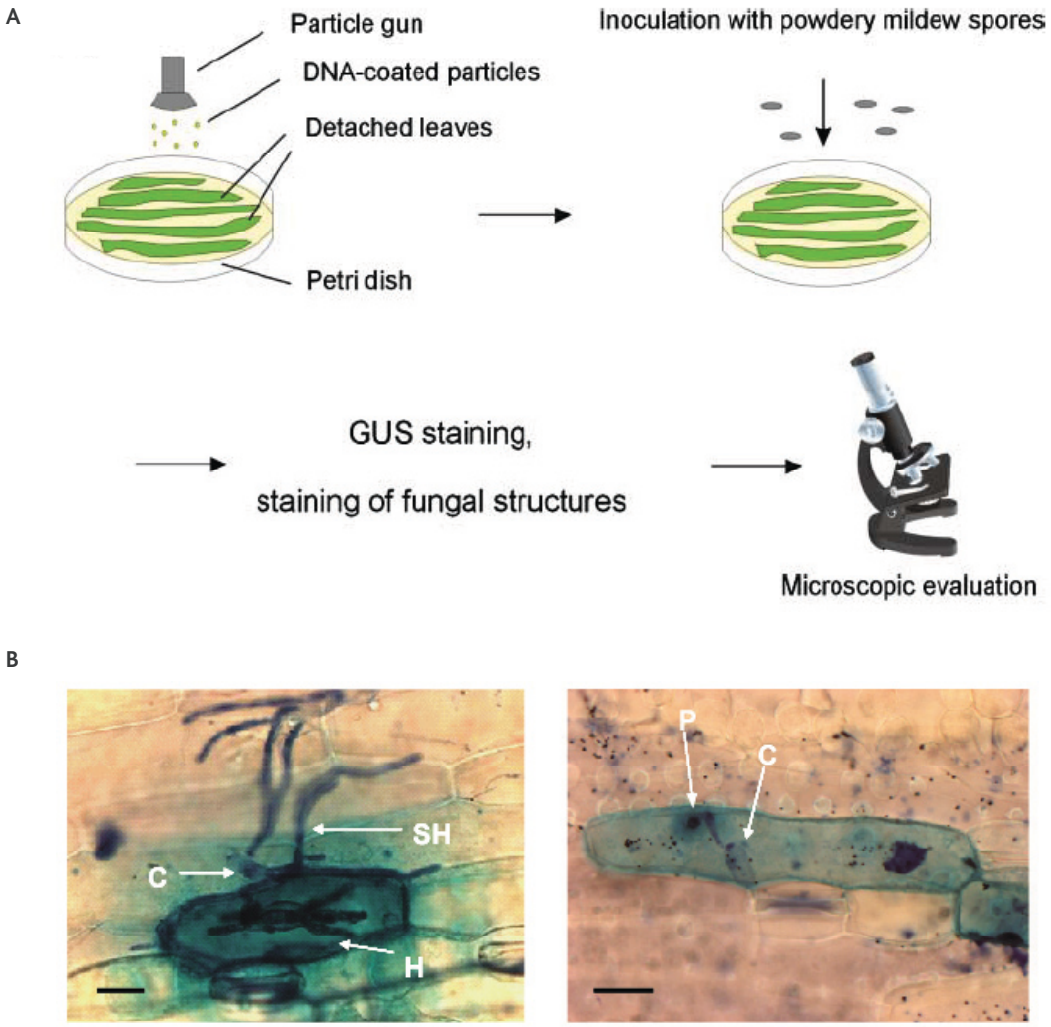


Figure 2.1. Schematic representation of the workflow used for functional characterization of barley NAC genes *in vivo*

(**A**) Plasmids harbouring full-length or inverted repeats of C-terminal sequences of individual NAC transcripts were coated onto gold particles for delivery into barley epidermal cells of detached leaves using a PDS-1000/He biolistic particle delivery system (Bio-Rad, München, Germany). All transformations included a plasmid encoding the β-Glucuronidase (GUS) reporter gene (*uidA*) for transformation control. Four hours post-bombardment leaves were inoculated with virulent *Bgh* spores. (**B**) After a further 48 hrs leaves were GUS stained and *Bgh* penetration attempts of GUS-transformed cells scored using a compound microscope (Nielsen *et al.*, 1999). Successful penetration is shown in the left panel, indicated by the presence of a haustorium and secondary hyphal growth. To the right, *Bgh* penetration attempt is halted by effective papilla formation beneath the site of attempted penetration. C, conidiospore; H, haustorium; SH, secondary hyphae; P, papilla. Scale bar = 20 µm. Figure and text adapted from Panstruga (2004) and paper III.

off-targets can only be addressed at whole-genome level, as expressed and processed (diced) fragments of your gene of interest may complement a range of transcribed elements in the tissue of use. This is not applicable for single-cell systems like that used in this study. However, both specificity and efficiency of the system can be quantified. In our approach, I have successfully addressed the specificity and efficiency of our transient gene silencing approach by the use of a chimeric reporter construct. Firstly, co-transforming target gene RNAi constructs and a vector carrying the full-length cDNA clone of the target gene fused with a reporter gene, allows for silencing efficiency to be calculated. Secondly, co-transforming the target gene RNAi construct with related targets fused with a reporter gene may reveal potential off-targets. Performing such experiments is crucial in order to couple RNAi vector design with obtained results (Panstruga *et al.*, 2003). Hence, though the transient single-cell system has proven efficient and specific as a functional genomics tool, questions concerning possible off-targets are hard to answer, but would add to the robustness of the approach. Additional concerns of the limitations of RNAi for the functional analyses of individual *Hv*NAC members (or any other large gene family), is the fact that individual members may have closely overlapping functions. This may hinder the genetic analysis of their respective roles as single loss-of-function mutants often have no obvious phenotype in the biological scenario of interest. Further limits to the use of RNAi concern the abundance of the transcript sequences to be targeted. Hence, if the gene is not induced or not predominantly expressed upon the relevant treatment there will be a chance that the gene product will be newly synthesized before the end of the respective treatment. Induced genes will, on the other hand be efficiently silenced, as you would expect a certain background level of expression to be of limited impact compared to the transcript abundance upon the studied stimulus. Finally, as the system relies on *in vivo* transcription of target and reporter genes, cells undergoing HR during the course of post-bombardment *Bgh* infection will not be identified. Additionally, a certain level of expression of wound responsive genes potentially compromises the RNAi effect of the same wound-inducible target genes.

2.5. Transformation: Stable vs. Transient

The transient expression system used in my studies has gained interest for its easy preparation and its feasibility by which it produces fast and reliable result suitable for selecting candidate genes for in-depth studies (Panstruga, 2004). In our hands, transient expression assays were performed and analyzed within 2-4 days, whereas generation of stable transformants in *Arabidopsis* and barley would require in excess of 3 and 6 months, respectively.

Though providing reliable information, the transient single-cell system used in our laboratories suffers from the fact that it mechanically stresses transformed cells and that the system only allows few options for understanding a displayed phenotype. In the case of significant changes in the outcome of the barley-*Bgh* interaction compared to control vector-transformed cells or organisms, researchers are often interested in the reason for the displayed phenotypical changes. Hence, small-scale or global transcriptomic and proteomic approaches can help address the underlying molecular changes observed in the transformants. Due to the restricted number of transformed cells in this single-cell system, this is not possible. At its best, it provides a high-throughput tool for the functional characterization of genes induced or repressed by a given stimulus of interest (Douchkov *et al.*, 2005). Furthermore it can be argued that technical artifacts, like titration effects of crucial endogenous elements of the transcriptional machinery can be linked to the 35*s*-based expression system used in my studies. However, for that reason, reporter and target constructs were expressed under the control of the *Ubiquitin* promoter or CaMV 35*s* promoter, respectively (Douchkov *et al.*, 2005;Schweizer *et al.*, 1999).

Due to limitations of time and resources, neither the generation of *HvNAC6* transgenic barley nor screening of barley TILLING lines was performed. Instead, we used a characterized T-DNA insertion line (SALK_067648) (Lu *et al.*, 2007) of the nearest *Arabidopsis HvNAC6* homologue, *ATAF1*, to complement our transient studies. In this respect, it deserves to be mentioned that *Arabidopsis* responses to barley *Bgh* inoculation predominantly includes efficient papilla formation, and that the use of this pathosystem is therefore well-suited for complementing the observed reduced penetration resistance in the transient *HvNAC6* gene silencing studies. Lu *et al.* (2007) show two independent *ATAF1* mutant alleles to have the same drought tolerant phenotype. Though it can be argued that the *ataf1-1* response towards *Bgh* is conferred by other trait loci, potentially accounted for by multiple mutations at other loci in the *ataf1-1* mutant line, such analyses have not been pursued. The current status is that two additional *ATAF1* mutant alleles (SALK_008110; SALK_557618) have been recovered from kan^R and genotyping screens. These mutant lines will be tested for responses to *Bgh* inoculation in the near future.

Furthermore, as detailed studies on stable *ATAF1* and *HvNAC6* over-expression transformants await, it is noteworthy that the reverse genetics tools used for functional analyses of individual barley NAC members, have been used successfully in several other studies (Schultheiss *et al.*, 2003;Zimmermann *et al.*, 2006). Schultheiss and co-workers furthermore used stable transformants of their RNAi/over-expression candidate genes for the verification of increased resistance of barley towards *Bgh* upon transient knock-down of small RAC/ROP-family G proteins encoding genes (Schultheiss *et al.*, 2005). This highlights the potency and robustness of the transient approach as a means for high-throughput preliminary functional characterization of genes of interest.

2.6. Statistics on Binomial Distributed Data.

Logistic regression analysis is an extension of multiple regression analysis to research situations involving categorical outcome variables (Wright, 1995). In this case the outcome is defined by the predominant dichotomous single-cell outputs of the compatible interaction between powdery mildew and barley, namely the susceptible haustorium-containing cells and the papillae-mediated penetration resistant cells. Using such categorical variables, it is straightforward to compare the outcome of transient modulations of individual transcript, *i.e.* modulation of different NAC genes, with respect to weighted mean of the individual treatments. For the same reason it is not appropriate to use a *t*-test when counting a one attribute variable with two values (papilla/haustorium), since the *t*-test looks at the difference in means of a continuous variable between two groups. Though the *t*-test requirements are not met in the raw data, other research groups have transformed the data and subsequently used the *t*-test to calculate the significance levels of the observed *penetration efficiencies* of at least four independent transformations, compared to the penetration efficiency of control transformed cells (Christensen *et al.*, 2004). In my hands, the transformation efficiency, however, varied by 10 fold, and for that matter weighted means of papillae formations of individual treatments of 8-9 leaves over 3-4 transformations provided a sound basis for logistic regression analysis (for data refer to Appendices I-IV). Additionally, using this approach, outputs of the variability of individual factors, for instance leaf and experiment number, can be incorporated to produce a robust measure of the reproducibility of the treatment on the included factors.

Recently, the laborious work of counting all transformed cells and scoring their interaction has been automated by simply allowing the automated movement of transformed leaves below a digitalized microscope snap-shooting transformed cells and calculating the ratio of haustorium-containing cells versus total number of transformed cells (haustorial index) (Douchkov *et al.*, 2005). Though cost-efficient it relies on even inoculum-density between all bombarded leaves to be analyzed. As the spread of particles used for bombardment does not always display similar distributions, leaves exhibiting lower transformation frequencies may give rise to non-normally distributed values of haustorial indices, and hence, not fulfilling *t*-test assumptions.

2.7. Conclusion

By the use of functional genomics approaches, attempts to elucidate gene functionality are made straight-forward. Though the transient RNAi and over-expression methods have some limitations and possible uncovered drawbacks, these methods have proven their worth as reverse genetics tools for the assessment of the contribution of individual *Hv*NAC members' contribution to papilla-mediated penetration resistance in barley towards *Bgh*. Furthermore, logistic regression modeling has proven adequate for the analysis of the binomially distributed data obtained.

3

Studies of Subcellular Protein Localization in Epidermal Cells Using Confocal Laser Scanning Microscopy

This chapter is based on the following manuscripts:

Jensen, M. K., Rung, J. H., Gregersen, P. L., Fuglsang, A. T. , Gjetting, T., Hansen, M., Joehnk, N., Lyngkjaer, M. F. and Collinge, D. B.
The *Hv*NAC6 Transcription Factor: A Positive Regulator of Penetration Resistance in Barley and *Arabidopsis*. Accepted for publication in ***Plant Mol. Biol.*** Appendix VII. Paper I.

Moeller, A. B., Jakobsen, M. K., Jensen, M. K., Holm, P. B. and Palmgren, M. G.
The Barley Type V ATPase HvP_5, a functional equivalent to *Spf1* and *MIA* secretory pathway pumps, is highly expressed during endosperm development. Manuscript in preparation. Paper IV

3.1. Abstract

The complete genome sequences of several living plant species represent an immense information resource. A central goal for the characterization of genome-wide information is to use functional genomics to describe the biological function of every gene product in a given organism. Apart from single-gene expression modulations and the identification of protein-protein interactions, the determination of subcellular localization of specific proteins will contribute significantly to an understanding of the function of each gene and to deciphering functional networks. In this chapter I will illustrate the use of onion and barley epidermal cells as hosts for the identification of subcellular localizations of three barley gene products.

3.2. Introduction

Eukaryotic cells are organized into a complex network of membranes and compartments enabling cells to physically separate vital processes. Defined subcellular localization of individual proteins within the overall cellular organization is intrinsic to their function and regulating mechanisms ensure the adequate spatio-temporal distribution of proteins and protein complexes (Donnes and Hoglund, 2004). To view *in vivo* compartmentalization of proteins, researchers have benefited greatly from the use of a range of reporter genes fused to gene products of interest (Prufer and Boudreaux, 2007;Saitoh *et al.*, 2001). The green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* is a widely used *in vivo* marker for studying protein localization (Chalfie *et al.*, 1994). Using GFP, researchers have systemically identified the localization of >75% of the *Saccharomyces cerevisiae* proteome and several hundred candidate gene products in *Arabidopsis thaliana*, *Drosophila melanogaster* and mammalian genomes (Escobar *et al.*, 2003;Huh *et al.*, 2003;Kelso *et al.*, 2004;Simpson *et al.*, 2000).

To monitor the subcellular localization of gene products of interest, a choice of expression platform has to be chosen. For this purpose the permissible time frame and additional downstream applications to be investigated comes into question. If tissue- or cell-cell specific transitions are to be studied, researchers often introduce the chimeric reading frame by an appropriate transformation procedure for the subsequent recovery of stable transformants using a tissue-specific promoter (Bellucci *et al.*, 2003;Hou and Hsu, 2005). In contrast, transient expression involves the non-durable *in vivo* processing of exogenously

or endogenously applied material. Studying large-scale GFP fusions in living cells (Koroleva *et al.*, 2005), regeneration of entire transformed organisms may delay the experiment unnecessarily. In such cases, cell culture or isolated tissues of interest have a number of advantages. Firstly, plant regeneration is not required, and each cell represents an independent transformation event. Secondly, cell cultures provide fast screening in a homogeneous cell population with a large proportion of dividing cells, advantageous for study of cell cycle-related proteins. A third major advantage is the speed, as, for instance, transformation and analysis of *Arabidopsis* protoplasts and onion epidermal cells can be assayed in 2-4 days. A natural drawback of the transient single-cell transformation technologies is that they rarely provide useful information for studies of intercellular distribution and dynamics within cell types and organelles other than in the tissue in question.

In this chapter, I summarize my studies on subcellular localization of three candidate genes originally isolated from barley cDNA libraries. I have used onion epidermal cell as a transformation platform due to easy handling and the large cells in this tissue, enabling high-resolution microscopy of individual compartments of eukaryotic cells (Varagona *et al.*, 1992). The easy delivery of foreign DNA into these cells (see Chapter 2) makes this one of the best methods for studying truly genotype-independent transformation in plants, bypassing *Agrobacterium* host-specificity and tissue culture-regeneration difficulties. In addition to onion epidermal cells, I have verified the localization of one candidate protein in barley epidermal cells.

3.3. Work Load

My study of the subcellular localization of three barley proteins was based on the full-length cloning of the individual cDNA sequences (table 3.1.) (papers I and IV, and appendix V (unpublished data)). In all cases, the gene product of interest was fused C-terminally to GFP, and GFP and chimeric full-length cDNA clones expressed under the cauliflower mosaic virus (CaMV) 35*s* promoter. For *Hv*NAC6::GFP and *Hv*RLK1::GFP constructs, cloning and transformation procedures were performed by me. Confocal microscopy in onion epidermal cells was performed in collaboration with Dr. Michael Hansen, University of Copenhagen using a Leica TCS SP2/MP confocal laser scanning microscope. HvNAC6::GFP localization in barley epidermal cells was performed by me using a Zeiss LSM Pascal 5 microscope. For the barley type V ATPase, *Hv*P_5::GFP, cloning procedures were performed by PhD student Annette Britt Moeller, University of Copenhagen, and subsequent transformation and confocal microscopy using a Zeiss LSM Pascal 5 microscope, performed by me.

3.4. Results

3.4.1. *Hv*NAC6 localization

The first candidate protein to be studied was the *Hv*NAC6 transcription factor. This protein was predicted to encode a single nuclear localization signal (NLS) in which the basic residues are closely associated. The predicted nuclear localization was confirmed in both onion and barley epidermal cells (figures 3.1. and 3.2.).

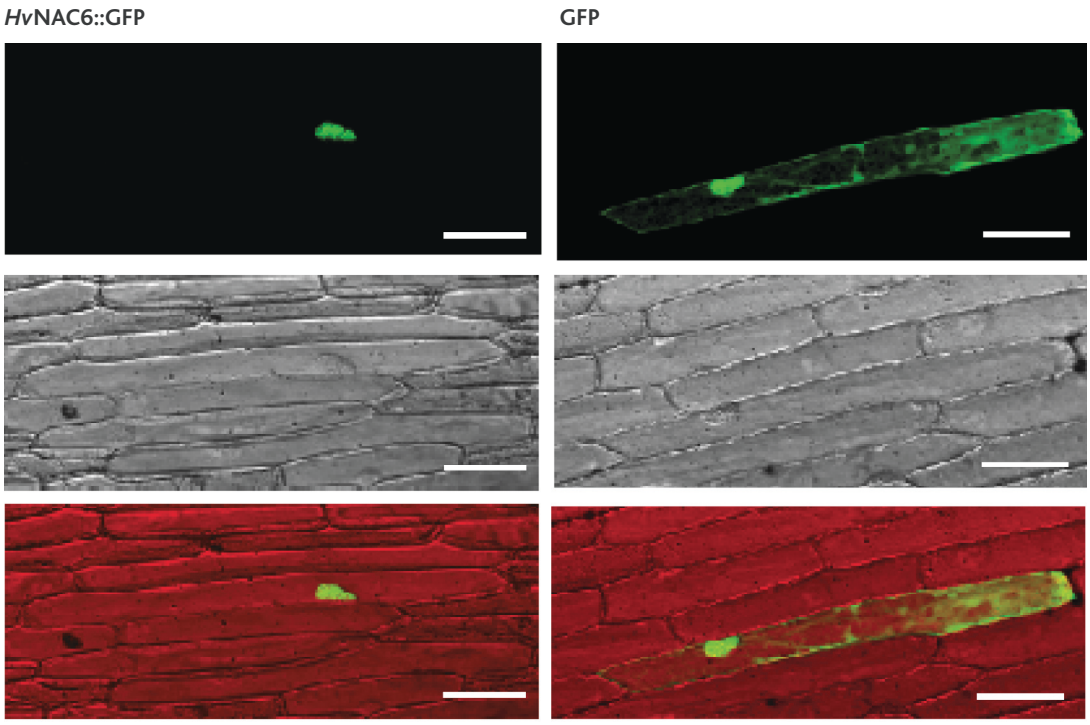


Figure 3.1. *Hv*NAC6 localizes to the nucleus in onion epidermal cells.
GFP alone (top row) or *Hv*NAC6::GFP (bottom row) was expressed under the control of the CaMV 35*s* promoter after biolistic delivery of vector DNA. Cells were analyzed for GFP fluorescence (top panel) by confocal microscopy. Differential interference contrast (DIC) images and merge images are shown in the middle and bottom panels, respectively. Pictures in the left and right columns are maximum intensity images of *Z*-stack projections. This figure is also presented in paper I. Scale bar = 50 μm.

Several NAC transcription factors have been observed to localize to the nucleus of plant cells to date (Fujita *et al.*, 2004;Guo and Gan, 2006;Hu *et al.*, 2006). Interestingly, on the

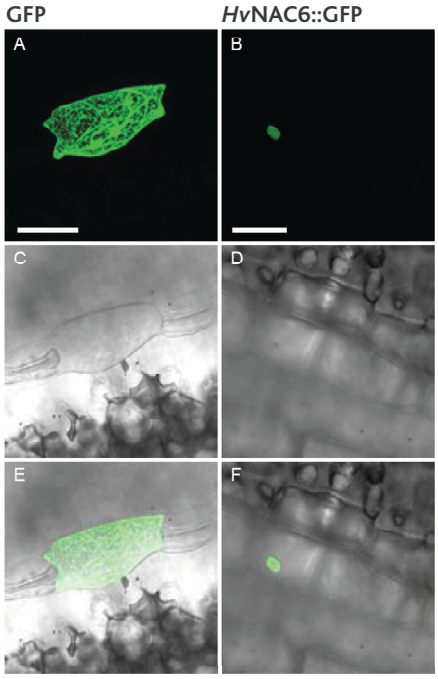


Figure 3.2. *Hv*NAC6 localizes to the nucleus of barley epidermal cells.
GFP alone (**A** and **C**) or *Hv*NAC6::GFP (**B** and **D**) was expressed under the CaMV *35s* promoter after biolistic delivery of vector DNA. Fluorescence (**A** and **B**) and bright field (**C** and **D**) images were captured 18-40 hrs after particle bombardment without any changes in fluorescence distribution. This picture is also presented in paper I (Supplementary figure S1). Scale bar = 25 µm.

basis of known nuclear localisation signals (NLS), not all are predicted to localize to the nucleus, though *in vivo* experiments point to nuclear accumulation (Guo and Gan, 2006). Particularly, querying predictors of cellular localization using full-length amino acid sequences of potentially membrane-associated NAC transcription factors of *Arabidopsis* and rice (summarized by Kim *et al.* (2007b)), points to a non-nuclear localization (typically mitochondrial or ER membranes) of members of this group of transcription factors (data not shown).

However, by deletion of the transmembrane (TM) region of the *Arabidopsis* NAC member; NTL8, Kim *et al.* (2007a) found that the protein accumulated in the nucleus, as predicted for the ΔTM version of NTL8. This illustrates the need for thorough sequence analysis of proteins of interest, to understand possible discrepancies between expected and observed subcellular localization.

Table 3.1. Candidate proteins for analysis of subcellular localization.
Sequences were analyzed using WoLF PSORT (Horton *et al.*, 2006) and fused C-terminally to GFP for expression under the CaMV *35s* promoter in onion and barley epidermal cells.

Name	Size (aa)	Predicted *	Observed
HvNAC6	304	Nucleus	Nucleus **
HvRLK1	645	Plasma membrane No. TMs: 1	ER
HvP_5	1174	Plasma membrane No TMs: 9	ER-like network Nucleus

* Top scoring localization as predicted by WoLF PSORT
** Observed in both onion and barley epidermal cells

3.4.2. *Hv*RLK1 localization

Studies on the barley receptor-like kinase 1, *Hv*RLK1, are unpublished and await further characterization. *HvRLK1* encodes a 645-aa protein with a predicted molecular mass of 70.7 kDa and a pI of 5.67. Computational analysis highlighted the five successive characteristic regions, from N- to C-terminus; a putative signal peptide, a bipartite domain of unknown function (DUF26), a transmembrane region, an Arg-based signal addressing proteins to the ER, and a Ser/Thr kinase domain. In appendix V, the full-length cDNA clone and deduced primary structure are shown.

*Hv*RLK1 is found to localize in a finely polygonal structured endoplasmatic reticulum (ER) network spanning the cytoplasm throughout the cells (figure 3.3). A local concentration of the ER network is observed aligning with, though distinct from, the nucleus, in agreement with the actual physiological orientation of this organelle in plant cells. Furthermore, GFP alone exhibited dense fluorescent staining in the nucleus; whereas *Hv*RLK1::GFP did not accumulate in this compartment. Transformants were studied during a time-span, commencing with the first visible fluorescent signal approx. 18 hrs after transformation until 72 hrs transformation. No changes in the localization patterns of GFP or *Hv*RLK1::GFP were observed during this period of time (data not shown), indicating that the observed accumulation of *Hv*RLK1::GFP is not transiently accumulating in the ER.

Traditionally, both luminal and membrane localized ER proteins have been predicted on the basis of classical C-terminus located retention signals ([KH]DEL) (Teasdale and Jackson, 1996). Other, non-canonical Arg-based signals addressing proteins to the ER have been identified (reviewed by (Michelsen *et al.*, 2005)). These trafficking signals conform to the consensus -R-R-X-R, in which -X- denotes a large neutral or positively charged amino acid, and have been found in both ion channels and G-coupled receptors (Ren *et al.*, 2003; Zerangue *et al.*, 1999). *Hv*RLK1 encodes an -R-R-L-R- motif adjacent to the transmembrane region.

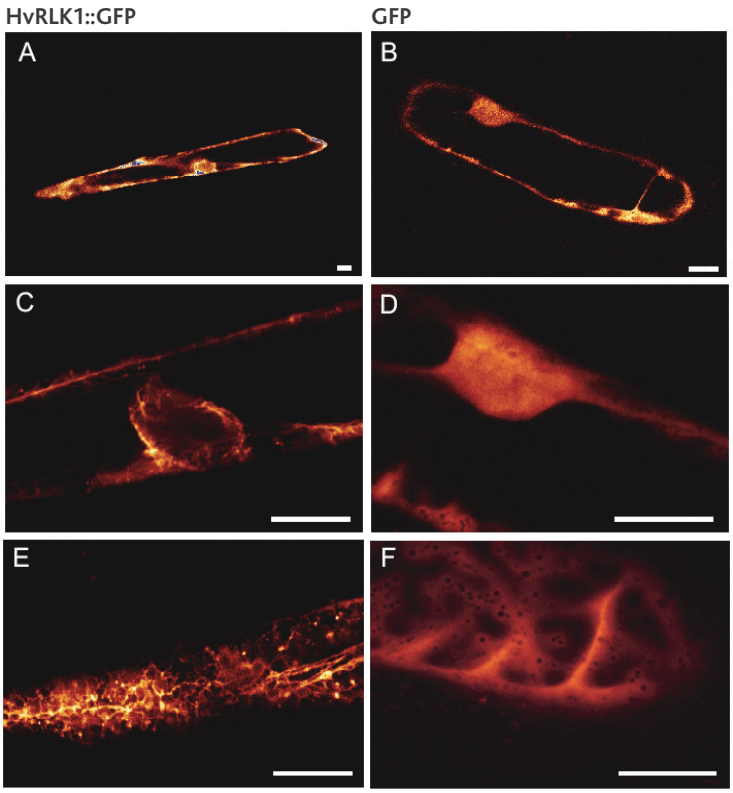


Figure 3.3. *Hv*RLK1 is localized in the ER of onion epidermal cells
GFP alone (right column) or *Hv*RLK1::GFP (left column) expressed under the control of the CaMV *35s* promoter after biolistic delivery of vector DNA. Cells were analyzed for GFP fluorescence by confocal microscopy. (**A**) and (**B**) show overall morphology of transformed cells. (**C**) and (**D**) highlight central part of the nuclei. (**E**) and (**F**) show abaxial part of transformed cells, visualizing the polygonal structures of the ER network (**E**) and characteristic diffuse cytoplasmic staining (**F**). Pictures are maximum intensity images of *Z*-stack projections. Scale bar = 20 μm.

3.4.3. *Hv*P5 localization

The final protein analyzed during my PhD study is the barley type V ATPase, HvP_5 (paper IV). This protein localized to the cortical cytoplasm and nucleus of onion epidermal cells (figure 3.4).

The protein was not observed to transverse the vacuole, as observed for the cytoplasmic strands, when viewing GFP alone. Furthermore, a modified GFP protein, harbouring a C-terminus located ER retention signal (HDEL), was included in this analysis. The GFP-HDEL protein localized exclusively to the ER polygonal network. Furthermore, the fluorescent

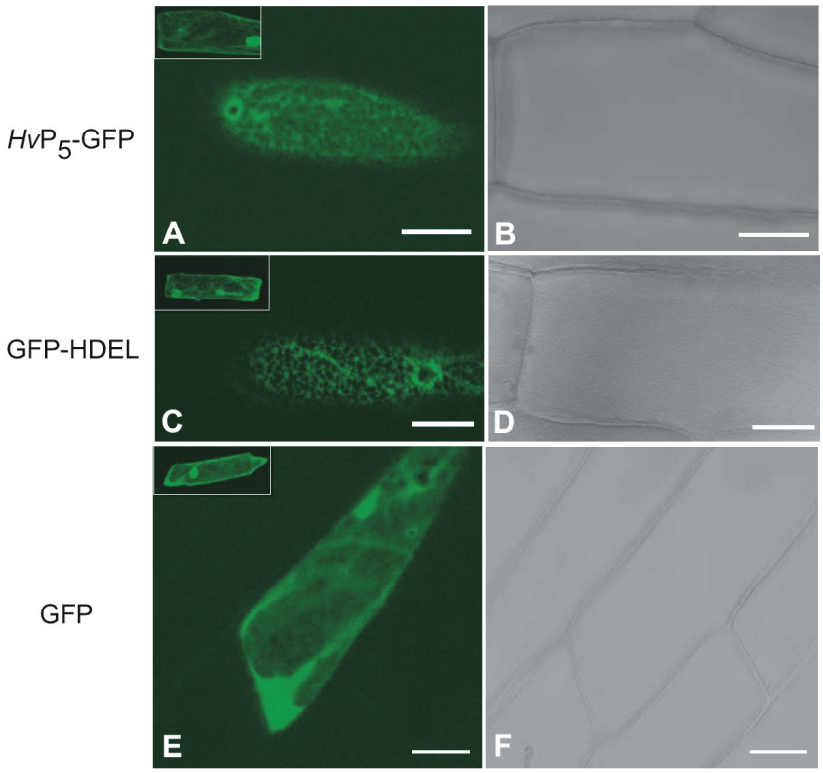


Figure 3.4. *Hv*P_5 localizes to ER-like structures in the cortical cytoplasm and to the nucleus of onion epidermal cells

HvP_5-GFP (**A**-**B**), GFP-HDEL (**C**-**D**) and GFP (**E**-**F**) were individually transiently expressed under the control of the CaMV *35s* promoter in onion epidermal cells. *Hv*P_5 and GFP-HDEL shows distinct fluorescent cortical networks, while GFP alone exhibits characteristic diffuse fluorescence throughout the cytoplasm and nucleus. (**A**), (**C**) and (**E**) are stacked images of the abaxial 10 μm (see paper IV for illustration) of transformed fluorescent cells. Inserts are maximum intensity images of *Z*-projections of all confocal planes of individual transformed cells. **B**, **D** and **F** are bright field images visualizing the circumference of the cells shown in (**A**), (**C**) and (**E**). This figure is also presented in paper IV. Scale bars = 50 microns.

signal of *Hv*P_5-GFP transformed cells resembled the fluorescence distribution in the cortical cytoplasm when transforming GFP-HDEL. Hence, *Hv*P_5 is believed to accumulate in close proximity of the plasma membrane in an ER-like network.

3.4.4. Prediction vs. observed localization

In the case of *Hv*NAC6, the expected and observed localization agreed (table 3.1, figure 3.1 and 3.2.). For *Hv*RLK1 and *Hv*P_5 the predicted (plasma membrane-tethered) and observed localizations disagree (table 3.1, figures 3.3 and 3.4). With respect to *Hv*RLK1, localization of a positive ER control, like the one used for *Hv*P_5 localization studies, should be incorporated for a qualified comparison to a positive ER-localizing control construct.

3.5. Concluding Remarks and Perspectives

The combination of sophisticated instrumentation and ongoing development of fluorescent probes and markers has transformed bioimaging into a powerful analytical tool, allowing for detailed real-time *in vivo* analyses of cellular compartments and the proteins associated with them. In this study, I have made use of a simple analytical platform for the elucidation of subcellular localization of three candidate proteins. Overall, my studies have confirmed the high quality of data gathered from studies in barley and onion epidermal cells, highlighting these tissues as potent and robust high-throughput platforms for subcellular localization studies (Shen *et al.*, 2007;Varagona *et al.*, 1992).

Two of the gene products studied here (*Hv*NAC6 and *Hv*RLK1) were isolated from barley epidermal tissue inoculated with *Bgh* (paper I and unpublished data). Acknowledging the cell-autonomous response of barley epidermal cells towards *Bgh* inoculation (Shirasu *et al.*, 1999), it is intriguing to pay attention to possible protein trafficking dynamics by the use of this transformation system. Indeed, the transient transformation of barley epidermal cells have proven perfectly suited for studies of defence-related aspects of protein trafficking in barley epidermal cells upon *Bgh* attack (Shen *et al.*, 2007). Shen *et al.* (2007) showed that the barley powdery mildew A10 (MLA10) R gene product interacts with a nuclear localized WRKY transcription factor upon recognition of the fungal avirulence A10 effector. Additionally, they found that nuclear localization of MLA10 was required for restricting *Bgh* growth in a race-specific manner, though MLA10 does not possess a known nuclear localization signal. Hence, future experiments on *Hv*NAC6 and *Hv*RLK1 would focus on their spatio-temporal localizations during defence responses to *Bgh* infection, and the effect on the interaction upon mutational analyses of these two candidate proteins. In this context, it is interesting that Arg-based ER-localization signals, like that found in *Hv*RLK1, have been found to disable egress from ER of mature subunits of multimeric protein complexes, only enabling further trafficking when the motif is sterically shielded in mature protein

complexes (Margeta-Mitrovic *et al.*, 2000). This could imply that *Hv*RLK1 is restricted to this compartment as part of a di- or multimerisation step. Future studies on *Hv*RLK1-interacting proteins could evaluate the possibility of the occurrence of the Arg-based sorting signal contributing to the trafficking behaviour of *Hv*RLK1, by co-transformation experiments. Alternatively, transformation assays using an identified and isolated *Hv*RLK1 promoter could be interesting.

The surprising accumulation of HvP_5 in the nucleus also calls for further studies on the functionality and possible trafficking of this membrane protein. Observing HvP_5-GFP transformed cells under changing ionic environments may shed light on the interesting compartmentalization observed, possibly affecting the egress of an 'inactive' HvP_5 nucleus-localized HvP_5 pool. The nuclear accumulation could also be due to over-expression artifacts using the CaMV 35*s* promoter. However, the general picture as observed from the commencement of fluorescence signal (approx. 18 hrs after transformation) and 24 hrs later included both nuclear and cortical cytoplasmic network accumulation (figure 3.4.).

Finally, in favour of the observed localization of *Hv*RLK1 and HvP_5, as opposed to their predicted localization, it should be noted that many *in silico* predictors assigns subcellular compartmentalization of a protein by mimicking the mechanism of cellular sorting and exploiting a variety of sequence and predicted structural features in its input (Donnes and Hoglund, 2004). Such assignments are complicated when it comes to membrane proteins, since the compositional properties of membrane proteins significantly differ from those of non-membrane proteins (Nair and Rost, 2005). Moreover, the *Gold Standard* used for training of *in silico* predictors is the Achilles' heel of the same prediction tools, offering poor information on the importance of dynamic protein-protein interactions and enzymatic reactions mediating the *bona fide,* often temporal, localization of proteins. This again highlights the importance of acknowledging possible protein trafficking dynamics, upon deciphering the subcellular localization of proteins of interest. Discovering and analyzing new signal 'addresses' will help understanding the functionality of proteins and protein complexes, and aid the optimization of *in silico* predictors.

4

Technical Prescript on Microarray Data Analysis

This chapter is based on the following manuscript:

Jensen, M.K., Hagedorn, P., Rung, J.H., Collinge, D.B., Lyngkjaer, M.F.
ATAF1 Negatively regulates Abscisic Acid Signaling for Efficient Penetration Resistance in *Arabidopsis* towards *Blumeria graminis f.sp. hordei*. Manuscript in preparation. Paper II.

4.1. Abstract

Microarray technologies provide a powerful technique suitable for measuring the expression of thousands of genes simultaneously. In order to obtain good quality data from microarray applications, researchers need to plan the experiment carefully in terms of the relevant question addressed and precisely sample and process material for use. This chapter serves as a technical prescript for the initial steps for planning microarray experiments and quality assessment of collected raw data. Hence, the aim of this chapter is to elucidate a general workflow used prior to data-driven generation of hypotheses that can explain the observed phenotype, in this case potentially explaining the compromised penetration resistance observed upon mutation of the *Arabidopsis HvNAC6* homologue, *ATAF1*. Examples of diagnostic plots, pseudo-images and probe-level methods will be presented for a thorough validation of our data quality.

4.2. Work Load

The design of the laboratory workflow, planning and execution of the experiment and extraction of total RNA was performed by me. Sampling was performed by Dr. Jesper Henrik Rung and me. For complementary RNA synthesis (cRNA), labelling and hybridization, DNase treated samples were shipped to AROS A/S at Skejby Hospital, Aarhus, Denmark (www.arosab.com/). For microarray data presented in Paper II, statistical analyses and visualizations were performed by Dr. Peter Hagedorn.

4.3. Experimental Setup

4.3.1. General Considerations

By the use of genome-wide experimental biology approaches, researchers have an unprecedented opportunity to explore system-oriented principles underlying a given genetic, regulatory or physiological condition. To gain optimal benefit from experiments involving the profiling of several thousands of genes, careful planning and performance needs to be arranged for, in order to have the opportunity to give a qualified answer to the question of interest. A laboratory workflow involving (i) a qualified question using a qualified experimental setup, (ii) exact sampling procedures and (iii) fine-tuned labelling and hybridization techniques, are required. Secondly, the *in silico* part of the setup needs to be carefully assessed for researchers to obtain meaningful and robust knowledge of the biology in question.

4.3.2. Multi-factor Experimental Setup

Multi-factor experiments are those experiments involving the analysis of two or more individual experimental dimensions and their interaction (Smyth, 2005). An example could be the application of treatments in combination to genotypes of model organisms. Such designs will not only be able to reveal the individual effects of treatment and genotype, but also the combination of any given treatment and genotype. Often such experiments are designed to investigate the perturbation of genetic networks by combinations of the effects of interest, thereby allowing the initial steps of genetic network reconstruction illuminating affected - and unaffected – biological processes. To obtain good data quality from factorial designs it is of key importance to decide what the contrasts of interest and constraints of biology are, given a specific problem formulation.

In the experimental design used in this thesis, my objective was to understand the transcriptional regulation underlying the phenotypic changes of reduced penetration resistance upon attack by the non-host pathogen *Bgh* in *ataf1-1* mutant plants compared to wild-type plants (paper I). This problem formulation could be answered by simply comparing wild-type and mutant transcriptomes upon *Bgh* inoculation. However, a thorough understanding of differential regulation upon infection should ideally refer to transcript profiles of untreated samples. From an experimental setup including both wild-type and mutant plants during control and *Bgh* inoculation conditions, we were able to address the questions of (i) which genes respond to *Bgh* inoculation, (ii) which genes respond to the *ataf1-1* mutation, and (iii) which genes respond differently in mutant compared to wild-type

in any of the two factors. The first two questions address the effect of *Bgh* inoculation and *ataf1-1* mutation, respectively. The third question relates to the difference of the differences, called the *interaction effect* (figure 4.1.) (Smyth, 2005).

In order to search for answers to the observed phenotypic changes of the *ataf1-1* mutant allele upon *Bgh* inoculation, compared to wild-type accessions, we decided to perform a genome-wide analysis of the transcriptome of mutant and wild-type lines at 12 hrs after *Bgh* inoculation. This time-point coincides with commences of *Bgh* penetration attempts and *ATAF1* induction. The analysis was based on the Affymetrix GeneChip®[3]. Though regulatory targets of ATAF1 may be temporarily induced at later time-points after *Bgh* inoculation compared to *ATAF1* expression induction, the effect of mutant vs. wild-type transcript accumulation at this time-point provided a sound starting point for the elucidation of the regulatory networks underlying the phenotypic changes observed in the *ataf1-1* mutant (paper I).

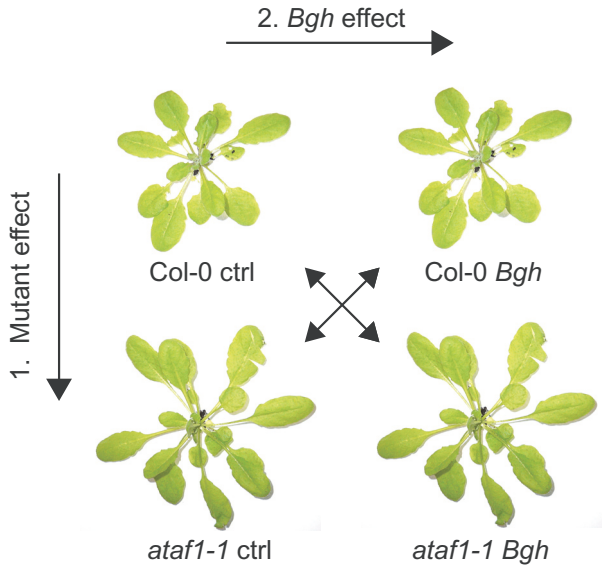


Figure 4.1. Experimental setup
A classical two-factorial design, each factor having two levels, was applied for analyzing the transcriptional changes in *ataf1-1* mutant and Col-0 wild-type accessions upon *Bgh* inoculation. From each of the four blocks, a total of eight 6-weeks old rosettes were harvested, each block including three biological replicates. The double-arrowed cross in the centre of the display indicates the potential of this setup, for the elucidation of interacting effects of genotype and treatment factors.

3 GeneChip® is a trademark of Affymetrix Inc. and will be referred to as 'GeneChip' throughout this chapter

4.4. The Affymetrix GeneChip®

The most popular microarray application to date is the measurement of genome-wide expression levels. For this purpose, GeneChips are among the best established and most widely used platforms (Ueda *et al.*, 2004). Without going into detailed technical aspects of GeneChip design, a few points need to be mentioned for a thorough understanding of the procedures described and used in this chapter. GeneChips each contain several hundred thousand oligonucleotide probes (for detailed information; www.affymetrix.com). A set of 11-20 different 25-mer oligonucleotides comprises a gene-specific probe-set used for expression index calculation. Individual probes from defined gene-specific probe-sets are located throughout the GeneChip. Hence, spatially localized artifacts on individual GeneChips will, at the most, affect only a few probes in a probeset (Bolstad *et al.*, 2005b). The obtained specificity from this design adds to the success of the GeneChip platform. In the Affymetrix system, the raw fluorescence scanning image containing probe-specific detections are stored in so-called DAT-files. Using GeneChip Operating Software (GCOS) estimates of single probe intensities are generated and stored in CEL files (Bolstad *et al.*, 2005b). CEL files contain the data on the intensity of all the probes on the GeneChip of a given sample and are often the starting point for data preprocessing and quality assessment (sections 4.5.2. and 4.5.3.).

4.5. Quality Assessment of Affymetrix GeneChip Data

4.5.1. Variation

Using delicate and costly resources like Affymetrix GeneChips, researchers are often only interested in the effects of planned experimental factors (figure 4.1.). However, different sources of variation originating from both technical and biological causes can contribute to the observed expression data (Churchill, 2002). The variation most biologists *per se* are interested in is the biological variation. This can be caused by different experimental factors like temperature, growth-stage, biotic stress treatment and so forth. The biological variation is referred to as stochastic noise, and we use statistics to deal with this issue (Huber *et al.*, 2005). It should be mentioned that stochastic noise can also arise from effects of cross hybridization to individual probes, which inevitably will negatively affect the strength of the output based on statistical analyses. The technical variation accounts for all sources of

variation during the sample processing, including RNA extraction, reverse transcription, hybridization and labelling efficiencies, and subsequent photo detection. These technical sources of variation all give rise to a global variation across the individual GeneChips used, and cannot be dealt with statistically. Therefore built-in quality-checks (QCs) are applied to avoid or minimize technical variations throughout the handling of the samples. In an optimized setup, these include visualizing spectrophotometric properties (A260/A280), verifying intact major ribosomal RNA products (28s/18s), and cRNA biotin-labelling efficiencies. Data from these procedures were generated as part of AROS A/S's commercially available Affymetrix service, and all of our 12 analyzed samples passed the standard quality check procedures. Data from these initial QCs will, however, not be presented in this chapter. Starting from raw hybridization data stored in CEL files, I will focus the attention on multiple 'post-hybridisation' quality assessment steps used prior to statistical data analyses presented in paper II.

4.5.2. Preprocessing

As part of the visual displays presented from my quality assessment will include normalized data, a short comment to what this includes will be given. Preprocessing is required to obtain gene-wise expression levels from observed probe-specific fluorescent intensity signals. This can be divided into defined tasks concerning background adjustment, summarization and normalization (Huber *et al.*, 2005). Background adjustment divides the measured hybridization intensities into a background, and a signal component. Summarization combines the individual probe-level data into an overall probe-set value (*i.e.*, gene-specific expression value), and, finally, normalization aims at the removal of all non-biological variation between arrays. These procedures have been compiled into various alternative preprocessing methods of which dChip (Li and Wong, 2001) and GCRMA (Wu *et al.*, 2004) are among the most prominent. The outcome of the preprocessing steps includes the raw probe-level data and the gene-specific overall probe-set values, which are used for subsequent quality assessment. A detailed description of the algorithms underlying the individual preprocessing methods is beyond the scope of this chapter and thesis in general.

In this project, all preprocessing and statistical data analyses were carried out using the open-source statistical language R (http://cran.r-project.org/) (Ihaka and Gentleman, 1996) using packages from Bioconductor (www.bioconductor.org) (Gentleman *et al.*, 2004). See table 4.1. for R-script and packages (libraries) used for preprocessing and quality assessment.

Table 4.1. R-Script for GeneChip quality assessment and visualization.

```
# load packages:
library(affy); library(affyPLM); library(IDPmisc)

#data import; robust probe-level fit:
CEL <- ReadAffy()
CEL.norm <- gcrma(CEL)
expr <- exprs(CEL.norm)
Pda <- fitPLM(CEL, model=PM~-1+probes+samples)

#image plots for raw and log-scaled data and residuals:
image(CEL[,4], transfo=F)
image(CEL[,4], transfo=log)
image(Pda, which=4, type="resids")

#boxplots of raw and normalized PM intensitites:
boxplot(CEL[,1:12])
boxplot(CEL.norm[,1:12])

#MvA plot of raw and normalized data:
par(mfrow = c(3, 4))
MAplot(CEL, cex = 0.75)
X11()
MAplot(CEL.norm, cex = 0.75)
```

4.5.3. Diagnostic plots of the data

Analyzing large amounts of data, including multiple factors, inevitably calls for means to assess the quality and tendencies of the data. In experiments including replicate samples, investigators are often interested in the reproducibility of the replicates, *i.e.*, are there any outliers that should be dealt with before carrying on more in-depth analysis based on the data. Quality assessment is therefore of outmost importance for detection of divergent measurements beyond the acceptable level of random fluctuations (Huber *et al.*, 2005). Three standard means to evaluate data quality will be described in the following section.

Upon successful data import into the R software, careful inspection of the *scan images* should be carried out. This exploratory visualization is an essential tool in detecting quality problems and for identifying possible outlying GeneChips. In this step individual GeneChips are scanned for regional biases and other spatial artifacts (figures 4.2A. and 4.2D.). As differences between hybridization intensities for individual probes can be orders of magnitude, log-transformed data are usually used to visualize potential artifacts (figures 4.2B. and 4.2E.).

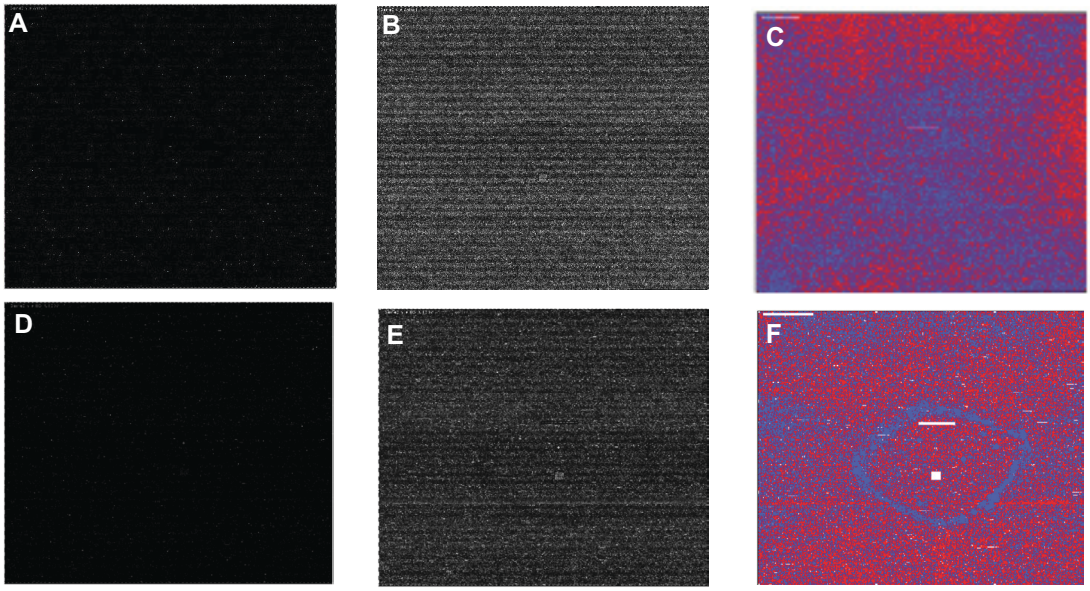


Figure 4.2. Image plots for quality diagnostics.
(A-C) A representative GeneChip from our data set, and, for comparison, (**D**-**F**) a GeneChip with a 'ring' artifact from the publicly available *AmpData* set (Bolstad *et al.*, 2005a). (**A**) and (**D**); raw probe intensities. (**B**) and (**E**); Log-scaled intensities. (**C**) and (**F**); negative (blue) and positive (red) signs of the residuals from a robust probe-level fit in the GCRMA procedure (Wu *et al.*, 2004) across all chips in the respective data sets.

Additionally, due to the structure of the probe-sets used for expression index calculation, a probe-level model (PLM) is required for the accurate summary (11-20 probes for each gene) of the expression level for a particular gene. Numerous useful tools can be derived from the output of PLM fitting procedures. One of these is the residuals plot (figures 4.2C. and 4.2F.). Due to the variability of the individual probe intensities it can sometimes be difficult to observe artifacts in the scanned image. The individual probe effect is however incorporated into most PLMs and therefore the large variability is not present in, for example, the residuals (Bolstad *et al.*, 2005b). Hence, a pseudo-image of the residuals on a representative GeneChip, from a robust PLM fit across all GeneChips has the potential to reveal spatial or whole-GeneChip artifacts of individual GeneChips. The negative (blue) and positive (red) residuals displayed randomly across the GeneChip in figure 4.2C. are not uncommon and usually inconsequential, due to the large spatial distribution of individual probes making up a gene-specific probe-set. Hence, in our data, the minor extensions of the observed artifacts from the used multi-chip PLM fit, are not believed to pose a problem to the quality of the output from subsequent data analyses. However, for comparison, a GeneChip with clearly visible local artifacts like the one shown in figures 4.2D.-F. can be an issue of concern to whether this GeneChip is of poorer quality relative to other arrays in the data set, and hence, careful considerations with respect to the origin of this artifact should be taken prior to subsequent statistical data analysis.

Another means of quality assessment is to look at the intensity distributions of individual GeneChips. The *boxplot* gives a simple summary of the distribution of probes, and provide an easy display for pinpointing outliers in spread and location (figure 4.3A.). Outliers detected from boxplots usually arise from differences in amplification and labelling reactions. In our data set no outliers are detected and smaller discrepancies are sufficiently removed by GCRMA normalization (Wu *et al.*, 2004) (figure 4.3B.).

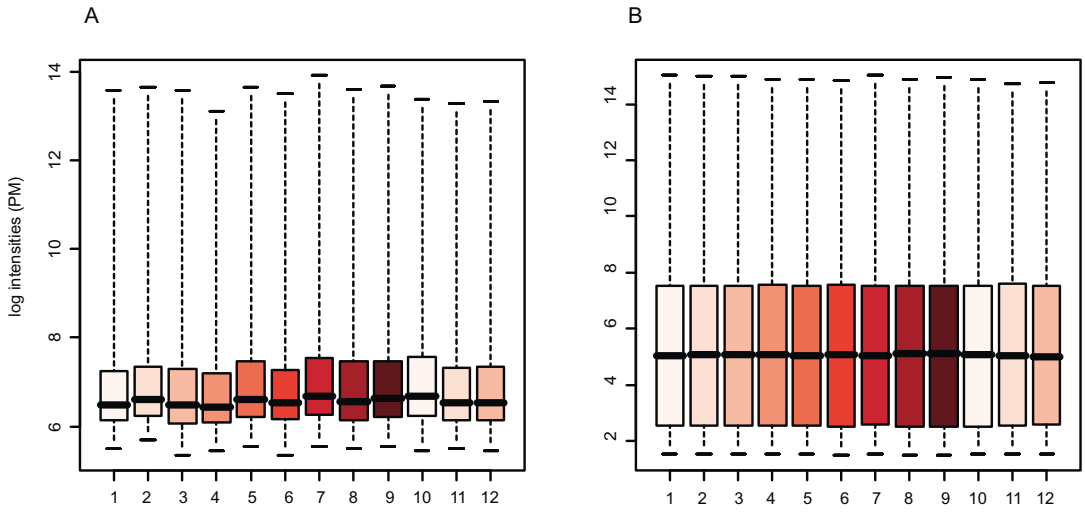


Figure 4.3. Boxplot representation of chip-wise perfect match (PM) log intensity distributions
(A) Raw data before normalization. Minor median deviations are observed. **(B)** After GCRMA (Wu ***et al.***, 2004) normalization, all 12 intensity distributions appear similar.

The *M versus A plot* (MvA) is a useful complement to the boxplots described above. MvA plots add information on the potential inter-dependencies between the quantitative levels of fold changes and the average gene expression level when comparing two GeneChips. An MvA plot of two arrays displays for each gene, the difference $M = E_2 - E_1$ of the log-expression value E_1 (Array1) and E_2 (Array2) on the y-axis against the average expression $A = (E_1 + E_2)/2$ of this particular gene on the x-axis (Knudsen *et al.*, 2003). From the correlation observed between fold changes and expression level intensities (for instance non-linear or linear), decisions concerning the choice of downstream normalization procedures can be made (Bolstad *et al.*, 2005b).

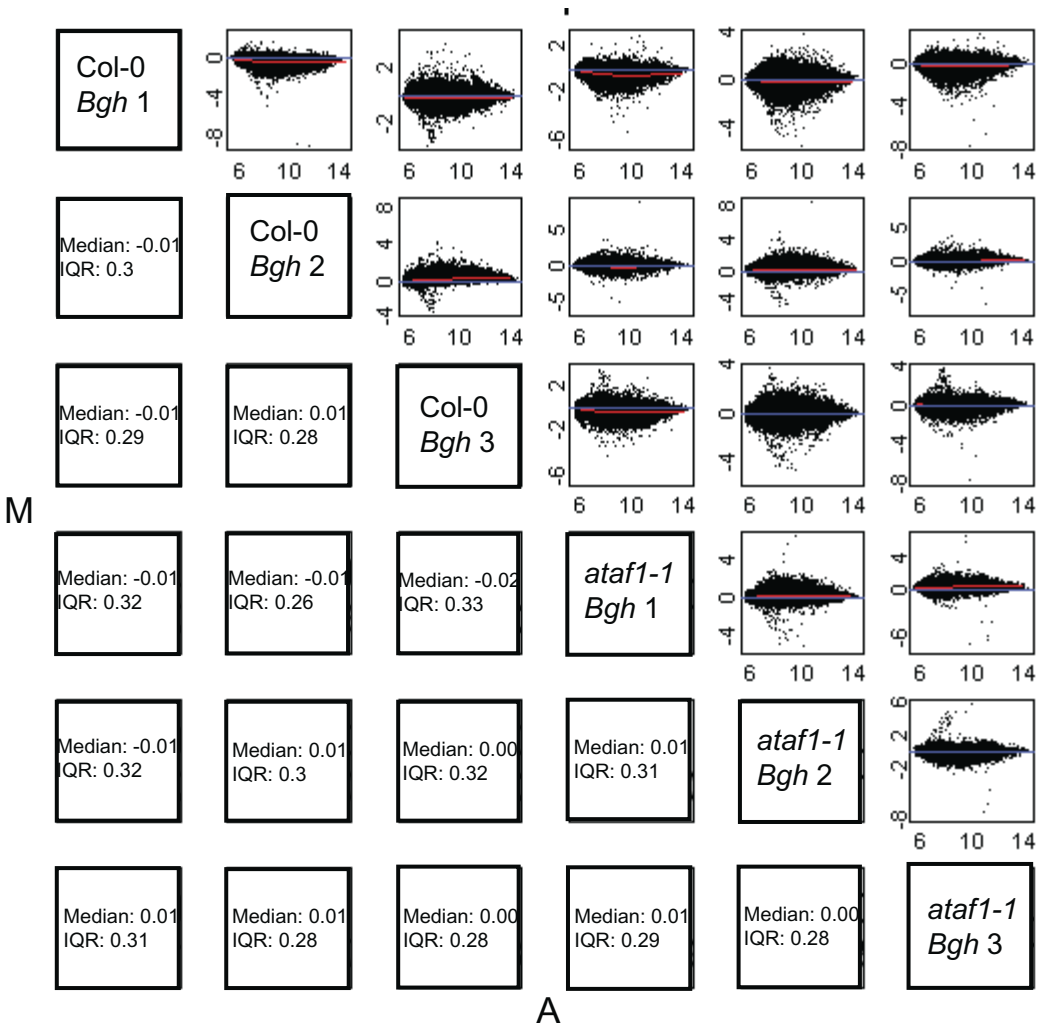


Figure 4.4.a MvA plots of raw data

MvA plots showing the inter-GeneChip intensity-dependent expression differences for six GeneChips before normalization. For each of the plots a loess curve (red) is fitted to the median M-value, highlighting deviations from the centred line at zero (blue). Additionally, the inter-quantile ranges (IQR) indicate M variability.

Figure 4.4.a and 4.4.b show the pair-wise MvA plots of 6 representative GeneChips before and after GCRMA normalization (Wu *et al.*, 2004), respectively. These figures show the plots originating from the 6 *Bgh* inoculated *Arabidopsis* samples (when only serving a representative purpose, data including all 66 pair-wise comparisons will not be provided). In each plot a loess curve (red) is fitted to the scatter plot to highlight non-centred or non-linear relationships between the individual gene expression intensities and ratios of the two GeneChips compared. Additionally, the median of all displayed gene expression values is given and the inter-quantile range (IQR), as a measure of M variability. Quality problems of

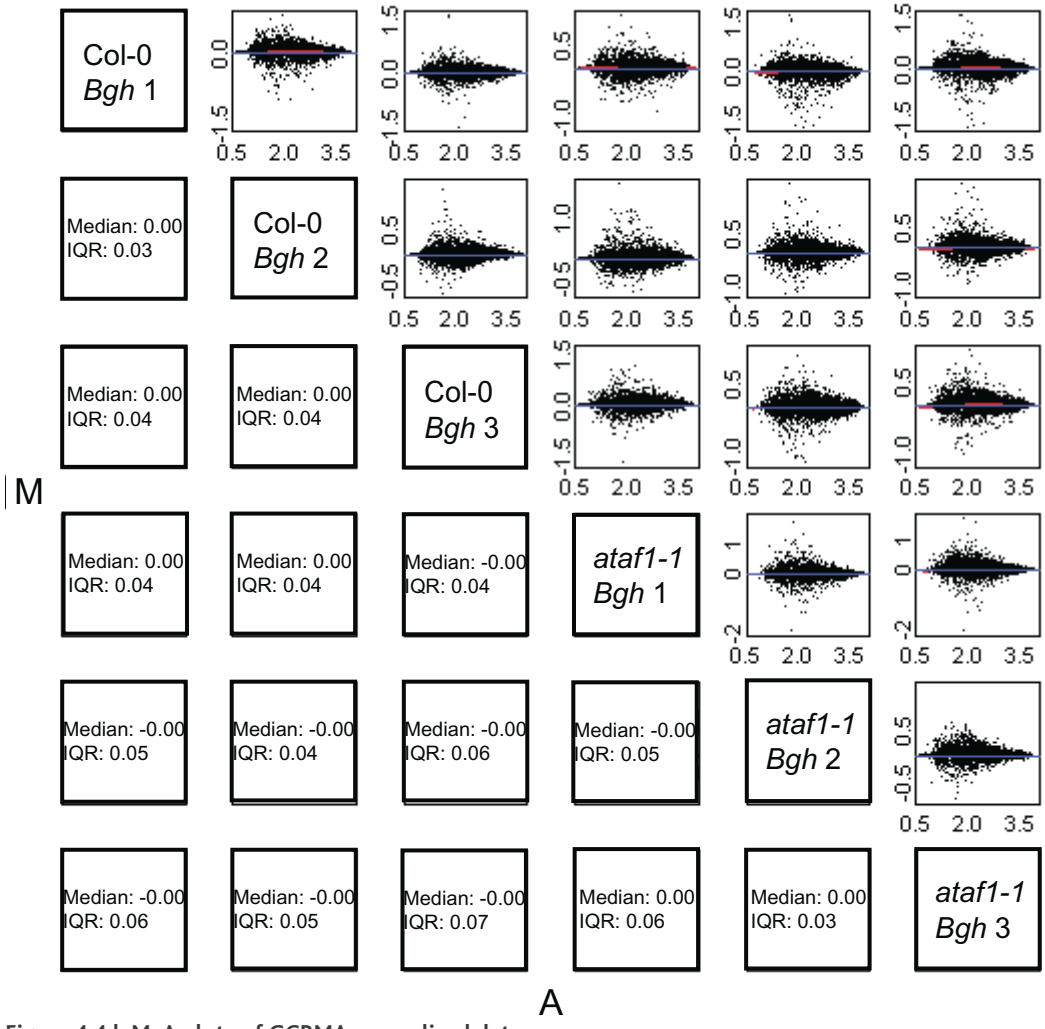


Figure 4.4.b MvA plots of GCRMA normalized data

MvA plots showing the inter-GeneChip intensity-dependent expression differences for six GeneChips after GCRMA normalization (Wu *et al.*, 2004). For each of the plots a loess curve (red) is fitted to the median M-value, highlighting deviations from the centred line at zero (blue). Additionally, the inter-quantile ranges (IQR) indicate M variability.

individual or multiple GeneChips are most apparent from an MvA plot where the loess curve oscillates a great deal or if the variability of the M values seems greater than those of other arrays in the data set. From pair-waise plots like the ones shown in figure 4.4., it is inferred that no single GeneChip suffers from inconsistent distributions of neither expression levels nor fold changes compared to the other GeneChips.

4.6. Conclusion

This chapter has highlighted the importance of experimental design and demonstrated the use of graphical displays for easy assessment of Affymetrix GeneChip data quality. Thoughtful use of summary plots such as boxplots provides useful information for preliminary assessment of array quality. MvA plots are used to visualize correlations between gene expression values and log fold changes, handy for decision-making regarding low quality arrays and normalization procedures. Further procedures involve PLM fitting and visualization of residuals from such fits. Despite the plethora of software and methods used for quality control, simple criteria which can be used to distinguish high- and low quality data are still awaited. However, the displays described in this chapter have all proved adequate for quality inspection of our array data. From these plots I conclude that there are no anomalous spatial artifacts on the chips and no corrupted single chip have been identified. Acknowledging good quality data will result in more meaningful biological results and better knowledge-based and data-driven hypotheses and decisions based on downstream statistical analyses. These matters are included in paper II.

5

Enclosed Manuscripts

I Jensen, M.K., Rung, J.H., Gregersen, P.L., Fuglsang, A.T., Gjetting, T., Hansen, M., Fuglsang, A.T., Joehnk, N., Lyngkjaer, M.F. and Collinge, D.B.
The *Hv*NAC6 Transcription Factor: A Positive regulator of Penetration resistance in Barley and *Arabidopsis*.
Accepted for publication in *Plant Molecular Biology* on the 15th of June. See appendix VII

II Jensen, M.K., Hagedorn,P., Rung, J.H., Collinge, D.B., and Lyngkjaer, M.F.
ATAF1 Negatively regulates Abscisic Acid Signaling for Efficient Penetration Resistance in *Arabidopsis* towards *Blumeria graminis* f.sp. *hordei*.
Manuscript in preparation

III Collinge, D.B., Jensen, M.K., Lyngkjaer, M.F., Rung, J.H.
How do we exploit functional genomics to understand the nature of plant defences? Barley as a case study.
Submitted for publication in *Eur. J. Plant Parthol*. Pending revision. See appendix VII

IV Moeller, A. B., Jakobsen, M. K., Jensen, M. K., Holm, P. B. and Palmgren, M. G.
The Barley Type V ATPase *Hv*P_5, a functional equivalent to *Spf1* and *MIA* secretory pathway pumps, is highly expressed during endosperm development.
Manuscript in preparation

The *Hv*NAC6 Transcription Factor: A Positive Regulator of Penetration Resistance in Barley and *Arabidopsis*

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Accepted for publication in Plant Molecular Biology on the 15th of June. See appendix VII
DOI: 10.1007/s11103-007-9204-5

Abstract Pathogens induce the expression of many genes encoding plant transcription factors, though specific knowledge of the biological function of individual transcription factors remains scarce. NAC transcription factors are encoded in plants by a gene family with proposed functions in both abiotic and biotic stress adaptation, as well as in developmental processes. In this paper, we provide convincing evidence that a barley NAC transcription factor has a direct role in regulating defence. The gene transcript was isolated by differential display from barley leaves infected with the biotrophic powdery mildew fungus, *Blumeria graminis* f. sp. *hordei* (*Bgh*). The full-length cDNA clone was obtained using 5'-RACE and termed *Hv*NAC6, due to its high similarity to the rice homologue, *Os*NAC6. Gene silencing of *HvNAC6* during *Bgh* inoculation compromises penetration resistance in barley epidermal cells towards virulent *Bgh*. Complementing the effect of *HvNAC6* gene silencing, transient overexpression of *HvNAC6* increases the occurrence of penetration resistant cells towards *Bgh* attack. Quantitative RT-PCR shows the early and transient induction of *HvNAC6* in barley epidermis upon *Bgh* infection. Additionally, our results show that the *Arabidopsis HvNAC6* homologue *ATAF1* is also induced by *Bgh* and the *ataf1-1* mutant line shows decreased penetration resistance to this nonhost pathogen. Collectively, these data suggest a conserved role of *Hv*NAC6 and ATAF1 in the regulation of penetration resistance in monocots and dicots, respectively.

Keywords ATAF1, *Blumeria graminis* f. sp. *hordei* (*Bgh*), expression profiles, *Hv*NAC6, NAC transcription factor, penetration resistance

Abbreviations

ABA	Abscisic acid
Bgh	*Blumeria graminis* f.sp. *hordei*
Bp	Base pair
EST	Expressed sequence tag
GFP	Green fluorescence protein
HR	Hypersensitive response
Hrs	Hours
Hv	*Hordeum vulgare*
NAC	NAM, ATAF1;2, CUC2
PGT	Primary germ tube
RACE	Rapid amplification of cDNA ends
RNAi	RNA interference
UTR	Untranslated region
X-GAL	β-D-galactopyranoside

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Introduction

Plants adapt to their environment by modulating a variety of physiological and biochemical mechanisms. Both abiotic and biotic stresses are perceived and stress signals transmitted through complex signalling pathways. The expression of genes that function in plant stress tolerance to a given environment can be induced or repressed by such stresses (Fujita *et al.*, 2006; Hu *et al.*, 2006) and this regulation is largely controlled by specific transcription factors. In recent years, increasing evidence has implicated a range of transcription factors in the process of directly or indirectly regulating plant defence responses towards attacking pathogens (Kim and Delaney, 2002; Li *et al.*, 2006). Transcription factors are grouped into different families on the basis of conserved structural domains involved with DNA-binding to *cis*-elements in the promoters of target genes, or other functional modular structures. One family of transcription factors is the plant-specific NAC (NAM, ATAF1,2, and CUC2) family. This large family has 106 and 149 predicted members in the *Arabidopsis* and rice genomes, respectively (Gong *et al.*, 2004; Xiong *et al.*, 2005). Members of this family share a conserved N-terminal NAC domain (Ernst *et al.*, 2004), originally characterized from the *NAM* gene from petunia and the *ATAF1*, *ATAF2* and *CUC2* genes from *Arabidopsis* (Aida *et al.*, 1997; Aida *et al.*, 1999; Souer *et al.*, 1996). The NAC family of transcription factors has been divided into several subfamilies according to sequence similarities both within and outside the conserved N-terminal NAC domain (Kikuchi *et al.*, 2000; Ooka *et al.*, 2003). One of these subfamilies, termed ATAF, has been shown to include important regulators of plants responses towards pathogen attacks (Delessert *et al.*, 2005; Selth *et al.*, 2005). An example is ATAF2, which was recently reported to repress the expression of pathogenesis-related genes in *Arabidopsis*, and *ATAF2* overexpressing plants showed a higher susceptibility towards the fungal pathogen *Fusarium oxysporum* (Delessert *et al.*, 2005). Additionally, the expression of rice, potato and *Brassica* genes, also belonging to the ATAF subfamily, have been reported to be induced by pathogen attack and wounding (Collinge and Boller, 2001; Hegedus *et al.*, 2003; Ohnishi *et al.*, 2005).

No barley NAC transcription factors of the ATAF subfamily have been characterized, though extensive work has been carried out on barley in response to biotic stresses. Especially, the interaction between barley and the biotrophic fungus *Blumeria graminis* f.sp. *hordei* (*Bgh*) has been well-studied for the characterization of physiological and transcriptional changes in barley upon pathogen attack (Caldo *et al.*, 2004; Mellersh and Heath, 2001; Thordal-Christensen and Smedegaard-Petersen, 1988). Several factors add to the success by which this economically important interaction has provided basic understandings of the mechanisms by which plants defend themselves upon pathogen attack. Firstly, the restriction of the *Bgh* interaction to the epidermal tissue, has made it ideal for performing detailed cytological and biochemical analyses of the interaction (Huckelhoven *et al.*, 1999; Zeyen and Bushnell, 1977). Secondly, the synchrony by which the biotrophic *Bgh* conidia develop upon infection of barley (Aist and Bushnell, 1991) has illuminated host spatio-temporal responses which differ between resistant and susceptible interaction (Gregersen *et al.*, 1997; Zierold *et al.*, 2005).

For the grass powdery mildew, fungal progression into plant epidermal cells is crucial for their biotrophy and asexual reproduction. In barley, *Bgh* conidia have to overcome basal penetration resistance to be able to establish their feeding apparatus, known as haustorium, and complete their asexual life cycle. Penetration resistance can be regarded as the plants first active line of defence and is conferred by cell wall appositions, known as papillae, directly beneath the sites of penetration attempts. As a second line of defence, successfully penetrated cells can undergo invasion-associated single-cell hypersensitive response (HR) (Trujillo *et al.*, 2004). Interestingly, penetration resistance and HR are typical features of both race non-specific and race-specific resistance in barley towards *Bgh* but are also features of barley nonhost resistance towards inappropriate pathogens. Nonhost resistance describes the immunity of an entire plant species against all genotypes within a given pathogen species (Thordal-Christensen, 2003), and due to the fact that plants defend themselves

against both host and nonhost pathogens by overlapping mechanisms, it is generally believed that appropriate host pathogens succeed in their infection both by avoiding recognition and suppressing basal host defences (Caldo *et al.*, 2004; Schulze-Lefert and Panstruga, 2003).

At the molecular level, considerable progress in the understanding of penetration resistance has been accomplished by the discovery of the barley *mlo*-mutant alleles conferring complete penetration resistance towards *Bgh* by effective papillae formations (Jørgensen, 1992). The mutant phenotype is dependent on *Ror1* and *Ror2* and mutations of *Ror* genes lead to partial loss of penetration resistance to *Bgh* (Freialdenhoven *et al.*, 1996). In *Arabidopsis*, resistance towards the nonhost pathogen *Bgh* is predominantly associated with the formation of papillae at sites of attempted fungal penetration and to a lesser extent, HR of individually penetrated cells (Zimmerli *et al.*, 2004). A recent screen for *Arabidopsis* mutants conferring limited penetration resistance to *Bgh* identified the *Arabidopsis Ror2* homologue *PEN1* gene which encodes a syntaxin believed to be involved in transport of defence compounds to sites of papillae formation (Assaad *et al.*, 2004; Collins *et al.*, 2003). Furthermore, Shen *et al.* have discovered that the *Hv*WRKY1 and *Hv*WRKY2 transcription factors of barley are sequestered to an MLA immune receptor, thereby relieving repression of basal penetration resistance upon virulent *Bgh* inoculation (Shen *et al.*, 2007). Mutant lines of the closest homologues in *Arabidopsis* agreed with the *HvWRKY1/2* gene silencing phenotypes (Shen *et al.*, 2007). Taken together, these discoveries have illustrated common mechanistic, genetic and possible regulatory elements of penetration resistance between monocots and dicots.

In this paper, we show an interesting candidate for a key positive regulatory element of penetration resistance. We present the isolation of three barley NAC full-length cDNA clones, and show for the first time the biological function of a barley NAC transcription factor induced by biotic stress. By performing gene silencing and overexpression of individual cDNA clones, we report that the barley *Hv*NAC6 transcription factor positively regulates penetration resistance in barley towards *Bgh*, and that a mutant allele of its cross-species homologue *ATAF1* from *Arabidopsis* phenocopies the transient *HvNAC6* gene silencing result. Additionally, we show that both of the genes are induced upon *Bgh* inoculation prior to the formation of haustoria within the epidermal plant cells. These results indicate that a NAC transcription factor from the ATAF subfamily plays an important role in regulating effective penetration resistance towards *Bgh* of host and nonhost plants.

Materials and methods

Oligonucleotides

Oligonucleotides used in this article are listed in supplemental Table S1

Plant Growth Conditions and Fungal Inoculations

Barley (*Hordeum vulgare*) P-01 Pallas near-isogenic line (Kølster *et al.*, 1986) was grown in pots of compost soil in growth chambers (16 hrs light; 70% relative humidity, approx. 20 °C constant temp.) for 7 days. *Blumeria graminis f. sp. hordei* race A6 [*Bgh*] was maintained on compatible barley P-01 by weekly transfer to fresh plants. For time-course and transient expression experiments, barley leaves were inoculated with 110 ± 18 and 80 ± 13 A6 spores mm^{-2}, respectively.

Arabidopsis thaliana wild-type Col-0 and *ataf1-1* (SALK_067648)(Lu *et al.*, 2007) mutants were grown in growth chambers at 21 °C with a 8-hrs photoperiod of 125 µE m^{-2} s^{-1}, for 6 weeks. Three-day stratification at 4 °C was used to synchronize germination. The *ataf1-1* mutant TDNA-line (SALK_067648) was obtained from NASC, Nottingham (Alonso *et al.*, 2003). Homozygotes were identified using kanamycin resistance selection and genotyping. For nonhost inoculations, *ataf1-1* and Col-0 plants were inoculated with *Bgh* using 120 ± 14 spores mm^{-2} and interaction phenotype assessed 48 hrs after inoculation. Individual *Arabidopsis-Bgh* interaction sites were characterized for (i) effective papilla formation and (ii) secondary hyphal growth formation using light microscopy, and (iii) hypersensitive-response-like cell death

using ultraviolet autofluorescence (excitation filter 365/12 nm) as described in Collins *et al*. (2003). For all inoculations, *Bgh* spores were air dispersed using a settling tower.

Fixation and clearing was carried out according to Prats *et al*. (2006a). For microscopy, inoculated leaves were treated with 0.25% Tryphan blue stain in lactoglycerol for easy visualization of fungal structures. Short epidermal cells (type A and B, (Koga *et al.*, 1990)) were used for all barley observations. For *Arabidopsis*, no restrictions were used, except for discarding attacks on trichomes.

Differential display and RACE procedure

Differential display of mRNA was performed essentially according to Liang and Pardee (Liang and Pardee, 1997), with oligodT primers end-labeled with γ-^{33}P-dATP. The mRNA was isolated from 7-days old P-02 leaves and epidermal strips, 72 hours after inoculation with *Bgh*, race C15, using Dynabeads (Invitrogen, WC, USA), following the manufacturer´s recommendations. RT-PCR was performed with oligoT_{12}CG and random hexamer primers according to Liang and Pardee (1997). Gel bands were eluted, re-amplified and cloned into the pGEM-Teasy vector (Promega, Madison, USA) and sequenced (MWG-Biotech, Ebersberg, Germany).

For RACE procedure, inner and outer *HvNAC6* gene specific primers, GSP (P12 and P13, respectively), were designed for the differential displayed 3'-end fragment. First strand synthesis was performed on mRNA using oligo-dT primers and SuperscriptII (Invitrogen, WC, USA) and the product purified using Dynabeads. For amplification of the RACE product an adaptor with a random four nucleotide 3' overhang (combination of primers P9 and P10) was added and ligated to the 3'-ends of the first strand cDNA. The PCR used primer P11 (an internal primer of P9) and the outer GSP primer (P13), followed by nested PCR with P11 and the inner GSP (P12). PCR products were cloned into the pGEM-T vector (Promega, Madison, USA)

Bioinformatic tools

Coding sequences were deduced for isolated cDNA clones, and closest homologues in rice, *Arabidopsis* and wheat were identified using tblastx (http://www.ncbi.nlm.nih.gov/BLAST/). For phylogenetic analysis of isolated full-length barley NAC members and other characterized NAC proteins, sequence alignment was performed with ClustalX (Thompson *et al.*, 1997) and a rooted phylogenetic tree built by the NJPlot software (Perrière and Gouy, 1996) using neighbour-joining (gap open and gap extension penalties of 10 and 0.2, respectively) with 1000 bootstrap trials.

Though a dynamic entity, UniGene Build #48 (http://www.ncbi.nlm.nih.gov/sites/entrez) was used for 'state-of-today' insights on (i) number of non-redundant UniGene clusters in *Bgh* infected cDNA libraries (Lib. IDs 6818, 5377, 9842, and HO) compared to total number of UniGene clusters, (ii) number of UniGene clusters with ESTs encoding NAC proteins, and (iii) number of UniGene clusters with ESTs encoding NAC proteins represented in *Bgh* infected barley libraries compared to total number of UniGene clusters with ESTs encoding NAC proteins.

Plasmid constructs

RNAi plasmids were GATEWAY cloned using pENTR1a (Invitrogen, Karlsruhe, Germany) as entry vector and plasmid pIPKTA30N (Douchkov *et al.*, 2005) as destination vector. This system allows easy generation of RNAi vectors for *in vivo* transcription of the contained inverted repeats in the destination vectors under the control of the CaMV 35s promoter. Primer pairs used for generation of individual *Hv*NAC RNAi constructs (P18/P19 for *Hv*NAC6, P20/21 for *Hv*NAC4 and P22/23 for *Hv*NAC1) amplified PCR products of 500-750 bp in size from the unconserved 3'-end of the cDNA clones. All constructs used for RNAi experiments were verified by sequencing of both inverted repeats using a combination of gene specific primers and pIPKTA30N vector specific primers P24 and P25.

For transient overexpression and subcellular localization assays, vector pIPKA9 (Dong *et al.*, 2006) was used. Overexpression vectors for *Hv*NAC1 and *Hv*NAC6 were obtained by subcloning individual full-length cDNA coding regions into *BamHI*/*PstI* digested vector using

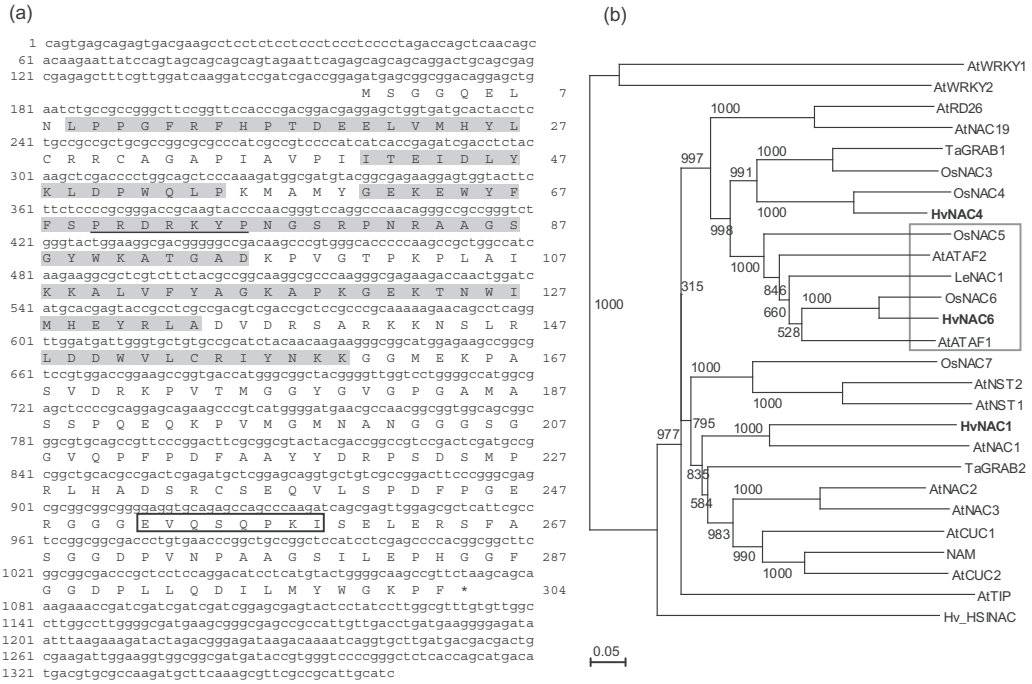

Fig. 1. Nucleotide sequence of *HvNAC6* cDNA and alignment of its putative translation product with other characterized NAC domain proteins. (a) Nucleotide and derived amino acid sequences of *HvNAC6* cDNA. The five motifs comprising the NAC domain are grey-shaded. A putative nuclear localization as predicted by PSORTII is indicated by a bold line under the sequence PRDRKYP. The ATAF subgroup-specific TAR motif is boxed. **(b)** The predicted amino acid sequence of HvNAC6 (A) and two other full-length barley cDNA clones (bold) were subjected to phylogenetic analysis together with a total of 22 other characterized NAC proteins. Additionally two WRKY proteins (AtWRKY1, AAL35282 and AtWRKY2, AAL13039) were included in the analysis as outgroup. Boot-strap values from 1000-replicates are indicated at each node. Scale bar indicates amino acid substitutions. The ATAF subfamily is highlighted in the dashed box. NAC nomenclature is as *follows: At, Arabidopsis thaliana; Hv, Hordeum vulgare; Os, Oryza sativa; Sl, Solanum lycopersicum*; Ta, *Triticum aestivum.*

primer sets P30/P31 and P1/P7, respectively. *Hv*NAC4 full-length cDNA coding region was subcloned into *XmaI* digested pIPKTA9 using primer set P32/P33. For subcellular localization studies, full-length *HvNAC6* was amplified using primers P1 and P2. Full-length GFP was amplified using primers P3 and P4. To generate *Hv*NAC6 C-terminal fusions with GFP, we used an overlap extension strategy (3x (94 °C, 4 min; 55 °C, 30 sec; 68 °C, 2 min); with subsequent addition of primers P1 and P4 followed by (94 °C, 3 min; 25x (94 °C, 45 sec; 58 °C, 45 sec; 68 °C, 2 min); 4 °C)). The chimeric product was digested with *BamHI* and *PstI* and ligated into pIPKTA9. For expression of GFP alone from pIPKTA9, primers P4 and P5 were used. The amplified product was digested with *BamHI* and cloned into pIPKTA9. All constructs were sequenced using vector specific primers for the 35S promoter and NOS terminator (primers P34 and P35, respectively).

Transient transformation assays in barley

Transient gene silencing and overexpression using a PDS-1000/He system (Bio-Rad, München, Germany) was performed essentially according

Douchkov *et al*., (2005), Coating of gold particles used for bombardment was performed according to Schweizer *et al*. (1999) except from using 2.5 M $CaCl_2$ instead of 0.5 M $Ca(NO_3)_2$. For individual treatments and replicates, 8 P-01 barley leaf segments were inoculated with virulent *Bgh* spores 4 hrs post-bombardment. Leaf segments were incubated according to Douchkov *et al*. (2005). For all transformations, plasmid pUbiGUS containing the β-Glucoronidase gene (*uidA*) under the control of the maize *Ubiquitin* promoter was used as a reporter for transformed epidermal cells (Schweizer *et al.*, 1999). Forty hours post-inoculation, leaf segments were GUS-stained and subsequently distained according to Douchkov *et al*. (2005). Using light and fluorescence microscopy, interaction phenotypes (haustorium or effective papilla formation) were scored by counting GUS-stained epidermal A and B cells (Koga *et al.*, 1990) according to Gjetting *et al*. (2004). A minimum of three independent sets of replicate transformations were used for both RNAi and overexpression assays. Differences in interaction outcome between individual treatments were analyzed using linear logistic regression analysis [proc genmod] using SAS software (SAS, Cary, USA). Inspection of residual plots confirmed in all cases that data conformed to normality.

The efficiency of RNAi was tested in barley by co-bombarding pIPKA9_*Hv*NAC6::GFP with pIPKTA30N_*Hv*NAC6 hairpin construct using a derivative of the F8 plasmid harbouring the coding sequence of DsRed (Clontech, Saint-Germain-en-Laye, France) as a reporter of transformed cells.

RNA extraction and cDNA synthesis

For barley transcript analyses, 20 abaxial epidermal peels, or 7 cm central part of 5 total first leaves, were prepared for each time-point of both control and inoculated plants for each biological replicate. Total RNA was isolated using the Aurum total RNA mini kit (Bio-Rad, Sundbyberg, Sweden) following the manufacturer´s instructions. For *Arabidopsis* transcript analysis, total RNA was isolated from three complete rosettes (numeric growth stage 3.90 according to Boyes *et al*. (2001)) for each time-point and treatment, using the RNeasy Mini kit (Qiagen, CA, USA). For all transcript analyses, a total of three biological replicates were sampled from non-overlapping growth periods. Total RNA from each sample was quality checked using the Experion® system (Bio-Rad, Sundbyberg, Sweden), verifying the presence of intact major RNA molecules (data not shown). One microgram of purified DNaseI-treated (Ambion, Austin, USA) quality-checked RNA was used for cDNA synthesis using the iScript™ cDNA Synthesis Kit (Bio-Rad, CA, USA) according to the manufacturer´s instructions.

Quantitative real-time PCR and analysis

Quantitative real-time PCR was performed according to Bedini *et al*. (2005). For analysis of *ATAF1* and *HvNAC6,* primer pairs P28/P29 and P14/P15 were used, respectively. *Ubiquitin conjugating enzyme 2* (*UBC2*, AY220735)(primers P16 and P17) and *18S* rRNA (X16077)(primers P26 and P27) were used to normalize for variation in quantity of barley and *Arabidopsis* cDNA, respectively. Relative gene expression difference (*R*) and statistical significance levels for *Bgh* inoculated samples compared to uninoculated control samples were quantified using the RESTγ software (Pfaffl, 2001). C_T-values included in the analyses were based on 3 biological replicate measurements, with two technical replicates for each time-point and treatment.

Transcriptional activation activity assay

A full-length fragment (*Hv*$NAC6_{1-304}$) of the *HvNAC6* cDNA was cloned into the *EcoRI* and *PstI* sites of the pBD-GAL4 Cam vector (Stratagene, La Jolla, CA, USA) to generate reporter plasmid, using primers P6 and P7. Additionally, a C-terminal deletion of *Hv*NAC6 (*Hv*NAC_{1-164}) was cloned into the *SmaI* and *PstI* sites of pBD-GAL4 Cam using primers P8 and P7. The obtained reporter plasmids, pBD-*Hv*$NAC6_{1-304}$ and pBD-*Hv*NAC_{1-164}, were individually transformed into *Saccharomyces cerevisiae* strain YRG-2 containing the *HIS3* and *LacZ* reporter genes, as described by the manufacturer (Stratagene, La Jolla, CA, USA). Transformants were dropped onto SD plates with or without histidine, for selection

of transactivation properties of the reporter constructs. γ-Galactosidase activity was assayed by colony-lift filter assay, using 5-bromo-4-chloro-3-indolyl γ-D-galactopyranoside (X-GAL) as substrate.

Subcellular localization

The pIPKTA9 vector (Dong *et al.*, 2006) harbouring the CaMV 35S promoter was used to transiently express GFP and *Hv*NAC6-GFP in onion (*Allium cepa*) epidermal cells (Varagona *et al.*, 1992). Onion epidermal strips on agar containing MS salt mixture (1 x MS salts (Invitrogen, WC, USA), 2% agar, 3% sucrose, pH 5.8) were bombarded using the PDS-1000/He biolistic particle delivery system (Bio-Rad, München, Germany). A total of 10 µg of each expression vector was coated onto 27.5 mg ml^{-1} 1 micron gold particles and transferred into the cells as described for transient gene silencing and OE experiments. After bombardment, petri dishes containing onion epidermal strips and placed in darkness at 22 °C for 18 hrs. Transformed cells were visualized using a Leica TCS SP2/MP confocal laser scanning microscope (Leica Microsystems, Mannheim, Germany). Excitation source for GFP was 488 nm. A total of 50 cells for each construct were observed and images processed using Leica Confocal Software. Transient studies in barley were performed as described above, and the leaves processed as with onion epidermal cells

Accession numbers

Isolated cDNA clones described in the article have been deposited to the EMBL database with the following accession numbers: *Hv*NAC6 (AM500854), *Hv*NAC1 (AM500855) and *Hv*NAC4 (AM500853).

Results

Isolation of the HvNAC6 cDNA

Using differential display of mRNA, we identified a barley NAC gene family member

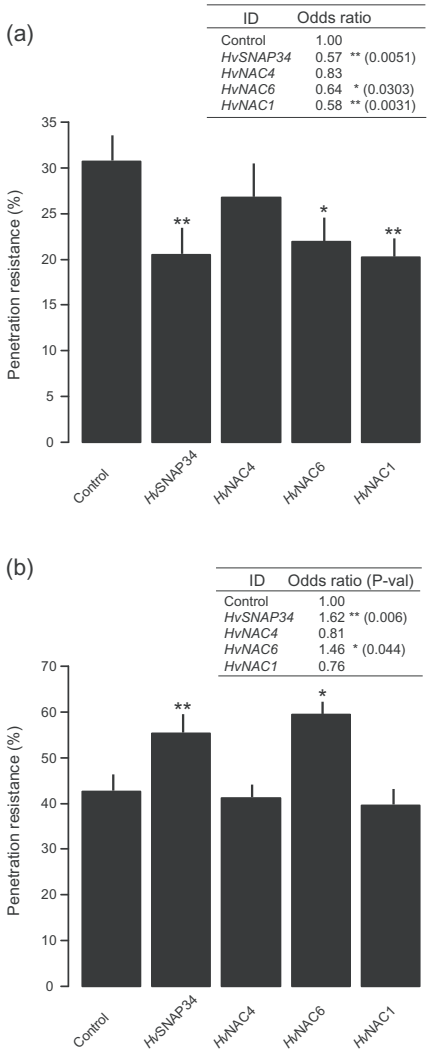

Fig. 2. *HvNAC6* regulates effective papilla formation in barley epidermis towards *Bgh*. Transient **(a)** RNAi and **(b)** overexpression of isolated NAC family members, respectively. *HvSNAP34* is a t-SNARE encoding gene, serving as a positive control (Douchkov *et al.*, 2005). Weighted mean values +/- SEM of at least three independent transformation experiments with approximately 2000 observed interactions in each panel. * and ** = statistically significant ($P < 0.05$ and $P < 0.01$, respectively) using logistic regression analysis against vector control.

Table 1. *HvNAC6* RNAi construct efficiently interferes with the accumulation of *Hv*NAC6::GFP fusion protein in barley epidermal cells.

	Fluorescent cells/shot [a]	
Constructs	**RFP + GFP**	**RFP**
pHvNAC6::GFP + pHvNAC6 RNAi	16	182
pGFP + pHvNAC6 RNAi	173	20
pHvNAC6::GFP + pHvNAC1 RNAi	152	21
pHvNAC6::GFP	163	17

[a] Mean value from two independent transformation experiments. All bombardments were performed using DsRed as transformation control.

showing differential gene transcript accumulation upon compatible interaction between barley and *Bgh* (Gregersen and Collinge, 2001). The full-length cDNA clone of 1363 bp was found to encode a single 915 bp long open reading frame, representing a complete coding region of 305 amino acids (Figure 1a)(AM500854). The encoded gene product was designated *Hv*NAC6 due to its high overall amino acid sequence similarity (87.8%) with rice *Os*NAC6 (AB028185). *Hv*NAC6 belongs to the ATAF subfamily (Figure 1b, boxed) of NAC transcription factors. In support of this classification, the C-terminal region of *Hv*NAC6 contains the transcriptional activation region (TAR) motif EVQS[E/x] PK[W/I] (Figure 1a, boxed), found only in ATAF subfamily members (Ooka *et al.*, 2003). Further analysis of the *Hv*NAC6 primary sequence using PSORTII (http://bioweb.pasteur.fr/seqanal/interfaces/psort2.html) identified a monopartite nuclear localization signal (PRDRKYP) in the third motif of the NAC domain (Figure 1a, underlined). This sequence is also found in the nuclear localized ATAF1, which is the closest *Hv*NAC6 homologue from *Arabidopsis*. Additionally, the C-terminal part of *Hv*NAC6 is Serine-rich, characteristic of activation domains of transcription factors (Sun *et al.*, 2003).

To search for additional barley NAC members expressed upon *Bgh* inoculation, a region spanning the conserved NAC domain of the *HvNAC6* cDNA (Figure 1a, grey-shaded) was used as a probe to screen a cDNA library prepared from *Bgh*-inoculated barley leaves. From this screen, two NAC clones were retrieved. Both clones contain 5′and 3′ UTRs, and poly-A tails. The two NAC clones show 88.9% and 72.2% overall sequence similarity to *Os*NAC4 (rice, AB028183) and *At*NAC1 (Arabidopsis, NM_104479), respectively. In this paper we will refer to them as *Hv*NAC4 and *Hv*NAC1 (acc. nr. AM500853 and AM500855, respectively). Phylogenetic analysis of the isolated full-length barley NAC members and previously characterized NAC members from *Arabidopsis*, rice, tomato and wheat (Figure 1b) revealed that *Hv*NAC1 and *Hv*NAC4 are related to the NAM and *Os*NAC4 subfamilies (Ooka *et al.*, 2003). These subfamilies include characterized transcriptional regulators of lateral root formation and meristem and cotyledon development, respectively (Souer *et al.*, 1996; Takada *et al.*, 2001; Xie *et al.*, 2000).

By data-mining public EST databases, including UniGene (Build #48) (http://www.ncbi.nlm.nih.gov/sites/entrez), we found that 17 out of a total of 27 NAC transcript containing UniGene clusters included transcripts from *Bgh* infected barley cDNA libraries. As *Bgh* infected barley cDNA libraries comprise approx. 38% of the total number of barley UniGene clusters (7331/19077), *Hv*NAC members could be overrepresented in *Bgh* infected libraries, making them interesting candidates for the understanding of the regulatory mechanisms involved in the barley-*Bgh* interaction.

Functional analysis of HvNAC members

As a means of determining the function of isolated *Hv*NAC members in host plant responses to *Bgh* inoculation, we performed gene silencing by RNAi and transient overexpression of individual isolated *Hv*NAC members. For RNAi studies, fragments encoding unconserved C-terminal parts of individual *Hv*NAC members were cloned into the RNAi vector pIPKTa30N (Douchkov *et al.*, 2005), to generate spliced hairpin dsRNAs *in vivo*. Individual constructs were transformed by particle delivery into barley epidermal cells together with a pUbiGUS reporter plasmid encoding β-Glucuronidase. Leaf segments were inoculated with virulent *Bgh* race A6, and a screen for possible biological effects of gene silencing of isolated NAC members was performed by scoring (i) interaction phenotypes (susceptible

or penetration resistant), (ii) haustoria number and (iii) secondary hyphae length of individual *Bgh* attacked GUS transformed cells. RNAi of *HvNAC6* and *HvNAC1* significantly reduced the frequency of penetration resistance upon powdery mildew infection of barley compared to control (Figure 2a). As a positive control, the Ror2-interacting tSNARE *HvSNAP34* (for synaptosome-associated protein of 34 kDa) gene product from barley (AY247208) (Collins *et al.*, 2003; Douchkov *et al.*, 2005), was silenced. The RNAi experiment was complemented by overexpression studies of the isolated full-length *Hv*NAC members. Using this approach, we found that *HvNAC6* significantly increased resistance of transformed epidermal cells compared to control cells, showing approx. 40% increased penetration resistance upon *Bgh* inoculation (Figure 2b). In contrast to *HvNAC6*, *HvNAC1* overexpression did not complement the observed RNAi phenotype. Overall, these results show that the ATAF subfamily member *HvNAC6* contributes to penetration resistance in barley upon inoculation with the virulent *Bgh* fungus. No significant changes were observed with respect to haustoria numbers or secondary hyphae length developed from *Bgh* penetrated cells transformed with any of our candidate genes (data not shown).

The RNAi efficiency and specificity was demonstrated *in vivo* by co-bombardment of (i) *HvNAC6::GFP* fusion construct, and (ii) *HvNAC6* or *HvNAC1* RNAi constructs. All transformations included a plasmid harbouring DsRed as the transformation control. The *HvNAC6* RNAi construct inhibited the accumulation of *Hv*NAC6::GFP fusion protein with an efficiency of approx. 90% but had no inhibitory effect on the accumulation of GFP alone (Table 1). Furthermore, no changes in *Hv*NAC6::GFP accumulation was observed when co-bombarded together with the *HvNAC1* RNAi construct, compared to cells transformed with *HvNAC6::GFP* alone. These results show that the significant changes in effective papilla formation observed for *HvNAC6* are conceived by efficient and target gene specific RNAi procedures.

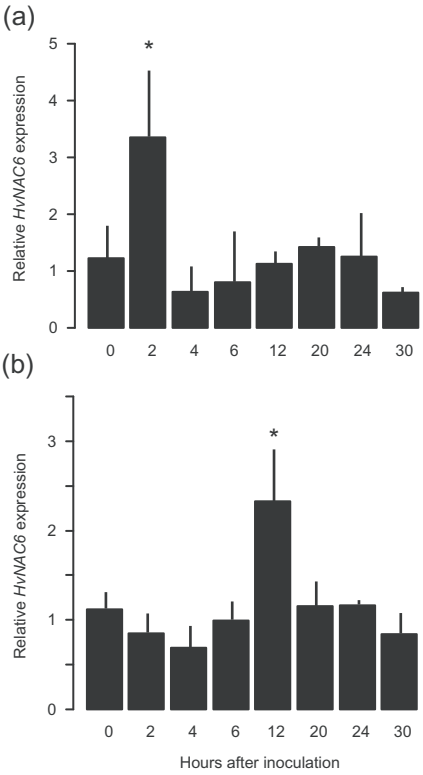

Fig. 3. *HvNAC6* is induced in barley leaves upon inoculation with virulent *Bgh* conidia. (a) *HvNAC6* is early induced in barley epidermal cells inoculated by *Bgh*, and at a later time-point in total leaf samples **(b)**. Expression values are relative to uninoculated control samples harvested at individual time-points. Three biological replicates were assayed using 20 epidermal strips **(a)** and 5 primary leaves **(b)** from each time-point and treatment. The expression values of *HvNAC6* were normalized using the expression level of *UBC2* as an internal standard. Error bars indicate standard deviation of mean normalized relative expression between inoculated and control samples. Data was analyzed using the RESTγ software to asses the significance level (Pfaffl, 2001). * = P< 0.05.

HvNAC6 expression is early induced in epidermal cells upon Bgh inoculation

Using an efficient functional approach of *HvNAC* genes expressed in *Bgh*-inoculated barley

epidermal cells, we identified *HvNAC6* as a positive regulator of penetration resistance. *Bgh* infection is restricted to the shoot epidermis and differential induction of defence related genes have been reported in the epidermis, compared to total leaf transcripts profiles (Gregersen *et al.*, 1997). Accordingly, epidermal and total leaf gene expression was investigated for the *HvNAC6* gene. Transcript profiles of *Bgh* inoculated epidermal samples showed that *HvNAC6* expression was significantly induced approx. 3-fold at 2 hrs after inoculation with *Bgh* (Figure 3a). In our studies, this time-point coincides with the first contact of the primary germ tubes of germinating *Bgh* spores with the epidermis (data not shown). The rapid and transient nature of *HvNAC6* expression indicates its potential as an early regulator of biotic stress response consistent with its role as a transcription factor. In total leaf samples (i.e. predominantly mesophyll cells), *HvNAC6* was induced approx. 2.5-fold, albeit at a later time-point (12 hrs after inoculation) compared to the epidermis-specific *HvNAC6* transcript profile (Figure 3b). The early induction in the epidermis of *HvNAC6* transcripts was presumably not resolved in the results from the total leaves due to simple dilution, since epidermal RNA probably comprises only around 5% of the total leaf RNA (Zierold *et al.*, 2005).

Self-activation assay

Several characterized NAC transcription factors have been reported to have a C-terminal transactivation region (Fujita *et al.*, 2006; Robertson, 2004). To determine whether *Hv*NAC6 encodes a functional transactivation domain in the C-terminal region, we fused both full-length (*Hv*NAC6$_{1-304}$) and a C-terminal deletion mutant (*Hv*NAC6$_{1-164}$) of the *HvNAC6* cDNA to a GAL4 DNA-binding domain expression vector and assayed each construct in yeast. The intact *Hv*NAC6 fusion activated transcription of *His3* and *LacZ* reporter genes (Figure 4), indicating that *Hv*NAC6 can function as a transcriptional activator. The C-terminal deletion mutant of the *HvNAC6* cDNA clone did not activate transcription of reporter genes. These results suggest that the transactivation region of *Hv*NAC6 is located in the C-terminal part of the

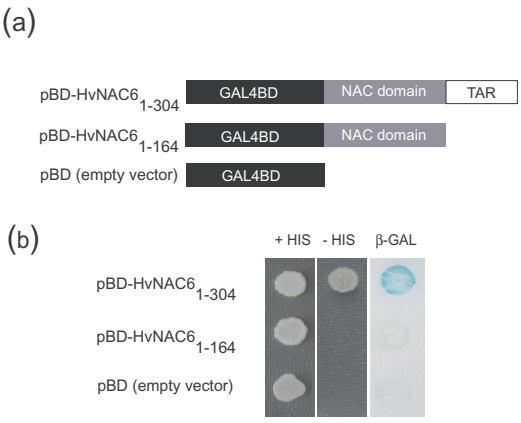

Fig. 4. Transactivation activiy of *Hv*NAC6 in yeast. (a) A schematic representation of the two *HvNAC6* cDNA clones fused to DNA sequence encoding the GAL4 DNA-binding domain in the pBD yeast expression vector. **(b)** Transactivation analysis of *Hv*NAC6 in yeast. Fusion proteins of pBD-*Hv*NAC6$_{1-304}$, pBD-HvNAC6$_{1-164}$ and pBD alone were expressed in yeast strain YRG-2. Transformatants were streaked onto SD/Trp- and SD/His- plates. Plates were incubated for 3 days and a γ-gal assay was performed to identify transactivation activity of transformants.

encoded gene product.

HvNAC6 is targeted to the nucleus

*Hv*NAC6, as a putative transcription factor, is presumably localized to the nucleus, and the presence of a nuclear localization signal predicted by the WoLF PSORTII program is consistent with this possibility (PRDRKYP, Figure 1a). To study the cellular localization of *Hv*NAC6 *in vivo*, we fused the open reading frame of *HvNAC6* cDNA to GFP and expressed it under the control of the CaMV 35S promoter. The fusion protein was transiently expressed in onion epidermal cells after biolistic delivery of vector DNA and analyzed by confocal microscopy (Varagona *et al.*, 1992). Cells expressing GFP alone displayed only diffuse cytoplasmic and nuclear staining (Figure 5, top row). In contrast, *Hv*NAC6::GFP localized exclusively to the nuclei of transformed cells (Figure 5, bottom row), which was clearly

visible as dense ovoid structures using Nomarski optics (middle column). The experiment was repeated in barley epidermal cells, with similar results obtained (Supplementary figure S1data).

The Arabidopsis mutant ataf1-1 is compromised in penetration resistance to the nonhost pathogen Bgh

The *Arabidopsis-Bgh* nonhost interaction is predominantly associated with penetration resistance associated with effective papillae formations (Figure 6a). Though a small fraction of penetration attempts succeed both in breaching the cell wall and in initiation of haustoria formation and secondary hyphal growth (Figure 6b), invasive growth is always aborted due to post-haustorial defences (Figure 6c-d) (Lipka *et al.*, 2005; Thordal-Christensen, 2003). Using this interaction, we were interested to determine whether stable mutation of the *Arabidopsis HvNAC6* homologue, *ATAF1*, would phenocopy the transient *HvNAC6* gene silencing result (Figure 2a). For this purpose, we used the SALK_067648 T-DNA line, recently reported to have a single T-DNA insertion in the third exon of the *ATAF1* ORF (Lu *et al.*, 2007). To differentiate pre- and post-haustorial defences, we quantified both (i) penetration and (ii) secondary hyphal growth frequencies, respectively. Interestingly, *ataf1-1* allowed *Bgh* to penetrate epidermal cells at a significantly higher frequency compared to wild-type plants (Figure 6e), suggesting that *ATAF1* contributes to the regulation of cell wall defences. Elevated penetration rates were associated with an increased incidence of invasion-associated cell death examined with ultraviolet light to monitor the autofluorescence resulting from hypersensitive-response-like cell death accompanying fungal penetration (Figure 6d). No changes in the initiation of hyphal development were observed comparing *ataf1-1* and wild-type plants (Figure 6b and e). Compromised nonhost resistance in the *ataf1-1* mutant line is thus inferred to arise from dysfunctional pre-haustorial defence. This result indicates that *ATAF1* is a functional homologue of *HvNAC6* conferring positive regulation of a penetration resistance mechanism, which is conserved between monocots and dicots.

ATAF1 is induced by Bgh inoculation

Several expression studies show *ATAF1* to be inducible by wounding, drought, ABA and necrotrophic pathogens (Collinge and Boller, 2001; Lu *et al.*, 2007; Schenk *et al.*, 2003). The proposed involvement of the *Arabidopsis* gene *ATAF1* in nonhost resistance prompted us to look at its expression pattern upon biotrophic *Bgh* inoculation. *ATAF1* transcript levels were > 3-fold induced at 12 hours after *Bgh* inoculation (Figure 6f). The observed expression pattern matches the *HvNAC6* expression profile in *Bgh*-inoculated total leaf samples both quantitatively and temporally. This time point coincides to *Bgh* penetration attempts of *Arabidopsis* epidermal cells (data not shown). We therefore conclude that *ATAF1* responds to several abiotic and biotic stimuli.

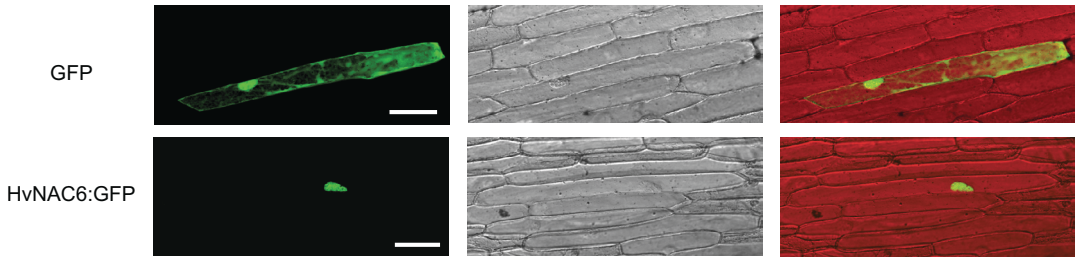

Fig. 5. *Hv*NAC6 localizes to the nucleus in onion epidermal cells. GFP alone (top row) or *Hv*NAC6:GFP (bottom row) was expressed under the control of the cauliflower mosaic virus (CaMV) 35S promoter after biolistic delivery of vector DNA. Cells were analyzed for GFP fluorescence (left column) by confocal microscopy. Differential interference contrast (DIC) images and merge images are shown in the middle and right columns, respectively. Pictures in the left and right columns are maximum intensity images of *Z*-stack projections. Scale bar = 50 mm

Discussion

Regulatory overlap between basal host resistance and nonhost resistance mediated by HvNAC6

Transcription factors are believed to play crucial roles in the transmission of pathogen-derived signals to either activate or suppress downstream defence gene expression, as well as in the regulation of synergies and antagonisms of different signaling pathways (Lorenzo *et al.*,

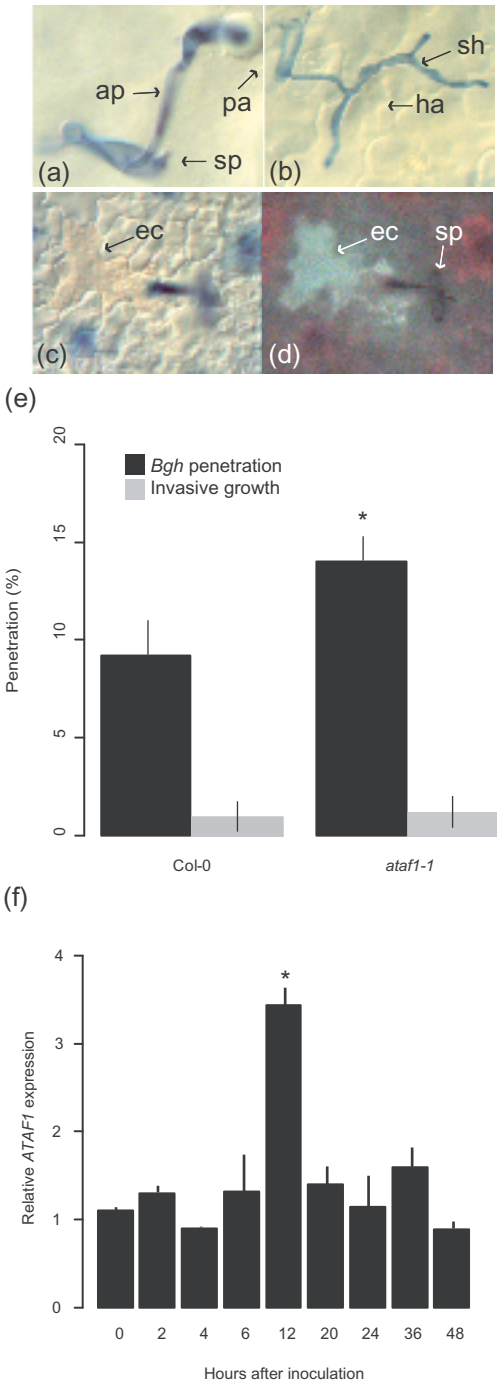

Fig. 6. ATAF1 in the *Arabidopsis-Bgh* interaction. (a) *Bgh* spores (sp) germinate and produce appresoria (ap) from which they attempt penetration. Most often penetration attempts fail due to the formation of papillae (pa). **(b)** Some *Bgh* germlings penetrating epidermal cell (ec) walls can establish a functional haustorium (ha), and secondary hyphal growth demonstrates invasive growth and nutrient uptake. **(c, d)** The hypersensitive response of penetrated cells represents the final part of nonhost resistance and is associated with the production of autofluorescent compounds. **(e)** The *ataf1-1* mutant shows increased penetration frequency towards *Bgh* attack. The figure shows weighted mean values +/- SEM of the *Bgh* penetration frequency in *Arabidopsis* 48 hours after inoculation, expressed as a percentage of total germinated spores. A total of 8 plants from each genotype were used. Asterisk denotes statistically significant difference in penetration frequency between wild-type and mutant (* = $P < 0.05$, n = 4000) using logistic regression analysis against wild-type Col-0. **(f)** *ATAF1* is induced in *Arabidopsis* leaves upon *Bgh* inoculation. Expression values are relative to uninoculated control samples. Three biological replicates were assayed with 5 plants from each time-point and treatment. The expression values of *ATAF1* were normalized using the expression level of *18S rRNA* gene as an internal standard. Error bars indicate standard deviation of mean normalized relative expression values. Statistically significant differences between inoculated and control plants were analyzed using the RESTγ software (Pfaffl, 2001). * = $P < 0.05$.

2004; McGrath *et al.*, 2005; Shen *et al.*, 2007). In this study, we carried out functional analyses of isolated barley NAC transcription factors, identified from differential display and cDNA library screening techniques of *Bgh*-infected barley epidermal tissue. Using both transient knock-down and ectopic expression approaches, we have provided evidence that *HvNAC6* positively regulates penetration resistance in barley upon inoculation with virulent *Bgh* spores. Interestingly, mutation of the *Arabidopsis HvNAC6* homologue, *ATAF1* was also compromised in penetration resistance against nonhost *Bgh*. The compromised resistance response was only observed at the level of penetration resistance, as no significant changes between *ataf1-1* mutant and wild-type lines were observed for the ability of the fungus to develop functional haustoria and initiate secondary hyphal growth. This result suggests the involvement of a NAC transcription factor in pre-haustorial defence to grass powdery mildew. Race non-specific resistance and nonhost resistance has been reported to share common genetic and mechanistic backgrounds (Trujillo *et al.*, 2004). Through this study, we speculate that a regulatory overlap between the two is conserved, though knowledge about the regulatory targets of *Hv*NAC6 and ATAF1 remains elusive. Future research will focus on possible regulatory targets using stable transformants.

Gene silencing of *HvNAC1* resulted in increased susceptibility towards *Bgh*. However, this biological effect was not complemented in our overexpression studies, maybe due to limitation of other host components needed for *Hv*NAC1 functionality in the epidermal cell layer.

Spatio-temporal expression pattern of HvNAC6

The rapid and transient appearance of *HvNAC6* transcript accumulation 2 hrs post-inoculation indicates its potential as an early regulator in the biotic stress response consistent with its role as a nuclear-localized transcription factor. This early time-point coincides with the attachment of the primary germ tube (PGT) with the barley epidermal cell (data not shown, and (Green *et al.*, 2002). PGTs have been proposed to function in water uptake through a small penetration peg exerted into the cell wall and in recognizing characteristics of the contact surface of epidermal cell (Carver *et al.*, 1995; Carver and Ingerson, 1987). Furthermore, samples extracted from total barley and *Arabidopsis* leaves 12 hrs after *Bgh* inoculation, revealed induction of *HvNAC6* and *ATAF1*, respectively. The postponed induction of *HvNAC6* in total leaf samples compared to the early epidermal induction could reflect a spatio-temporal transmission of the inducing signal, *i.e. Bgh* elicitors or endogenously generated defence-related compounds, from epidermal to mesophyll cells. Additionally, Zierold *et al.* (2005) recently proposed 5% transcript content in barley epidermal cells compared to mesophyll cells. This dilution of epidermis-specific transcripts is sufficient to explain why we do not see significant accumulation of *HvNAC6* transcripts at 2 hrs after *Bgh* inoculation in the total leaves sample. On the other hand, as penetration resistance is generally considered a single cell-autonomous event in the attacked epidermis (Nielsen *et al.*, 1999; Panstruga, 2004), the late induction in total leaf samples could indicate that *Hv*NAC6 additionally prepositions barley for defence upon detection of *Bgh* conidia. We speculate that *Hv*NAC6 show tissue-specific regulation of host physiological processes thereby attenuating *Bgh* virulence.

As is it is not practical to work with abaxial epidermal tissues of *Arabidopsis* leaves due to contamination by underlying mesophyll cells which inevitably attach to the epidermal cells, no epidermis-specific *ATAF1* transcript profile upon *Bgh* inoculation of *Arabidopsis* was obtained.

ATAF1; A node of convergence

Lu *et al.* (2007) have shown recently that the same *ATAF1* mutant allele (*ataf1-1*) exhibits a drought tolerant phenotype. They also showed that *ATAF1* gene expression is induced at early time points by both drought and abscisic acid (ABA) (Lu *et al.*, 2007). Additionally, Ohnishi *et al.* (2005) have shown that *OsNAC6* also is induced by drought and ABA. Here we show that *ATAF1* is also induced by biotic stress. Interestingly, both drought and *Bgh* attack on barley induces rapid alkalinization of the xylem and apoplast of epidermal cells, respectively (Felle *et al.*, 2004; Wilkinson and Davies, 1997). The apoplastic alkalinization of

Bgh inoculated epidermal cells peaks about 2 hrs after inoculation with *Bgh* spores and is believed to be a non-specific response to biotic stress, as it was observed for all compatible and incompatible interactions tested (Felle *et al.*, 2004). Felle *et al.* speculate that the alkalinization could obstruct fungal growth, and find that, at later time-points (approx. 20 hrs after inoculation), a more drastic alkalinization is recorded in the incompatible interactions compared to the compatible (Felle *et al.*, 2004). Drought is also a known inducer of ABA biosynthesis leading to stomatal closure in leaves (reviewed by Finkelstein, 2006). Additionally, exogenously applied ABA has been shown to suppress basal defence against the necrotrophic fungal pathogen *Fusarium oxysporum* in *Arabidopsis* and biotrophic *Bgh* in barley (Anderson *et al.*, 2004; Edwards, 1983). In this context, it is interesting that *Bgh* induces stomatal closure within 2-4 hrs after inoculation (Prats *et al.*, 2006b). This, furthermore, implies that the ATAF1 transcription factor may operate as a node of convergence in the delicate molecular and biochemical regulation of plants perception of abiotic and biotic stresses. Taken together, we hypothesize that *ATAF1*, and possibly *HvNAC6*, act downstream of ABA biosynthesis, mediating early nonspecific biotic and abiotic stress sensing signals, possibly influencing stomata movement. With respect to this, it would be interesting to study the conductance of *ataf1-1* mutants compared to wild-type plants upon *Bgh* inoculation as a measure of transpiration activity.

Recently, ATAF2 was reported to be a repressor of pathogenesis-related genes of *Arabidopsis* and stable ATAF2 overexpression mutants were more susceptible towards the necrotrophic fungal pathogen *Fusarium oxysporum* (Delessert *et al.*, 2005). ATAF2 belongs to the same phylogenetic clade as ATAF1, *i.e.* the ATAF subfamily, which comprises seven members in *Arabidopsis*. The fact that *ataf1-1* is compromised in a basal component of plant defence towards a biotrophic fungus could indicate complementing rather than redundant functions of these two close homologues. Also, it could provide further evidence for the fine-tuned and often inverse relationship between plant defence signaling towards necrotrophic and biotrophic microbes (Glazebrook, 2005; Govrin and Levine, 2002). In this context, it is interesting that Delessert *et al*., (2005) do not see any induction of *ATAF2* by ABA, demonstrating putative complementing biological effects of *ATAF1* and *ATAF2* with respect to this plant hormone. Since *Arabidopsis* is a nonhost to *Bgh,* we speculate that ATAF1 serves as a direct or indirect transcriptional activator, as opposed to transcriptional repressor like ATAF2, of defence related genes. Alternatively, the *ataf1-1* mutant limits the components allocated to maintain penetration resistance by attenuating processes affecting this line of defence.

Acknowledgements

Authors would like to thank Drs. Patrick Schweizer and Dimitar Doutchkov for the empty and *Hv*SNAP34 overexpression pIPKA9 vectors, Michael Næsby for optimization of RACE amplification procedure, NASC for providing the T-DNA insertion line, and Hans Thordal-Christensen for fruitful discussions and for reading the manuscript. MKJ was supported by a PhD scholarship from the University of Copenhagen, Faculty of Life Sciences (formerly the Royal Veterinary and Agricultural University) and research financed by a Danish Research Council grant "Cell specific analysis of host-plant responses to pathogens using a functional genomic approach" SJVF 23-03-0167 (to MFL and DBC).

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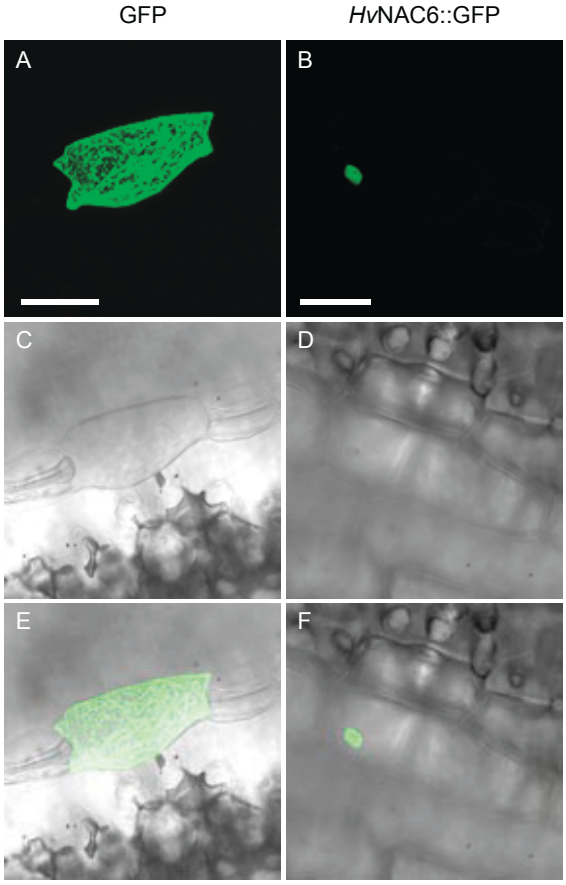
Table S1. Oligonucletide sequences

Name	Sequence
P1	5'-AGGATCCATGAGCGGCGGACAGGAGC-3'
P2	5'-CTTGCTCACCATGAACGGCTTGCCCCAGTA-3'
P3	5'-GGCAAGCCGTTCATGGTGAGCAAGGGCGAG-3'
P4	5'-AAACTGCAGTTACTTGTACAGCTCGTCCATG-3'
P5	5'-AGGATCCATGGTGAGCAAGGGC-3'
P6	5'-TATGAATTCATGAGCGGCCGACAGGAG-3'
P7	5'-ATACTGCAGTTATTAGAACGGCTTGCCCCA-3'
P8	5'-TATCCCGGGATGAGCGGCCGACAGGAG-3'
P9	5'-TCAGTGAGCAGAGTGACNNNN-3'
P10	5'-^{P}GTCACTCTGCTCACTGA-NH_2-3'
P11	5'-CCAGTGAGCAGAGTGACG-3'
P12	5'-CGGCGAACGCTTTGAAGCATCTTGG-3'
P13	5'-GATGATGCAATGCGGCGAAC-3'
P14	5'- GCAGAGCCAGCCCAAGATCAG-3'
P15	5'- GGTCGCCGCCGAAGCC-3'
P16	5'- TCTCGTCCCTGAGATTGCCCACAT-3'
P17	5'- TTTCTCGGGACAGCAACACAATCTTCT-3'
P18	5'- CACCATGTACGGCGAGAAGGAGTG-3'
P19	5'-TTGCCCCAGTACATGAGGAT-3'
P20	5'-CACCAGGCGCTCGTCTTCTACTCC-3'
P21	5'- CATCTCAGAATGGTGGCAAG-3'
P22	5'-CACCCACTCAAGAAACATTGCACC-3'
P23	5'- TCATGCACGAATACAGGTTGGAG-3'
P24	5'-TCCTTCGCAAGACCCTTCCTCT-3'
P25	5'-TTATAACACATGTGCAGGTGACATG-3'
P26	5'-CGGCTACCACATCCAAGGAA-3'
P27	5'- GCTGGAATTACCGCGGCT-3'
P28	5'-GCCTCTCGGTAGCTCCTTTTTTGT-3'
P29	5'-TCAGGCTGGATGATTGGGTTCTCT-3'
P30	5'-AGGATCCATGTCGATGAGCTTCTT-3'
P31	5'- AAACTGCAGCTAGTTGTGGTTCCA-3'
P32	5'- AACCCGGGATGACGGCGGAGGCGG-3'
P33	5'-AACCCGGGTCACCATCTGCCCCATGG-3'
P34	5'- TGGTCCCAAAGATGGACCCC-3'
P35	5'- TACCCACTGGATTTTGGTTTTAGG-3'



Supplementary figure S1. ***Hv*NAC6 localizes to the nucleus of barley epidermal cells**. GFP (**A**, **C**, and **E**) and *Hv*NAC6::GFP (**B**, **D**, and **F**) was expressed under the CaMV *35s* promoter after biolistic delivery of vector DNA. Fluorescence (**A** and **B**), briegth field (**C** and **D**), and merged (**E** and **F**) images were captured 18-40 hrs after particle bombardment without any changes in fluorescence distribution. Scale bar = 25 μm.

II

ATAF1 negatively regulates ABA signalling for efficient penetration resistance in *Arabidopsis* towards *Blumeria graminis* f.sp. *hordei*

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Manuscript in preparation

Abstract

ATAF1 is a member of the large gene family encoding plant-specific NAC transcription factors. *ATAF1* is induced in response to various abiotic and biotic stimuli in *Arabidopsis thaliana*. We have previously shown that modulation of ATAF1 levels compromises penetration resistance in *Arabidopsis* towards the non-host pathogen *Blumeria graminis* f.sp. *hordei* (*Bgh*). In this study, we have used genome-wide transcript profiling for the characterization of signalling perturbations in the *ataf1-1* mutant upon *Bgh* inoculation. Comparative transcriptomic analyses identified sets of ABA-responsive genes to be significantly differentially regulated in the *ataf1-1* mutant compared to wild type plants, particularly upon *Bgh* inoculation. Furthermore, we show that the *ataf1-1* mutant allele confers hypersensitivity to the phytohormone abscisic acid (ABA) and that ABA is a negative regulator of penetration resistance in *Arabidopsis* towards *Bgh*. We therefore propose *ATAF1* as a novel negative regulator of ABA signalling in *Arabidopsis*, required for effective penetration resistance.

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Introduction

Being sessile organisms, plants have to adapt to changing environmental conditions in order to survive. The wide variety of abiotic and biotic stresses plants are subjected to calls for a multitude of plant responses to adapt to suboptimal environments. Among the components involved in early sensing of environmental stresses are the endogenous plant phytohormones, abscisic acid (ABA), jasmonic acid (JA), ethylene and salicylic acid (SA). Several studies have identified components of the hormone-dependent responses of plants to challenging environmental perturbations (Anderson *et al.*, 2004;Lorenzo *et al.*, 2004). Generally, biotic stresses from necrotrophic and biotrophic microbes are perceived in plants by JA/ET- and SA-dependent signalling cascades, respectively (reviewed by Glazebrook, 2005). ABA, on the other hand, has been studied primarily with respect to its important roles in abiotic stresses, in particular the regulation of responses to drought, low temperature and salinity, as well as in plant growth and development (reviewed by Shinozaki *et al*., 2003).

Though generalizations highlight important correlations, the mechanisms controlling biotic and abiotic stress responses do not confer linearly isolated signalling pathways, but rely on complex regulatory circuits of feed-back loops, as well as antagonistic and synergistic interactions (Gupta

et al., 2000;Spoel *et al.*, 2003;Turner *et al.*, 2002). Recent studies suggest that, apart from abiotic stress responses, ABA influences biotic stress responses and, moreover, in doing so interferes with SA- and JA-signalling (Anderson *et al.*, 2004;Mohr and Cahill, 2006). This has been illustrated by strong ABA-dependent reduction of transcript levels of JA/ET-responsive defence genes indicating antagonistic signalling mediated by ABA on JA/ET-mediated biotic stress perception (Anderson *et al.*, 2004). Additionally, *Arabidopsis jasmonic acid resistant1* (*jar1*) and *jasmonic acid insensitive4* (*jin4*) show hypersensitivity to ABA inhibition of germination (Berger *et al.*, 1996;Staswick *et al.*, 1992), adding evidence to the notion that interactions between ABA and JA signalling are antagonistic. Furthermore, ABA treatment prior to infection increased the susceptibility of *Arabidopsis* to avirulent *Pseudomonas syringae* pv *tomato*, presumably by interference with SA-mediated defence responses and SA hormone levels (Mohr and Cahill, 2003;Mohr and Cahill, 2006).

More recently, increasing evidence supports the function of ABA in direct or indirect responses towards a range of pathogens (Adie *et al.*, 2007;Kaliff *et al.*, 2007;Mohr and Cahill, 2006;Torres-Zabala *et al.*, 2007). On the basis of exogenous application of ABA, inhibition of ABA-biosynthesis, or the use of ABA-deficient mutants, several studies highlight the positive correlation between enhanced ABA levels and increased susceptibility to pathogens (Audenaert *et al.*, 2002;Edwards, 1983;Gupta *et al.*, 2000;Mohr and Cahill, 2003;Ward *et al.*, 1989). Most notably, Torres-Zabala and co-workers recently showed that bacterial infection by *Pseudomonas syringae* pv. *tomato* manipulates endogenous ABA biosynthetic and signalling pathways as a virulence mechanism (Torres-Zabala *et al.*, 2007). Thus numerous studies indicate that ABA exerts a negative role in plant defence responses. However, reports from tobacco and *Arabidopsis*, show a positive correlation between ABA levels and plant defence towards virus and necrotrophic fungi, respectively (Adie *et al.*, 2007;Kaliff *et al.*, 2007;Ton and Mauch-Mani, 2004;Whenham *et al.*, 1986), suggesting ABA as a regulator of defence responses in a stimuli-dependent manner. Overall, the nature of the presumable regulatory dual-functionality of ABA with respect to plant defence remains to be deciphered.

One way to approach ABA functionality is by genome-wide transcript profiles of ABA-responsive genes (Huang *et al.*, 2007;Rabbani *et al.*, 2003;Seki *et al.*, 2002). Huang *et al*. (2007) estimated that 14% of *Arabidopsis* genes are ABA-regulated in aerial tissues, highlighting ABA multipotency (Huang *et al.*, 2007). One gene that has been identified as ABA-responsive is the NAC transcription factor–encoding gene *ATAF1* (Lu *et al.*, 2007). The plant-specific NAC transcription factors comprise on of the largest families of transcription factors (Riano-Pachon *et al.*, 2007;Riechmann *et al.*, 2000), and functional studies have characterized members involved in both development and stress related responses (Delessert *et al.*, 2005;Fujita *et al.*, 2004;Takada *et al.*, 2001). The NAC family has been divided into several subfamilies (Ooka *et al.*, 2003), of which the ATAF subfamily is implicated in the regulation of biotic stress responses. Over-expression of members of this family has resulted in increased plant resistance towards the biotrophic fungal pathogens *Fusarium oxysporum* and *Blumeria graminis* f.sp. *graminis* [*Bgh*] (Delessert *et al.*, 2005;Jensen *et al.*, 2007). Additionally, *ATAF1* and its orthologues are known to be induced by wounding and attack by pathogens (Collinge and Boller, 2001;Jensen *et al.*, 2007;Schenk *et al.*, 2003). Furthermore, the *ataf1-1* mutant allele represents a T-DNA insertion in the third exon of the *ATAF1* ORF, and *ataf1-1* plants exhibit drought tolerance, presumably by relieving the repression of stress-responsive genes in *Arabidopsis* (Lu *et al.*, 2007). Overall, these studies demonstrate the regulatory potential of *ATAF1* in both abiotic and biotic stress sensing.

Previously, we have shown that the barley *ATAF1* homologue *HvNAC6* is a positive regulator of basal resistance towards *Bgh* (Jensen *et al.*, 2007). Basal, or non-host, resistance in *Arabidopsis* towards *Bgh* attack is predominantly associated with deposition of callose-containing appositions at sites of *Bgh* penetration sites leading to penetration resistance (Zimmerli *et al.*, 2004), which is a widely recognized early response of plants to both virulent and avirulent microbial attack, potentially impeding fungal entry to the

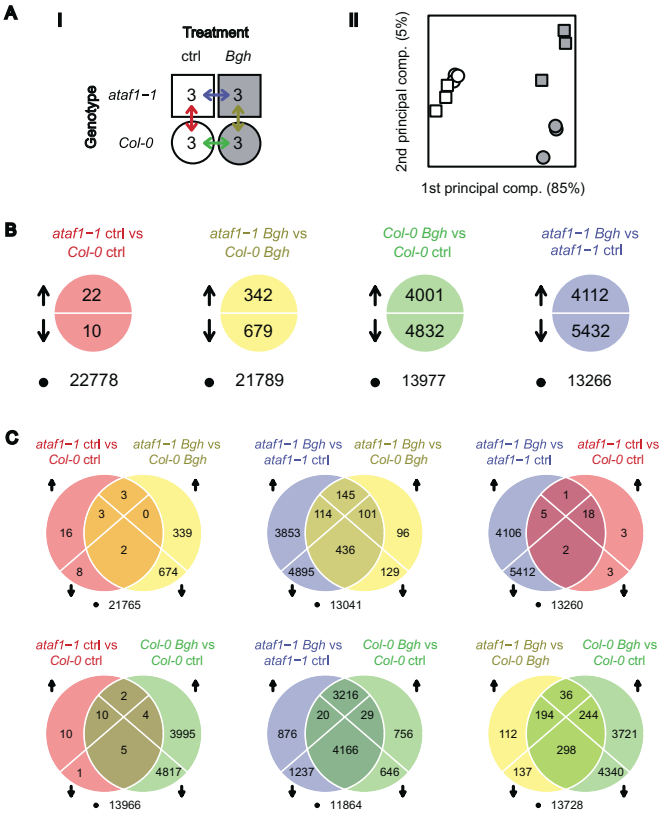

Figure 1. Overview of microarray analysis results. (**A, I**) Illustration of the 2*2 factorial design: Three independent biologica samples are taken from *ataf1-1* mutants and from Col-0 wildtype plants, both when inoculated by barley powdery mildew (*Bgh*) anc without inoculation (ctrl). The double-arrows denote the contrasts investigated. (**A, II**) PCA plot showing the first two principa components using all genes on the microarray. The percent variance explained by each component is shown on the axis label Squares refer to *ataf1-1* mutant samples and circles to Col-0 wildtype samples. Grey fill indicate *Bgh* inoculation and white fil indicate no inoculation. (**B**) Significantly (FDR < 0.05) up- or downregulated genes for each of the four contrasts investigated. (**C** Venn diagrams showing the overlapping and non-overlapping sets of genes between any two contrasts presented in (**B**).

plant (Thordal-Christensen, 2003). In *Arabidopsis*, a small percentage of successful *Bgh* penetration attempts will evoke a subsequent post-invasive hypersensitive response and development of all *Bgh* germlings will come to a complete arrest (Collins *et al.*, 2003;Zimmerli *et al.*, 2004). We have shown that *ATAF1* is induced by the non-host pathogen *Bgh* and that *ataf1-1* phenocopies the transient gene silencing of *HvNAC6* in barley, exhibiting compromised penetration resistance towards *Bgh* (Jensen *et al.*, 2007).

In this study, we have undertaken expression profiling as a means of elucidating the possible regulatory changes underlying the observed phenotype of compromised penetration resistance in *ataf1-1* mutant plants compared to wild-type plants. Building upon transcriptome changes during *Arabidopsis-Bgh* interaction, we now present results supporting *Bgh*-mediated manipulation of ABA biosynthesis and signalling pathways as a core mechanism for *Bgh* to overcome *Arabidopsis* penetration resistance in the *ataf1-1* mutant. We also demonstrate that alteration of *ATAF1* expression modulates

ABA responsiveness on germination and seedling development. Furthermore, we show that exogenous application of ABA mimics the *ataf1-1* mutant phenotype upon *Bgh* inoculation, compromising penetration resistance in *Arabidopsis* towards *Bgh*. This implicates *ATAF1* as a novel negative regulator of ABA signalling which is required for maintenance of effective penetration resistance towards *Bgh*. The *ATAF1*-dependent perturbations in transcripts of SA- and JA-signalling marker genes upon *Bgh* inoculation furthermore highlights the complex interplay between ABA, and JA and SA signalling in the *Arabidopsis*-*Bgh* interaction.

Results

Compromised penetration resistance in Arabidopsis ataf1-1 mutants towards non-host Bgh is associated with the coordinated regulation of ABA-responsive genes.

To identify components of reduced penetration resistance in *ataf1-1* mutant plants infected with the non-host pathogen *Bgh* (Jensen *et al.*, 2007), we investigated global changes in gene expression by microarray analysis. RNA was isolated from rosettes of 6-week old control (ctrl) and *Bgh* inoculated (*Bgh*) Col-0 and *ataf1-1* plants 12 hours after inoculation (hai) with *Bgh*. At this time-point, the *ATAF1* transcripts accumulates in *Bgh* inoculated plants and the synchronously developed *Bgh* germlings are commencing penetration attempts of *Arabidopsis* epidermal cells (Jensen *et al.*, 2007). This setup therefore allows determination of transcriptome changes during mechanical commence of penetration resistance and modulation of *ATAF1* levels.

Three biological replicates were sampled for each of the four conditions (Figure 1a, I). To visualize the relationships between the 12 samples without imposing any structure on the data beforehand, we reduced the dimensionality of the data to two dimensions using principal component analysis (Figure 1a, II). The two first principle components accounted for approx. 90% of the variation in the dataset and clearly separate *Bgh*-treated samples from ctrl samples (first principal component), as well as wild-type samples from *ataf1-1* mutant samples (second principal component).

Using a Bayes moderated hypothesis test (Smyth, 2005), we identified genes differentially expressed genes between conditions as shown in Figure 1b. To elucidate the differences in *ataf1-1* transcriptional regulations compared to wild-type regulations, the first two contrasts in Figure 1b are of primary interest. Notice that a lot fewer genes (32) are differentially regulated between Col-0 and *ataf1-1* plants without *Bgh* treatment (first contrast), than between Col-0 and *ataf1-1* plants with *Bgh* treatment (1021 genes, second contrast), correlating with induced *ATAF1* levels upon *Bgh* inoculation. In total, 1045 genes differ in expression between Col-0 and *ataf1-1* plants, only 8 of which overlap between the two contrasts (Figure 1c, top-left). This clearly indicates focused *ATAF1*-dependent transcript regulation in response to *Bgh* inoculation.

ATAF1 is acknowledged as an ABA-inducible gene, implicated in drought tolerance (Lu *et al.*, 2007, and our studies appendix VI). Interestingly, among the 1045 genes that the above analysis shows to be associated with *ataf1-1* (Figure 1c, top-left), around 30% of them (294) are ABA-responsive genes, which is a clear over-representation compared to full-genome ABA-responsiveness (Fisher's exact test; $P < 2.2e\text{-}12$). For these 294 genes we furthermore identified their regulatory behaviour in a well-studied ABA-mediated biological process from publicly available GeneChip data on drought responses. Using all three datasets (the *ataf1-1*/*Bgh* dataset generated in the present study, the ABA treatment study, and the drought study), we clustered the 294 genes to group genes with similar expression profiles together (Figure 2).

Five main clusters of genes can be identified in Figure 2. Focusing on cluster II consisting of 45 genes significantly up-regulated in both *ataf1-1* mutant plants and ABA treated plants, we found that it includes the rate-limiting ABA biosynthetic aldehyde oxidase gene product (AAO3), which catalyzes the ABA biosynthesis rate-limiting step from abscisic aldehyde to ABA (Barrero *et al.*, 2006). Additionally, a polyubiquitin (UBQ3) was identified. Interestingly, ubiquitin has been shown to be involved with the post-translational destruction of the ABA signalling regulator ABI3 (Zhang *et al.*, 2005). Apart from these ABA-related gene products, several unknown

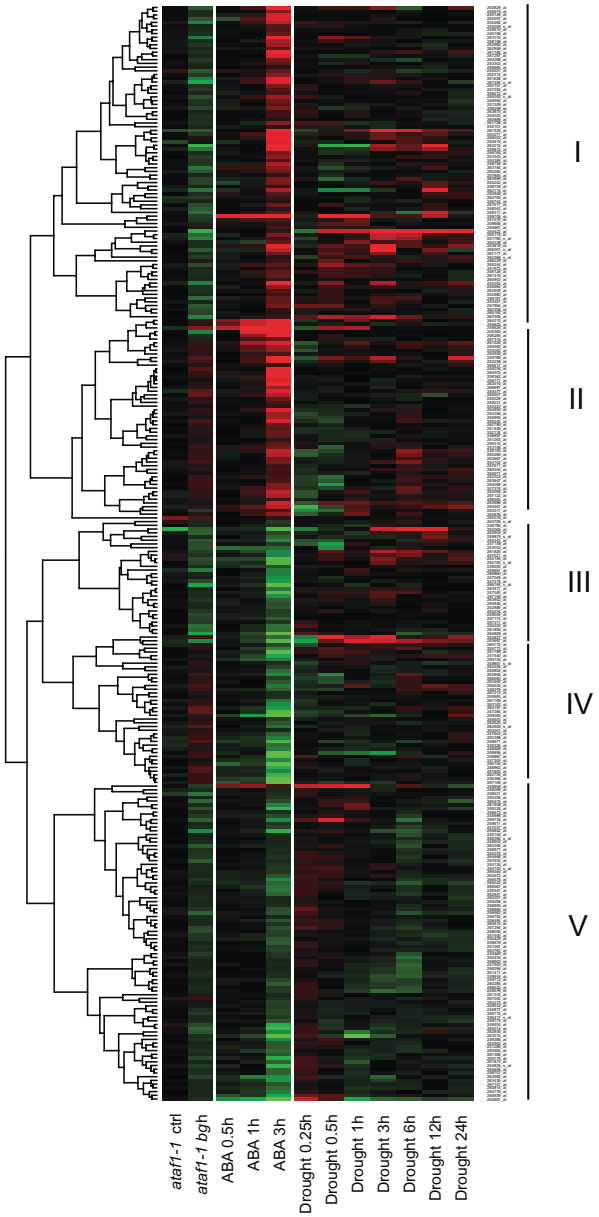

Figure 2. Hierarchical clustering (complete linkage, centered Pearson correlation as distance measure) of the 293 genes that are both differentially regulated between the *ataf1-1* mutant and the Col-0 wildtype, and significantly induced or repressed by ABA. Data for both of these conditions as well as in response to drought are included in the clustering. For a given sample, genes induced relative to their control are coloured red, those repressed are coloured green, and genes unchanged in their expression levels are coloured black. To the right, major co-expressing clusters are designated I-V.

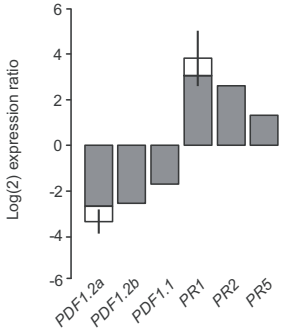

Figure 3. JA/ET- and SA-signaling marker genes show *ATAF1*-dependent expression perturbations upon *Bgh* inoculation. Log(2) expression ratios of several defence related genes in the *ataf1-1* mutant compared with Col-0, both inoculated with *Bgh*. Mean values from three biological replicates are shown. All genes comprise to FDR < 0.05 cut-off. White boxes added to *PDF1.2a* and *PR1* bars refer to mean expression ratios as measured by QRT-PCR. Error bars represents standard deviation of the mean from three biological replicates. *PDF1.2a*: At5g44420; *PDF1.2b*: At2g26020; *PDF1.1*: At1g75830; *PR1*: At2g14610; *PR2*: At3g57260; *PR5*: At1g75040.

and putative expressed proteins were identified (supplementary table I). Analysis of the proximal 1 kb promoter region of the 45 co-regulated genes (cluster II) using TOUCAN MotifScanner (Aerts *et al.*, 2003) identified that approx. 45% contain one or more ABA responsive elements (ABRE: ACGTG[GT]C) and more than 90% contain one or more NAC core binding sites (NACBS: [TA][GT][TACG]CGT[GA])(Olsen *et al.*, 2005). The significant over-representation of these motifs was confirmed using POBO (Kankainen and Holm, 2004). Furthermore, as this cluster includes several genes which are down-regulated rapidly under drought conditions, a known inducer of ABA production, it is inferred that *ataf1-1* indeed affects the expression of genes associated with both biotic and abiotic stress responsive genes. In summary, our analysis suggested that one mechanism of ATAF1 action is to attenuate components of the ABA signalling and biosynthesis pathways, *i.e. ataf1-1* mutant plants significantly de-repress, or induce, the expression of these components.

ATAF1-dependent expression perturbations of SA- and JA/ET-signalling marker genes upon Bgh inoculation

Antagonistic interplay of ABA- and JA/ET-mediated signalling in *Arabidopsis* under biotic stress has been described by Anderson *et al*. (2004). From our analyzed microarray transcript profiles (Figure 1c), we were interested to see whether this holds true for the non-host *Arabidopsis-Bgh* pathosystem. Twelve hours after inoculation correlates with the timing of early penetration attempts from *Bgh* appressorial hyphae of *Arabidopsis* epidermal cells. The epidermal cell wall breaching induces JA/ET-activated transcription of plant defensins in wild-type plants. Interestingly, we observe that induction of JA/ET-signalling marker genes is abolished in *ataf1-1* mutant compared to Col-0 upon *Bgh* inoculation (Figure 1c, genes included in mid bottom panel), whereas SA-signalling marker genes are de-repressed, or induced, in the *ataf1-1* mutant upon *Bgh* inoculation. *PDF1.2a* and *PR1* gene expression ratios were confirmed by QRT-PCR (Figure 3, white bars added). Hence, we infer *Bgh*-dependent induction or de-repression of PR genes in mutant plants compared with wild-type plants, whereas JA/ET signalling marker genes only were induced in wild-type plants.

The ataf1-1 mutant is hypersensitive to ABA

Lu and co-workers have shown that the mutant allele *ataf1-1* confers increased drought tolerance compared to wild-type accessions, and that *ATAF1* is induced by ABA (Lu *et al.*, 2007). These results were verified under our conditions (data not shown, see appendix VI). Previous reports have demonstrated that high concentrations of ABA inhibit the germination of seeds (Leung and Giraudat, 1998). To evaluate the effect of the *ataf1-1* mutation on ABA sensitivity, we germinated Col-0 and *ataf1-1* mutant plants in various concentrations of ABA (Figure 4). Compared to wild-type seeds, the *ataf1-1* seeds were more sensitive to ABA inhibition of germination (Figure 4), especially at 0.5 µM ABA. At higher concentrations, the germination of both wild-type and *ataf1-1* mutant seeds was greatly inhibited. In addition to germination,

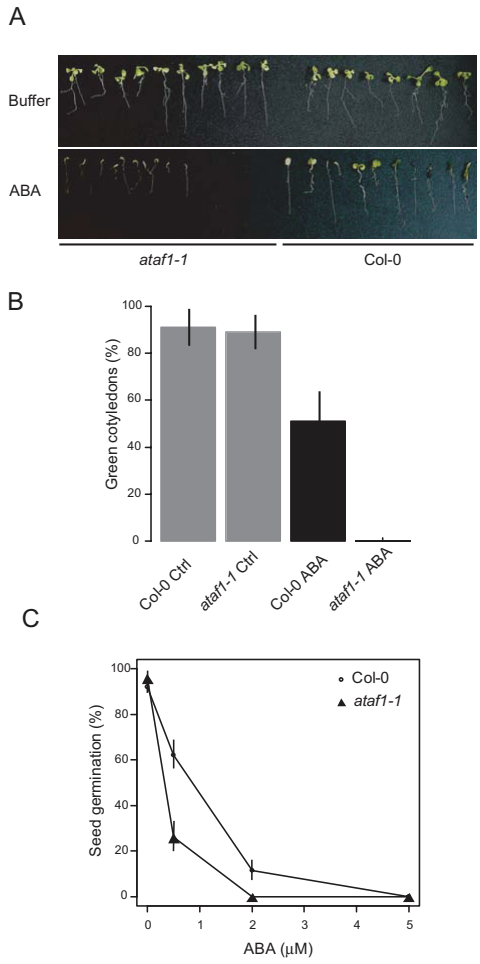

Figure 4. Modulation of *ATAF1* alters seedling development and germination in the presence of ABA. (**A**) Seeds of Col-0 wild-type and *ataf1-1* mutant allele were germinated on MS + 0.5 μM ABA plates for 14 days. (**B**) Percentage (+/- SEM) of green cotyledons after 14 days with 0 (grey-shaded) or 0.5 μM ABA (black) is shown for Col-0 and *ataf1-1* mutant (**C**) Percentage germinated seeds (+/- SEM) after 3 days. In all cases, results were based on three independent experiments.

both seedling growth, including root growth and cotyledon greening, and expansion were severely inhibited in the *ataf1-1* mutant, when applied 0.5 M ABA. In contrast, seeds of wild-type plants germinated and seedlings grew normally, although at a slower rate compared to those on ABA-free medium (Figure 4). Thus, *ATAF1* modulates the response of ABA in germination and seedling development.

The ataf1-1 mutant mimics ABA growth promoting effect

Low concentrations of exogenously applied ABA exerts a vegetative growth promoting effect in *Arabidopsis* (Barrero *et al.*, 2005). Additionally, ABA-deficient mutants show reduced vegetative growth and early flowering (Barrero *et al.*, 2005). Interestingly, we have observed that the *ataf1-1* mutation mimics the effect of exogenous ABA application of *Arabidopsis* plants, increasing both fresh and dry weight by 74% and 39%, respectively, compared to wild-type plants (Figure 5).

Lowered width/length ratio of stomatal pores in ataf1-1 mutant

Promoting closure and inhibiting opening of stomatal pores under low water stress is one of the crucial ABA-regulated processes (Leung and Giraudat, 1998;Schroeder *et al.*, 2001). Since the *ataf1-1* mutant is hypersensitive to ABA (Figure 4a-b), we expected to observe accelerated closure of stomatal pores, thereby minimizing water loss and enhance survival under dehydration (Jensen *et al.*, 2007). For this purpose, we looked at ABA-induced stomatal closure. Interestingly, the stomata of the *ataf1-1* mutant had a markedly lowered width/length ratio upon both control and ABA treatments, whereas wild-type plants exhibited ABA-induced closure of stomatal pores (Figure 6a-b). This data suggest that the enhanced drought tolerance of this mutant could be attributed to lowered water loss from stomata on the abaxial leaf side. Indeed, water loss rates were markedly lower in the *ataf1-1* mutant compared to wild-type plants (Figure 6c).

ABA compromises penetration resistance in Arabidopsis towards the non-host pathogen Bgh

With the proposed acceleration of ABA signalling on the *ataf1-1* mutant, we speculated that this could explain the reduced penetration resistance towards *Bgh* of the *ataf1-1* mutant compared to wild-type accessions (Jensen *et al.*, 2007). To test this hypothesis, we scored *Bgh* responses in

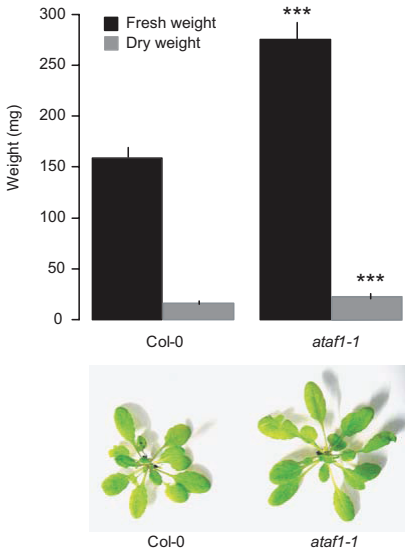

Figure 5. Increased vegetative growth of the *ataf1-1* mutant compared to wild-type plants. Mean values +/- SEM of fresh and dry weight 10 rosette measurements of each genotype from 6-weeks old *Arabidopsis* plants. *** = P < 0.001 using student's *t*-test.

wild-type plants after exogenous application of ABA. ABA-induced compromise of penetration resistance in *Arabidopsis* towards the non-host pathogen *Bgh* was indeed observed (Figure 7). The effect of ABA application in *ataf1-1* plants was furthermore increased compared to ABA-treated wild-type plants, allowing approx. 35% of *Bgh* germlings to penetrate epidermal cells, compared to approx. 23% in wild-type plants. In addition to verifying our previous results on compromised penetration resistance of *ataf1-1* (Jensen *et al.*, 2007), ABA-induced *Bgh* responses of *Arabidopsis* delayed the hypersensitive response of successfully penetrated cells, thereby enabling ectophytic mycelial growth at a higher level compared to control treated plants (data not shown). In other words, ABA compromises and delays pre- and post-invasive non-host defences, respectively.

Discussion

In this study, we have investigated the underlying transcriptional changes observed in the *Arabidopsis-Bgh* interaction upon mutation of the *ATAF1* gene. From this data, we have shown that ABA-responsive genes are differentially regulated in the *ataf1-1* mutant compared to Col-0. This prompted us to investigate ABA responsiveness and ABA-induced *Bgh* responses of *Arabidopsis* Col-0 and mutant plants. The latter studies showed that *ataf1-1* is an ABA-hypersensitive mutant line, with reduced germination and seedling development upon ABA application. Furthermore, we have shown that application of ABA mimics the effect of *ataf1-1* mutation, conferring reduced penetration resistance in wild-type *Arabidopsis* towards the biotrophic fungus *Bgh*. This ABA-induced manipulation of efficient penetration resistance was even stronger in the *ataf1-1* mutant than wild-type plants. We therefore infer that *ATAF1* is a novel negative regulator of ABA signalling, required for efficient penetration resistance towards *Bgh*.

Recent studies have focused on the possible cross-talk between biotic and abiotic stress perception and responses (Anderson *et al.*, 2004;Xiong and Yang, 2003), though results are conflicting. However, when inferring cross-talk, it is important to consider whether the cross-talk is a product of the factors modulated, or whether the physiological response or phenotypic changes themselves are products of ubiquitous cross-talk abundantly present throughout plant life (Mundy *et al.*, 2006). Referring to the latter, endogenous ABA levels increase in plants in response to a variety of abiotic stresses (Leung and Giraudat, 1998;Schroeder *et al.*, 2001). Furthermore, ABA levels have been shown to increase upon biotic stress and to correlate with reduced disease resistance (Kariola *et al.*, 2006;Torres-Zabala *et al.*, 2007). Exogenous ABA has furthermore been shown to suppress JA/ET-activated induction of defence genes, whereas ABA-deficient mutants showed a corresponding increase (Anderson *et al.*, 2004). Hence, several studies have elucidated a positive correlation between ABA and susceptibility. From our study, we infer that a possible *ATAF1*-dependent repression of ABA signalling has a positive effect on penetration

resistance towards biotrophic *Bgh*. In relation to ABA signalling, several lines of evidence suggest that ABA hormone levels are elevated in the *ataf1-1* mutant plants. Firstly, the *ataf1-1* mutant mimics ABA-associated vegetative growth-promoting effects (Barrero *et al.*, 2005) compared to wild type plants. Secondly, and most importantly, the gene encoding aldehyde oxilase (AAO3), conferring the last and rate-limiting step in ABA biosynthesis (Barrero *et al.*, 2006), is specifically up-regulated in mutant plants upon *Bgh* inoculation. Thirdly, the de-repression of ABA-signalling and possible elevated ABA levels, may have accumulating effects in the *ataf1-1* mutant during *Bgh* stress and possibly antagonize JA/ET-mediated signalling (Anderson *et al.*, 2004) as judged by the expression of JA/ET-marker genes in *Bgh* inoculated *ataf1-1* plants compared to wild-type plants. However, as individual JA/ET signalling mutant alleles, which abolish the induction of defensins, do not compromise *Bgh* entry to the epidermal cells of *Arabidopsis*, it is believed that JA/ET-regulated defences work in conjunction with other defence mechanisms to restrict *Bgh* growth (Zimmerli *et al.*, 2004). Overall, we hypothesize that the above mentioned observations directly or indirectly reflect acceleration of ABA biosynthesis and signalling, with the effect of compromising penetration resistance in *ataf1-1* mutant plants. In this respect, improved understanding of the observed stimulus-specific outcome of ABA-dependent signalling upon pathogen attack may be obtained by looking into the ABA accumulation in both host and pathogen during their interaction, as it has been shown that several microbes produce ABA themselves (Hirai *et al.*, 2000;Kitagawa *et al.*, 1995;Siewers *et al.*, 2006), whereas others corrupt ABA signalling in their host as a deadly virulence mechanism (Torres-Zabala *et al.*, 2007). Future studies on hormone measurements in *ataf1-1* and Col-0 plants upon *Bgh* inoculation will be necessary before this hypothesis can be verified.

In contrast to the observed negative correlation between ABA and plant resistance (Anderson *et al.*, 2004;Kariola *et al.*, 2006), both the non-protein β-amino-butyric acid (BABA) and ABA have been shown to induce callose depositions in *Arabidopsis* following inoculation with the

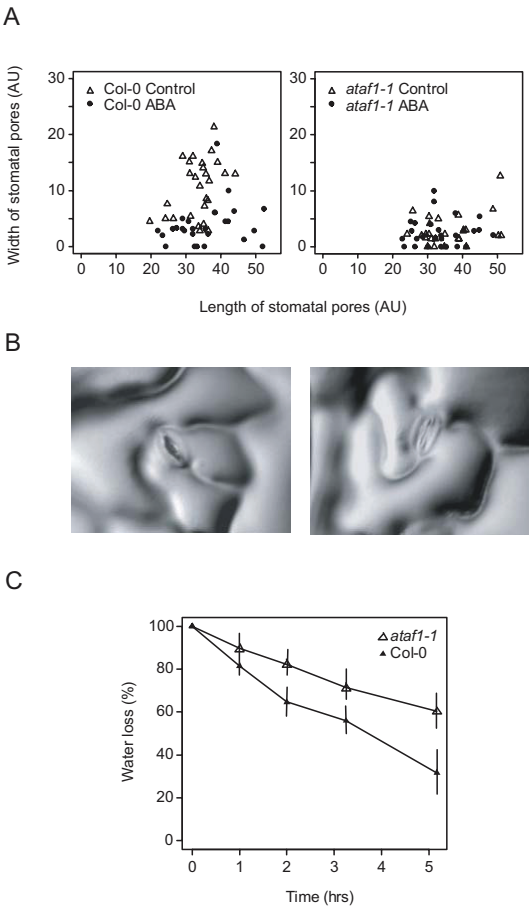

Figure 6. Lowered width/length ratio of stomatal pores correlates with reduced water loss in *ataf1-1* mutant plants. (**A**) The width and length of stomatal pores measured 3 hrs after ABA (100 μM in 0.2 % MeOH) or buffer (0.2 % MeOH) treatment. Plots are representative of results obtained from three independent plants from each treatment. AU; arbitrary units. (**B**) Positve replica of *Arabidopsis* adaxial leaf epidermis, showing open (left) and closed (right) stomata. (**C**) Water loss rates of Col-0 and *ataf1-1* plants. Each data point represents the mean +/- SEM of four rosette leaves from three independent measurements.

necrotrophic pathogens *Altenaria brassicicola* and *Plectosphaerella cucumerina* (Ton and Mauch-Mani, 2004). Furthermore, Jacobs *et al.* (2003) have shown that depletion of callose from papillae in the callose synthethase-deficient mutant *gsl5* marginally enhanced the penetration of *Bgh* on the non-host *Arabidopsis* (Jacobs *et*

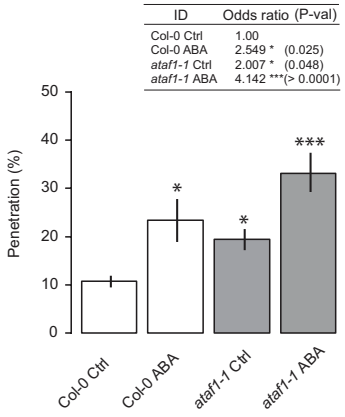

Figure 7. ABA compromises *Arabidopsis* penetration resistance towards *Bgh*. Weighted mean values +/- SEM of *Bgh* penetration frequency in *Arabidopsis* 48 hours after inoculation, expressed as a percentage of total germinated spores. Three hours prior to *Bgh* inoculation plants were sprayed with 100 μM ABA or control buffer (0.2% MeOH). Leaves from a total of 4 plants from each genotype and treatment were analyzed. Approx. 2.800 interactions were scored. Asterisks denote statistically significant difference in penetration frequency between Col-0 control plants and ABA- and mutant-specific treatments (* and *** = P < 0.05 and < 0.0001, respectively) using logistic regression analysis against wild-type Col-0 penetration frequencies.

al., 2003). Surprisingly, our studies show that efficient effective papilla formation in *Arabidopsis* towards the biotrophic *Bgh* is impeded, or overruled, by ABA application. *GSL5* is, however, not differentially regulated in *ataf1-1* mutant vs. Col-0 upon *Bgh* inoulation (data not shown), indicating that *GSL5*-downstream ABA-dependent manipulations account for the observed compromised penetration resistance of *ataf1-1*. Overall, these seemingly conflicting results highlight the intricate regulatory network of stimulus-dependent ABA signalling. Indeed, the possible contrasting outcomes of ABA interference on the signalling components which comprise resistance towards necrotrophic and biotrophic fungi deserve future attention.

From our study, an intriguing question arises, concerning the mechanism of ABA action. The widely recognized ABA-dependent stomatal closure during drought has also been shown to be induced by PAMPs of *Pseudomonas syringae* in a SA-dependent manner (Melotto *et al.*, 2006). From our study, the observed perturbations of ABA biosynthesis and ABA-responsive genes by *Bgh,* represents a powerful strategy for the fungus to impede penetration resistance in plants, as hormone homeostasis has wide impact on several biochemical processes. One obvious physiological benefit for the pathogen would be ABA-mediated stomatal closure and subsequent reduction in water loss. Prats *et al*. (2006) showed recently that, following epidermal cell death due to HR caused by avirulent *Bgh*, resistant barley lines were unable to close their stomata in darkness, whereas stomatal opening in response to light was permanently impaired 24 hai in susceptible barley lines (Prats *et al.*, 2006). The authors suggest that, though *Bgh* does not rely on stomatal opening for epidermal cell entry, the opening of stomata could evoke an inhospitable intracellular environment, compromising *Bgh* pathogenesis. Interestingly, we have shown that water loss rates are indeed diminished in *ataf1-1* mutant plants due to a constitutively low level of stomatal opening. It could be speculated that the accumulated effects of ABA signalling manipulation, including reduction of water loss from abaxial stomatal pores could provide beneficial high water availability within the apoplast of mutant plants compared to wild-type plants.

Finally, our results strongly indicate the possibility of *Bgh* attempting penetration by boosting ABA production and signalling in plants. However, in doing so ATAF1, and possibly the barley functional homologue *Hv*NAC6 (Jensen *et al.*, 2007), are induced, thereby attenuating ABA-induced susceptibility-signalling. Hence, in *ataf1-1* mutants *Bgh* potentates ABA-signalling without the interference of ATAF1, ultimately providing a means for increased penetration rates compared to wild-type plants. From this hypothesis, it could furthermore be speculated that induced levels of *ATAF1* prior to *Bgh* attack would help alleviate the plants from successful penetration attempts. However, this is not the case. At least not when combining the positive and negative effects of ABA treatment and *ATAF1* induction, respectively, on the *Arabidopsis*-*Bgh* single-cell outcomes. In

this scenario, ABA treatment (a known *ATAF1* inducer, appendix VI) of *Arabidopsis* prior to *Bgh* inoculation overruled the ATAF1-dependent negative regulations on ABA signalling. However, this may not come as a surprise as ABA have been shown to overrule plant responses to biotic stresses to ensure adequate adaptation to ABA-associated abiotic stress responses, *e.g.* drought (Anderson *et al.*, 2004).

In summary, we have found that *Bgh* targets phytohormone responsive genes upon infection attempt of the non-host *Arabidopsis*. The success of the penetration attempts seems to be correlated with the elevation of endogenous ABA signalling and biosynthetic pathways, and highlight intricate *ATAF1*-dependent perturbations of expressions of both SA and JA/ET-signalling marker genes. Hence, we conclude that ABA negatively regulates defensive cell wall alterations towards *Bgh*, though the ABA-stimulated regulatory network of *ATAF1*-downstream targets remains to elucidated for improved understanding of the regulations conferring pre-invasive non-host resistance towards microbes. Furthermore, ABA is a hormone that modulates a variety of agronomically important growth and developmental processes and various stresses responses. However, so far ABA-mediated signal transduction pathways have remained poorly understood to date. From our studies, we highlight the pleiotropic effects associated with mutation of *ATAF1*; a new negative regulator of ABA biosynthesis and signalling, for improved understanding of biotic and abiotic stress perception.

Experimental procedures

Plant material and growth conditions

Arabidopsis (*Arabidopsis thaliana*) ecotype Columbia-0 (Col-0) wild-type and *ataf1-1* T-DNA insertion line SALK_067648 (Alonso *et al.*, 2003;Lu *et al.*, 2007) were grown in growth chambers at 21 °C with a 8-hrs photoperiod of 125 $\mu E\ m^{-2}\ s^{-1}$, for 6 weeks, as previously described (Jensen *et al.*, 2007). Plants corresponding to numeric growth stage 3.90 (Boyes *et al.*, 2001), were used for all analyses.

Microarray studie

Three biological replicates of control and *Bgh* inoculated *Arabidopsis ataf1-1* mutant (SALK_067648) and Col-0 wild-type plants were harvested at 12 hai with *Bgh*. Eight rosettes of 6-week old plants were frozen in liquid nitrogen and pooled for each replicate sample. Total RNA was isolated using the RNeasy Mini kit (Qiagen, CA, USA). Three microgram of purified DNaseI-treated (Ambion, Austin, USA) Agilent Bioanalyzer (Agilent Technologies, CA, USA) quality-checked RNA was used for labelling. Labelled cRNA was prepared and hybridized to Affymetrix ATH1 GeneChips, containing 22,810 *Arabidopsis* genes, according to the manufacturer's guidelines (Affymetrix, Santa Clara, CA) by AROS Applied Biosystems A/S. The GeneChips were scanned with an Affymetrix GeneArray 2500 scanner and data acquired via the Microarray Suite software MAS 5.0.

Statistical analysis

All data analysis was performed using the R language (R Development Core Team, 2007) and selected packages from the Bioconductor software project (Gentleman *et al.*, 2004). To ensure gene expressions comparable across microarrays, intensities were normalized using GCRMA (Wu *et al.*, 2004). A log-base-2 transformation of the normalized intensities resulted in a virtually constant variability at all intensity levels (see chapter 4 of this thesis). The normalized and transformed intensity of each gene is denoted the expression of that gene. Differential expression was assessed using LIMMA (Smyth, 2005) – a multivariate approach that uses all 12 arrays simultaneously to judge statistical significance. Briefly, for each gene, empirical Bayes moderated F-statistics were used to test for changes between (1) inoculated and non-inoculated wild type plants, (2) inoculated and non-inoculated mutant plants, (3) wild type and mutant plants without inoculation, and (4) inoculated wild type and mutant plants. Using the step-up procedure proposed by Benjamini and Hochberg (1995) we corrected for multiple testing by controlling the false discovery rate (the expected proportion of false discoveries amongst the differentially

expressed genes) at 5%.

To identify ABA-inducible genes we used AtGenExpress consortium GeneChip expression data on wild-type seedlings treated with 10μM ABA for 30 min, 1 hour and 3 hours as well as control seedlings given mock treatment for the same durations; two biological replicates per condition (submission number ME00333). The dataset was preprocessed and analyzed using the same procedures and tools described above. Specifically, 4417 genes differentially expressed between ABA and mock treated plants at one or more time-points were identified.

For clustering, an additional time-course dataset on drought was included (AtGenExpress consortium submission number ME00338) and preprocessed as described above. Within each of the three datasets biological replicates were averaged. To make genes comparable across datasets, ratios of expression values were calculated between each sample type and the relevant controls for that dataset. From the combined dataset, selected sets of genes were clustered hierarchically using centered Pearson correlation and complete linkage.

Pathogen inculation

Blumeria graminis f. sp. hordei [race A6] (*Bgh*) was maintained on compatible barley (*Hordeum vulgare)* Pallas near-isogenic line P-01 by weekly transfer to fresh plants. For ABA-induced non-host response to *Bgh* inoculation, *Arabidopsis* plants were inoculated with 34 ± 6 spores/mm^{-2}. *Bgh* spores were air dispersed using a settling tower (Thordal-Christensen and Smedegaard-Petersen, 1988). For non-host inoculations, interaction phenotypes were assessed 48 hrs after inoculation. Individual *Arabidopsis-Bgh* interaction sites were characterized for (i) effective papilla formation and (ii) secondary hyphal growth formation using light microscopy, and (iii) hypersensitive-response-like cell death using ultraviolet autofluorescence (excitation filter 365/12 nm) as described in Collins *et al*. (2003).

Stomatal width/length ratio

Assessment of stomatal movement on the abaxial side of rosette leaves upon ABA-treatment and *Bgh* inoculation plants, was conducted as described by de Torres-Zabala *et al*. (2007), except from using cellulose acetate solubilized in acetone (2% v/v) for making replicas of leaf surfaces, instead of Lastic latex and nail varnish. Once dried, impressions were mounted dry on microscope slides and analyzed using DIC optics at 50x long-distance magnification using a Zeiss Axioplan2 compound microscope. Five replicate leaves of both wild-type and mutant were examined and width/length ratio measured on 25 stomata in each leaf.

Abscisic acid response assays

ABA (+/- *cis*,*trans*, Sigma, Dorset, UK) was solubilized in methanol diluted in water. This ABA solution (100 μM, 0.2% MeOH) was sprayed onto *Arabidopsis* plants. Control plants were treated identically with a solution of 0.2% MeOH (Buffer). For QRT-PCR analyses, 6-weeks old complete rosettes of control and ABA-treated wild-type and *ataf1-1* mutant plants were harvested 3 hrs after spraying. For each of the 4 biological replicate samples used for scoring ABA-induced *Bgh* responses, one rosette leaf from each plant was detached and analyzed by microscopic evaluation (Jensen *et al.*, 2007). For seed germination and root elongation assays, seeds were surface sterilized, placed on MS media containing 0, 0.5 μM, 2 μM or 5 μM ABA in 0.2% methanol, and stratified as previously described (Foster and Chua, 1999). The seeds were then incubated at 22 °C under a 23-h light/1-h dark cycle for 10 days. For water loss assay, young leaves from each plant at the same developmental stage were detached and fresh weights were measured. The water loss was expressed as the percentage of water lost over the initial fresh weight (Zheng *et al.*, 2002).

RNA extraction, cDNA synthesis and QRT-PCR analysis

For *Arabidopsis* transcript analysis, total RNA was isolated from three complete rosettes for

each time-point and treatment, using the RNeasy Mini kit (Qiagen, CA, USA). For all transcript analyses, a total of three biological replicates were sampled. One microgram of purified DNaseI-treated (Ambion, Austin, USA) quality-checked RNA was used for cDNA synthesis using the iScript™ cDNA Synthesis Kit (Bio-Rad, CA, USA) according to the manufacturer´s instructions. Quantitative real-time PCR was performed according to Bedini *et al*. (2005). For analysis of *ATAF1* (At1g01720), reverse 5'-TCAGGCTGGATGATTGGGTTCTCT-3' and forward 5'-GCCTCTCGGTAGCTCCTTTTTT-GT-3' primers were used. For *PR1* (At2g14610), reverse 5'-ACTTTGGCACATCCGAGTCT-3' and forward 5'-GTGGGTTAGCGAGAA-GGCTA-3 primers were used. For *PDF1.2a* (At5g44420), reverse 5'-ACTTGGCTTCTCG-CACAACT-3' and forward 5'-TCACCC-TTATCTTCGCTGCT-3' primers were used. For reference, *18S* rRNA (X16077) forward 5'-CGGCTACCACATCCAAGGAA-3' and reverse 5'- GCTGGAATTACCGCGGCT-3' primers were used. Relative gene expression difference (*R*) and statistical significance levels for ABA treated samples compared to buffer-treated control samples were quantified using the REST® software (Pfaffl, 2001). C_T-values included in the analyses were based on 3 biological replicate measurements, with two technical replicates for each time-point and treatment. Standard deviations were based on three biological replicates.

Acknowledgements

MKJ was supported by a PhD scholarship from the University of Copenhagen, Faculty of Life Sciences (formerly the Royal Veterinary and Agricultural University) and research financed by a Danish Research Council grant "Cell specific analysis of host-plant responses to pathogens using a functional genomic approach" SJVF 23-03-0167 (to MFL and DBC).

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AGI	Annotation	NACBS	ABRE
AT4G16190	cysteine proteinase	2x	2x
AT4G37470	putative protein beta-ketoadipate enol-lactone hydrolase	5x	2x
AT5G03240	polyubiquitin (UBQ3)		1x
AT1G29395	expressed protein	3x	
AT4G34480	putative protein	2x	1x
AT2G25520	putative phosphate/phosphoenolpyruvate translocator protein	2x	1x
AT5G53870	putative protein similar to phytocyanin/early nodulin-like protein	2x	
AT1G02470	hypothetical protein	4x	
AT3G14280	unknown protein	2x	1x
AT3G13700	unknown protein	3x	1x
AT3G13710	unknown protein	3x	1x
AT2G27150	aldehyde oxidase (AAO3)	1x	
AT2G45820	remorin, involved in intercellular communication	3x	1x
AT5G40690	putative protein 39	3x	
AT4G01130	putative acetyltransferase	2x	2x
AT5G40960	putative protein	3x	
AT5G42030	putative protein	2x	
AT3G47590	putative protein putative hydrolyse	2x	
AT1G23040	unknown protein	4x	
AT4G21980	symbiosis-related like protein	3x	
AT1G29760	hypothetical protein	2x	
AT3G03870	hypothetical protein	1x	
AT1G13090	putative cytochrome P450 monooxygenase	3x	
AT3G60180	URIDYLATE KINASE-like protein		
AT3G50830	putative cold acclimation protein	1x	1x
AT5G25560	putative protein	1x	
AT3G62190	putative protein DNAJ PROTEIN	5x	
AT1G30360	unknown protein	3x	
AT4G35780	putative protein kinase 6	4x	1x
AT5G54080	homogentisate 1,2-dioxygenase	4x	1x
AT2G42890	putative RNA-binding protein		
AT1G62810	putative amine oxidase	3x	1x
AT4G24450	putative protein R1	1x	
AT3G26580	unknown protein	2x	
AT2G22660	unknown protein	4x	
AT4G00355	expressed protein		
AT3G48530	putative transcription regulator protein	4x	1x
AT2G04690	unknown protein	3x	
AT5G18630	triacylglycerol lipase-like protein	5x	1x
AT5G63190	topoisomerase-like protein	3x	
AT3G51000	epoxide hydrolase-like protein	1x	1x
AT3G20250	RNA-binding protein	1x	2x
AT2G46260	unknown protein	3x	
AT2G42790	putative citrate synthase	4x	1x
AT2G15960	unknown protein	3x	1x

Supplementary table S1. Genes significantly induced by *ataf1-1* mutation and ABA treatment (3 hrs). Analysis of proximal 1 kb promoter region identified significant over-representation of NAC transcription factor core binding site (NACBS: [TA][TG][TACG]CGT[GA]) (Olsen *et al.* 2005) and core ABA responsive elements (ABRE: ACGTG[GT]C) using POBO (Kaikanen and Holm, 2004)

How can we exploit genomics for the understanding of defence responses? Barley as a case study.

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III

Submitted for publication in Eur. J. Plant Parthol. Pending revision. See appendix VII

Summary

The development of functional genomics technologies offers new opportunities for studying the nature of disease resistance in plants. The analysis of gene expression data is of key importance prior to thorough functional characterization of individual gene products. Many genes are transcriptionally activated following attack by pathogens and these often contribute to the defence mechanisms which underlie disease resistance. The use of large-scale cDNA library constructions and genome-wide transcript profiles of plants exposed to biotic stress provide the data required to drive hypotheses concerning the function of newly identified genes. In this paper, we illustrate how publicly-available has proved valid; enabling a cost-effective workflow starting from isolated gene transcripts to elucidation of biological function upon biotic stress.

Introduction

Plants are constantly under attack by microorganisms. However, only a few of these are potential pathogens capable of causing disease on a particular plant species. Even for pathogen species which can cause disease on the plant species in question, only few of the infection events from a spore (or other infectious structure) actually develop to cause a successful infection. More often than not, the plant succeeds in repelling attack through deployment of its defences, with disease resistance as the result. Even, in compatible interactions, plant defences also play a role in limiting the rate at which the pathogen invades the host tissues (Schulze-Lefert, 2004; Trujillo *et al.*, 2004).

Plant defences comprise the production of antimicrobial compounds (Field *et al.*, 2006; Hammerschmidt, 1999) and proteins (van Loon *et al.*, 2006), chemical and physical changes to secondary cell walls (Mörschbacher and Mendgen, 2000), and the induction of programmed cell death, known as the hypersensitive response (HR) (Jabs and Slusarenko, 2000). Some defence mechanisms are essentially constitutive, that is, they are always produced at a particular stage in the host's development. Others are first induced or activated when a pathogen attacks the host. Many of the same defences are activated in a particular host by different pathogen species. However, the particular mechanisms which are effective against a specific pathogen will depend on physiology of the pathogen, which in part reflects its taxonomic group, *e.g.*, fungus, bacteria or virus.

The employment of different life style strategies

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by different pathogens also plays a role in the efficacy of specific defence mechanisms used by the attacked host plant. The extremes are represented by necrotrophy, in which the pathogen destroys and consumes the hosts' tissues, and biotrophy, in which the pathogen parasitizes living tissue. Hemibiotrophs utilise both strategies at different phases of their life cycles. Defence mechanisms, for example the hypersensitive response, differ in their effectiveness against pathogens using these different strategies, and the regulation of the activation of these mechanisms differs too; thus, salicylic acid signalling and jasmonic acid signalling are associated, at least in Arabidopsis, with defence against biotrophs and necrotrophs, respectively (Glazebrook, 2005a).

In this article, we will look at the means by which plant defences are studied and the tools which can be used for determining whether a particular defence mechanism has a role in disease resistance towards a specific pathogen. As a case study, we will focus on the interaction between barley, *Hordeum vulgare*, and the biotrophic barley powdery mildew, *Blumeria graminis* f.sp. *hordei* (*Bgh*).

Why barley and *Blumeria*?

Bgh is the causal agent of powdery mildew; one of the most important diseases of barley worldwide. The barley – *Bgh* interaction has evolved as a model system for several reasons, both biological and practical (Collinge et al., 2002). Firstly, a very large number of race-specific resistance genes have been described (Jørgensen, 1994) – and many of these have been incorporated into near-isogenic lines of barley *e.g.*, (Kølster et al., 1986). The resistant phenotype for majority of these disease resistance genes is associated with the HR. Recently, mutant lines have also become available with TILLING lines (Mejlhede et al., 2006) and single-nucleotide polymorphisms (SNP) populations (Rostoks *et al.*, 2005), which offer great potential for exploitation in disease resistance studies. Secondly, the development of the fungus on the host is synchronised, facilitating meaningful experiments where physiological and molecular responses of the barley host can be correlated perfectly with the development of the fungus, using bioimaging analyses and transcript profiles among other methods (Caldo *et al.*, 2006c; Caldo *et al.*, 2006b; Gjetting *et al.*, 2007; Gregersen *et al.*, 1997a; Zierold *et al.*, 2005). Collectively, this has made the barley-*Bgh* interaction among the best-studied systems for investigating plant responses towards pathogen attack.

Historical perspective

The majority of studies have been performed using barley with *Bgh*. The first molecular studies to assay changes in the transcriptome used *in vitro* translation products by 2D-PAGE (Collinge *et al.*, 2002; Gregersen *et al.*, 1990; Manners and Scott, 1985). The next phase, in the 1990's, was the utilisation of various differential and subtractive hybridisation techniques to isolate cDNA clones (Collinge *et al.*, 2002; Gregersen *et al.*, 1997b; Hein *et al.*, 2004), and the differential expression suggested by the screening method was confirmed by northern blotting. In some cases, sequence-based identification was supported by biochemical evidence. For the barley-*Bgh* interaction, these approaches for gene discovery have been superseded largely by the use of EST libraries which provide a vast open resource of partial – and full-length – cDNA sequences (representing roughly 500,000 individual cDNA clones) which reflect gene expression in specific tissues and physiological states. We illustrate this in table 1 with the NAC transcription factor family of barley (see below). EST data bases are now providing a corroborative effort to assemble contigs (sequences constructed from individual clones) encoding full length or near full-length gene products. The UniGene database (http://www.ncbi.nlm.nih.gov/sites/entrez?db=unigene) uses part of a coding sequence to extract all sequence clusters including transcripts exhibiting sequence similarities to the query domain (Boguski and Schuler, 1995). Though dynamic entities, UniGene clusters can be used to (1) obtain an indication of the level of transcript accumulation for a given UniGene member for a specific tissue and (2) perform intercluster comparisons for the possible discovery of expressed genes responding to a particular environmental stress factor or physiological stage (Zhang *et al.*, 2004).

EST data (*e.g.*, Zhang *et al.*, 2004) also provided

the basis for the design of an Affymetrix GeneChip®, which in barley carries 22,792 gene sequences (Close *et al.*, 2004a; Shen *et al.*, 2005c). Parallel to the development of the Affymetrix barley GeneChip®, dotted filter array technology has also been utilised for barley-*Bgh* interactions (Zierold *et al.*, 2005). Each of these technologies offers its advantages and disadvantages which we will discuss below. However, all hybridization-based transcriptome techniques suffer the limitation that a specific gene will not be present unless the cDNA is prepared from a tissue in physiological state where it is expressed. This problem is essentially solved once the entire genomic sequence is available for the species in question. Although the barley genome is large (5,000 Mb), it is predicted that a draft sequence for barley will be available within a few years.

Transcriptomics in barley today

The barley1 Affymetrix GeneChip® microarray, has been used for several studies of gene expression in barley after *Bgh* inoculation (Caldo *et al.*, 2004; Caldo *et al.*, 2006e; Caldo *et al.*, 2006a) as well as for other interactions in barley involving biotic (Boddu *et al.*, 2006) and abiotic (Svensson *et al.*, 2006) stress. The dotted filter array has likewise also been utilised for barley-*Bgh* interactions (Eichmann *et al.*, 2006; Gjetting *et al.*, 2007; Zierold *et al.*, 2005). The advantage of the dotted filter array compared to the microarray is that it is straightforward to add new sequences to the study as they are discovered. A disadvantage lies both in sensitivity and specificity, and therefore ability to distinguish closely related and less predominantly expressed gene sequences from each other, respectively. The advantage of both array technologies is that it is possible to study the expression of a large number of genes simultaneously. Thus detailed time course studies with appropriate biological replicates using array technologies have been conducted (Caldo *et al.*, 2004; Caldo *et al.*, 2006f) and much of the data from these and other studies can be accessed through the public BarleyBase (Close *et al.*, 2004b; Shen *et al.*, 2005b). However, with a price tag of up to €800 per replicate, this approach is still prohibitively expensive for many laboratories which instead chose to make a more limited microarray experiment and support this with more detailed expression studies of individual candidate genes using northern blotting or real-time PCR.

Case study: EST libraries and their exploitation for studying the NAC transcription factors of barley

Members of the plant-specific gene family encoding NAC transcription factors share a common N-terminal domain, comprised of five highly conserved motifs. The domain is termed NAC from its first identified members NAM, ATAF and CUC2 (Aida et al., 1997; Souer et al., 1996). Genes encoding NAC transcription factors have been reported to be induced by both abiotic and biotic stresses, and over-expressing individual members has resulted in improved salt, drought and resistance towards *Fusarium oxysporum*, in rice and Arabidopsis (Hu et al., 2006; Lu et al., 2006). We have isolated several NAC gene members from barley, using differential display and cDNA library screening techniques of transcripts expressed in barley upon *Bgh* inoculation (Gregersen and Collinge, 2001; Jensen *et al.*, 2007). We have shown subsequently that *Hv*NAC6 (*Hordeum vulgare* NAC6) has a positive role in penetration resistance against *Bgh* (Jensen *et al.*, 2007). In the following, we will present how public transcript data repositories can be used in a data-driven approach for developing hypotheses on the functionality of specific genes of interest. We will use expression profiles from NAC gene members as a case-study, but any gene of interest can be exploited, as long as a transcript sequence originating from the gene of interest is present on the array platform to be analyzed.

A BLAST search of EMBL and Genbank databases using a nucleotide sequence encoding a conserved NAC domain yields approx. 600 putative barley NAC derived transcript sequences. However, as most of these are partial sequences, they are grouped as 27 UniGene clusters (Table 1). Each UniGene cluster comprises several partial transcript sequences, ideally making up a contig (*i.e.* contributing to a composite and complete gene sequence), deciphering the full-length mRNA sequence of the individual gene. Table 1 show that approx. 60% of the current

Table 1. Gene-oriented UniGene clusters of NAC transcript sequences

UniGene	Transcripts	cDNA Source
Hv.6550	16	*Bgh* inoc. leaf, seed, stem, root
Hv.1425	90	*Bgh* inoc. leaf, seed callus, root
Hv.984	6	Root
Hv.6308	38	*Bgh* inoc., seed, callus
Hv.5295	29	*Bgh* inoc. leaf, callus, flower
Hv.5097	8	Seed, leaf, root
Hv.5147	10*	*Bgh* inoc. leaf, callus, seed
Hv.877	10	Seed, leaf
Hv.13165	5*	*Bgh* inoc. leaf, callus
Hv.2292	62	*Bgh* inoc. leaf, root, seed, flower
Hv.17199	3	*Bgh* inoc. leaf, seed
Hv.2154	5	Seed
Hv.4825	26	*Bgh* inoc. leaf, stem, callus
Hv.1254	30	*Bgh* inoc. leaf, callus, root
Hv.15755	70*	*Bgh* inoc. leaf, root, shoot, seed
Hv.19392	14	Seed
Hv.19815	9	Seed, flower, Fusarium inoc. leaf
Hv.6910	10	Seed
Hv.18811	4	Seed, Fusarium inoc. leaf
Hv.18323	18	*Bgh* inoc. leaf, root, callus
Hv.17687	10	Seed, Fusarium inoc. leaf
Hv.5282	47*	*Bgh* inoc. leaf, seed, flower
Hv.21351	7	*Bgh* inoc. leaf, callus, seed
Hv.2526	23	*Bgh* inoc. leaf, seed
Hv.21779	3	*Bgh* inoc. leaf, stem, root
Hv.19852	26	*Bgh* inoc. leaf, root, stem
Hv.19865	14	Stem, leaf

* For which a full-length cDNA clone has been isolated. Apart from a clone represented in the Hv.5282 UniGene cluster, all full-length clones have been isolated in our laboratory and submitted to the EMBL data bank (Gregersen and Collinge, 2001; Jensen *et al.*, 2007).

NAC domain-containing UniGene clusters include ESTs originating from *Bgh* inoculated barley cDNA libraries. Obviously, the presence of a specific sequence in a given library does not mean that the encoded protein has any relevance to the pathogenesis of the microbe or defensive properties of the host. However, as transcripts from *Bgh* infected barley cDNA libraries are included in approx. 35% of the total number of barley UniGene clusters, NAC members seem to be over represented in *Bgh* infected barley cDNA libraries, making them interesting candidates for the understanding of the regulatory mechanisms involved in the barley-*Bgh* interaction.

Upon identification of transcripts of interest, subsequent laboratory experiments will provide a more detailed knowledge of spatio-temporal changes in the accumulation of transcripts of interest, and elucidating their possible function using down-stream reverse genetics approaches. Firstly, to obtain further details of the expression profile of genes of interest, a wealth of public repositories enable free download of large-scale transcript data (see table 1 & Shen *et al.*, 2005a). These databases therefore enable researchers to verify and/or supplement their own experimental data. In the case of barley NAC genes, we supplemented proposed transcript profiles of our differentially displayed candidate genes with transcript data from the *Bgh* infected barley cDNA library 'HO' available from IPK in Gatersleben, Germany (Zierold et al., 2005). In barley, the recessive loss-of-function alleles of the *Mlo* gene mediate durable and race-nonspecific resistance towards *Bgh* (Jørgensen, 1992). By comparing the transcript responses of *mlo* mutant plants with wild-type *Mlo* plants upon *Bgh* inoculation, Zierold and colleagues aimed at identifying candidate genes mediating durable resistance towards *Bgh* in barley (Zierold et al., 2005). Investigating the origin of the approx. 600 transcripts representing 27 NAC UniGene clusters, for transcripts originating from the *Bgh* inoculated DNA library used for spotting the nylon filter used by Zierold and co-workers, we identified 11 gene-oriented NAC clones, of which 8 had been successfully spotted on the cDNA array (Figure 1). Among the spotted clones, two belonged to UniGene clusters *Hv*.13165

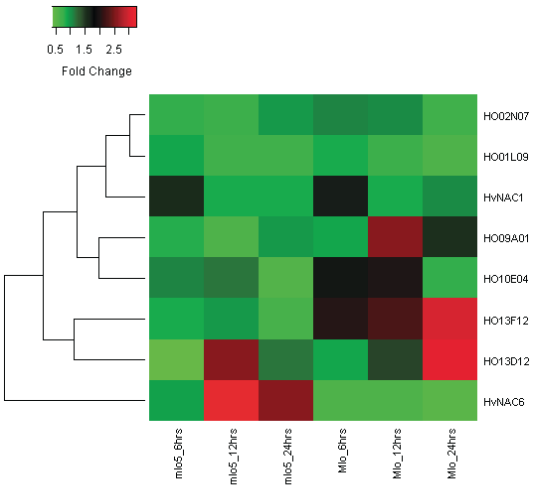

Figure 1. Transcript accumulation of 8 barley NAC genes and UniGene members upon Bgh inoculation and modulation of Mlo. Querying the epidermis-specific *Bgh*-inoculated cDNA library spotted onto nylon membranes (Zierold et al., 2005) identified 8 NAC encoding transcripts. The bottom panel displays the experimental conditions; genotype and hrs after *Bgh* inoculation. Gene names and UniGene cluster gene-oriented names are given to the right. Colours refer to mean ratios of gene centred signal intensities of inoculated samples versus corresponding control samples. Hierarchical clustering was performed using unscaled correlation and complete linkage clustering. Colour key displays correlation between colour and fold changes of *Bgh* inoculated vs. control samples.

and *Hv*.15755, of which we have isolated full-length cDNA clones (*HvNAC*1 and *HvNAC*6, respectively, Jensen *et al.*, 2007). Interestingly, from our data-mining we observed the *mlo5*-specific up-regulation of *HvNAC6* upon *Bgh* inoculation. In the susceptible *Mlo* wild-type background, no *HvNAC6* induction was observed (Figure 1), possibly due to a *Mlo*-dependent negative control of *HvNAC6* transcription (Zierold *et al.*, 2005). Another interesting transcript profile is depicted by the HO13D12 cDNA clone (UniGene cluster *Hv*.1425). HO13D12 abundance showed delayed accumulation in wild-type plants compared to *mlo5* plants upon *Bgh* attack. As the outcome of race non-specific lines of defence are believed to depend on the timing of host responses towards attacking pathogens (Caldo *et al.*, 2006d), the observed delayed induction of HO13D12-specific transcripts in *Mlo* plants could affect the delicate timing of effective race non-specific resistance.

We verified the *HvNAC6* expression pattern of a *Bgh*-challenged Pallas near-isogenic line using quantitative real-time PCR, and continued with functional studies to examine the possible importance *HvNAC6* for resistance towards *Bgh* (Jensen *et al.*, 2007). For this purpose, we made use of the particle-bombardment transformation assay of barley epidermal cells (Shirasu et al., 1999). Individual NAC gene constructs for *in vivo* gene silencing or over expression were co-transformed with the β-Glucuronidase (GUS) reporter gene (*uidA*), providing a perfect reverse genetics tool to study the cell-autonomous interaction outcomes between barley and *Bgh* of transformed cells (Figure 2). Our studies show that *HvNAC6* transcript abundances indeed affect the defence responses in barley by positively regulating penetration resistance (Jensen *et al.*, 2007).

To summarize, the wealth of data deposited in publicly available repositories provide a free and cost-effective tool for bench-top analyses of transcripts of interest. Though data should be thoroughly inspected with respect to their origin and relevance to the research in question, it can accommodate new hypothesis to be tested in the laboratory or field. In the case of barley NAC transcription factors, interesting hypothesis have been tested and verified, partially based on publicly available EST resourcesand simple functional genomic tools.

The role of individual genes in defence

Much of the effort to understand the defence mechanisms of plants concerns the identification of components of defences rather than understanding the role of the individual defences. It is abundantly clear from the literature that mutational approaches aimed at identifying genes necessary for disease resistance rarely lead to the identification of defence genes *per se*, *i.e.* those encoding antimicrobial proteins or enzymes involved in biosynthesis of antimicrobial phytoalexins (Field *et al.*, 2006; Hammerschmidt, 1999; van Loon *et al.*, 2006). Instead, mutations

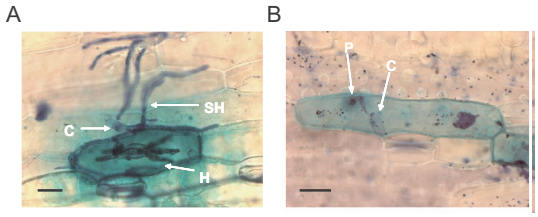

Figure 2. Barley epidermal single-cell interaction outcomes with Bgh provides a well-established system for transient expression studies of genes of interest using GUS as a transformation control. (A) Susceptibility. An epidermal cell penetrated by a *Bgh* conidia spore (C) and subsequent development a feeding organ, known as a haustorium (H) and secondary hyphae (SH) elongation. (B) Race-non-specific resistance. A penetration resistant epidermal cell showing race non-specific resistance towards *Bgh* penetration attempts by formation of a papilla (P).

affecting resistance are generally in genes involved in the regulation of defence mechanisms, including race-specific resistance genes themselves, and are often associated with signal transduction pathways (Glazebrook, 2005a; Panstruga and Schulze-Lefert, 2002; Takken *et al.*, 2007). They therefore fall outside the subject of this review. The lack of mutants in defence genes which exhibit compromised resistance implies that individual components of the defence response have an incremental, rather than determinative, role on the outcome of an interaction with a pathogen. The approach which has to date given the most extensive data set for understanding of the impact that individual genes encoding components of defence mechanisms have on resistance to *Bgh* is the use of transient RNA interference, itself a defence mechanism which operates against viruses (Lindbo and Dougherty, 2005; MacDiarmid, 2005). An inverse repeat RNA structure is produced in the host cell (Waterhouse *et al.*, 2001). This RNA folds to make a double-stranded RNA molecule which stimulates the host cell's defence against viruses with the result that both extraneous and endogenous copies of the transcript are essentially eliminated, and, as a consequence, in essence, the host gene product is no longer produced. This can be achieved by direct particle bombardment using plasmid constructs which contain an inverted repeat of the sequence of interest, or by infecting with a virus containing the sequence – virus-induced gene silencing (VIGS). Most studies have used the former approach (Christensen *et al.*, 2004; Douchkov *et al.*, 2005; Jensen *et al.*, 2007; Schweizer *et al.*, 2000; Shen *et al.*, 2007b). To date, few studies have used VIGS successfully to investigate the role of individual defence and disease resistance genes in the barley-*Bgh* interaction (Hein *et al.*, 2005; Shen *et al.*, 2007a). A common feature of the results obtained is that the effect of silencing is usually partial. This is in accordance with results obtained with mutational studies where mutations in regulators provide the major phenotypic effects and no defence-related genes have been identified (Glazebrook, 2005b).

Concluding remarks

In this review we have demonstrated the use of the genomics resources available in barley, a species where the genomic sequence is not yet available, for the identification and validation of the roles of specific defence mechanisms. Arabidopsis, poplar and rice have the advantage of full sequenced genomes and many tools have been developed which are not available in barley. For example, T-DNA insertion lines are available for many *Arabidopsis* genes, and stable transformation experiments are technically more effective in both *Arabidopsis* and rice.

Although microarrays are available for many species, it is a major undertaking to design and prepare microarrays carrying a significant part of a genome. What do you do if there is no microarray in your biological system? In these cases, the best approach is to use more classical gene discovery techniques such as the use of subtractive libraries (van den Berg et al., 2004) or differential display techniques, such as cDNA-AFLP (Liang and Pardee, 1997; Ramonell and Somerville, 2002). An alternative, though more costly would be to prepare a customized array, designed from the cDNA library of interest, for example for specific families of genes, or from subtractive libraries. One technology uses the Affymetrix GeneChip® platform, for example, through the collaboration between NimbleGen Systems and Affymetrix. Moreover laboratories world-wide are set up to make dotted oligonucleotide arrays, which may be a cheaper option.

For what can the knowledge gained from these studies be used for in the context of developing sustainable agriculture? The answer is currently not much yet! However, studies made to date have made it clear that the idea of taking an antimicrobial protein and using it to make a transgenic plant which has gained effective disease resistance is now largely discredited as the effect observed is at best, partial resistance (see Collinge *et al.* in this issue). This is in itself valuable knowledge. A second achievement from the study of defence mechanisms is the realisation that the regulation of plant defence mechanisms is more complex than previously imagined. It is now becoming clear that antagonistic regulations and interlinked signalling pathways are involved in both biotic and abiotic stress signalling. This may mean that the dream of making a universally disease resistant plant may remain that for the foreseeable future. The real challenge, and therefore our efforts, needs to be concentrated on understanding the nature of the regulatory networks underlying host defence responses against biotic and abiotic stress and through this be able to manipulate them to achieve resistance.

Acknowledgements

Authors would like to thank Drs. Patrick Schweizer for fruitful discussions and access to data. MKJ was supported by a PhD scholarship from the University of Copenhagen, Faculty of Life Sciences (formerly the Royal Veterinary and Agricultural University) and research financed by a Danish Research Council grant "Cell specific analysis of host-plant responses to pathogens using a functional genomic approach" SJVF 23-03-0167 (to MFL and DBC). This paper is based on two oral contributions held at the EFPP conference held in Copenhagen in August 2006.

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The barley Type V P-type ATPase *HvP5*, a functional equivalent to *Spf1* and *MIA* secretory pathway pumps, is highly expressed during endosperm development

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Manuscript in preparation

IV

Abstract The internal environment of the endoplasmic reticulum (ER) is regulated to accommodate essential cellular processes, including secretion, yet our understanding of this regulation remains incomplete. Barley HvP_5 belongs to the widely conserved, uncharacterized type V branch of P-type ATPases, a large family of ion pumps. Our previous work suggested that the related Arabidopsis protein MIA may function in the ER and, consistent with this hypothesis, we localized HvP_5 to the ER membrane. The cloned *HvP_5* cDNA functionally complements a yeast mutant carrying a deletion of *Spf1*, a P_5 ATPase involved in the secretory pathway by maintaining ion homeostasis in the ER. Furthermore, *HvP_5* was found to functionally complement an Arabidopsis *MIA* mutant, which is defective in secretory pathway homeostasis during development of pollen grains. *HvP_5* was expressed in all major tissues of barley, but in particular in the endosperm, where the expression level correlated with that of storage protein biosynthesis in the grain. We propose that *HvP5* is important for ensuring ER function and accordingly of importance for ER mediated processing and secretion of storage proteins.

Key words P-type ATPase - P_5 ATPase - secretory pathway - UPR - grain development - endosperm

Introduction P-type ATPases are ATP fuelled cation pumps characterised by having a phosphorylated reaction cycle intermediate (Møller et al. 1996). The pumps form an ancient family of distantly related transporters present in almost all living organisms studied to date (P-type ATPase database). Based on substrate specificity they are divided into five subfamilies (Axelsen and Palmgren 1998) of which the one encompassing P_5 ATPases is the least studied.

Several reports suggest that P_5 ATPases are localized to ER membranes and are involved in the unfolded protein response (UPR). UPR is a stress response pathway observed in all eukaryotic organisms from humans to yeasts, and it is activated when unfolded proteins accumulate (Kaufman 1999; Sidrauski et al. 1998). UPR results in the transcriptional induction of various genes that act to improve protein folding and

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transport, degrade unwanted proteins and limit the entry of secretory protein into ER (Martínez and Chrispeel 2003). A mutant in the *Saccharomyces cerevisiae* gene encoding the ER localized yeast P_5 ATPase, Spf1p, is deficient in regulating the ubiquitin-dependent degradation of the ER resident enzyme Hmg2p, a yeast isoform of hydroxy-methyl-glutaryl Coenzyme A Reductase (HMGR) in the mevalonate biosynthetic pathway and therefore hypersensitive to lovastatin, an inhibitor of the reductase (Cronin et al. 2000; Cronin et al. 2002). Furthermore, *spf1* mutants are sensitive to tunicamycin (Suzuki 2001) as well as DTT (Rand and Grant 2006), both compounds that cause accumulation of misfolded proteins in the ER and activation of UPR (Cox et al. 1997). Expression of bovine chymosin can also activate UPR, and it has been shown to increase the expression of the *Spf1* ortholog, *AN3146.2*, in *Aspergillus nidulans* (Sims et al. 2005).

A single P_5 ATPase homologue was identified in the genomic sequence of Arabidopsis termed *MIA* (*Male gametogenesis Impaired Anthers*) (Jakobsen et al. 2005) and one in rice termed *OsP5* (Baxter et al. 2003). *MIA* complements the lovastatin sensitive *spf1* mutant suggesting a function in maintaining the functionality of ER, secretory processes like UPR and vesicle transport. Homozygous *mia* mutants show highly reduced fertility as well as severely reduced silique length and seed yield compared to wildtype. MIA is localized to the ER of developing pollen grains and tapetal cells as well as small vesicles that probably are ER derived. Both cell types have high secretory activity; the tapetal tissue has a secretory role providing essential nutrients required for microspore and pollen grain development, while germinating pollen grains have a high metabolic activity and protein trafficking through the ER to sustain the growth of pollen tubes (Bedinger 1992; González-Sánchez et al. 2004**;** Wang et al. 2003**)**.

Higher plants accumulate large quantities of storage proteins, such as globulins, albumins and prolamins in the protein storage vacuoles of dry seeds to be used as a nitrogen source for growth after germination. Cereal seed storage proteins are synthesized on rough ER as precursor forms and co-translationally transported into the lumen of ER where after they are moved to the Golgi compartment for deposition into storage vacuoles or deposited as aggregates in ER derived vesicles. Eventually, Golgi and ER derived vesicles are moved to and fused with the protein storage vacuole (Müntz 1998; Harasaki et al. 2005; Jolliffe et al. 2005; Vitale and Hinz 2005). However, despite much effort, the molecular mechanism underlying the vacuolar targeting of storage proteins has not yet been revealed (Hanton et al. 2005; Hanton et al. 2006; Matheson et al. 2006).

The barley grain consists of a number of compartments, such as embryo, aleurone layer and endosperm, with high metabolic activity and specialized function in transport and deposition of nutrients (Hoh et al. 1995; Swanson et al. 1998). The endosperm stores compounds like starch, cell wall carbohydrates and storage proteins that subsequently are used for sustaining the growth of the germinating embryo and early seedling (Berger 2003; Lai et al. 2004; Lopes and Larkins 1993; Olsen 2004). The mature seed contains 10-12% protein on a dry weight basis and a substantial part of its metabolic machinery is accordingly devoted to the biosynthesis, transport and deposition of proteins (Shewry and Halford, 2002).

In the present study we report on the cloning of the first monocot P_5 ATPase gene, barley HvP5, and, as HvP_5 functionally complements both yeast *spf1* and Arabidopsis *mia* mutations, we demonstrate that the function of P_5 ATPases is conserved across distant evolutionary lineages within eukaryotes. Finally, we find that HvP_5 is localized to the ER and expressed in all tissues of the barley plant with a significantly high expression in the endosperm that reaches its maximum at the stage of intense storage protein, starch and cell wall biosynthesis where after it decreases toward the end of grain maturation.

Methods

Cloning of HvP_5 full-length cDNA

Basic Local Alignment Search Tool (BLAST) at TIGR (http://www.tigr.org) was used to extract sequence information from barley to generate primers for cloning. TIGR contains 370.546 ESTs from barley. This information was supplemented with the wheat EST information (580.155 ESTs

are available). BLAST was performed with the protein sequence of rice P_5 ATPase obtained by using PlantsT (http://plantst.genomics.purdue.edu/). ESTs used for cloning were: CA497971 (from ATG), BU980814 (596-765 amino acids within OsP_5) and CB877529 (3' UTR).

*Y*oung leaves were isolated from *Hordeum vulgare (*barley) variety Golden Promise and total RNA was extracted using FastRNA Pro green kit (Qbiogene). HvP_5 gene specific primers were designed from ESTs and used to generate two gene specific DNA fragments with SuperScript III First Strand Synthesis System (Invitrogen). HvP_5.1 was generated with Oli35 5'ATGGCGCGGTTCGAG 3' (forward) positioned from ATG and HvP_5.2 was generated with oli36 5'AGTCCGACAAAATTCTCCTA AACTAC3' (reverse) positioned 291 bp after the stop codon in the 3' UTR. PCR on HvP_5.1 and 2 yielded two partial HvP5 clones that were sub cloned into the TA cloning vector 2.1-TOPO (Invitrogen) for sequencing. HvP_5 from ATG to 1615 bp downstream was amplified from HvP_5.1 with primer pair Oli35 and oli37 5'GGTCACCAACCAGCTTGTTG 3' and the segment of HvP_5 from 1615 bp to 291 bp after the STOP codon was obtained with primer pair oli36 and oli38 5'GGTGACCCCCTTGAAAA AGCTGC 3' from HvP_5.2. A BstEII restriction site 1620 bp within the HvP_5 sequence was used to ligate the two cDNA clones together to generate a HvP_5 full-length cDNA clone in the 2.1-TOPO vector (pAM1). The fill length clone is hereafter referred to as HvP_5.

Bioinformatic sequence analysis of HvP5

The sequence of HvP_5 was subsequently analysed with bioinformatic tools. ClustalW (http://www.ebi.ac.uk/clustalw/) generated alignment of different P_5 ATPases. Three different topology prediction programs were used; TMHMM (http://www.cbs.dtu.dk/ services/TMHMM), DAS (http://www.sbc.su.se/ ~miklos/DAS/) and TMPRED (http://www. ch.embnet.org/software/TMPRED_form.html).

Functional complementation of yeast spf1 mutant with HvP_5

A fragment containing the HvP_5 full-length clone was excised from plasmid pAM1 and inserted into yeast expression vector pYES 2.0. with a galactose inducible promoter (pAM2). The phosphorylation site of the HvP_5 was destroyed by an amino acid substitution generated by overlapping PCR where a point mutation was introduced into pAM2 to substitute aspartate (D) (488) with asparagine (N) generating plasmid pAM3 containing HvP_5:D488N ATPase. A his-tag (RGSH6) was introduced to the C-terminal of HvP_5 and HvP_5:D488N by PCR, generating plasmid pAM5 (HvP_5, RGSH6) and pAM6 (HvP_5:D488N, RGSH6).

Plasmids pYES2.0 (empty control), pAM5 and pAM6 were expressed in two yeast strains (Euroscarf) BY4741 (wild-type; *Mat a, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0*) and YOO272 (*spf1; Mat a, his3Δ1, leu2Δ0, met15Δ0, ura3Δ0, YEL031w::kanMX4*). Experiments were performed at 30°C in synthetic minimal media supplemented with galactose (2%), Histidine (3 mg/mL), Methionine (3 mg/mL) and Leucine (3 mg/mL) with and without 150 µg/mL Lovastatin. A western blot was performed to investigate expression level of the different plasmids in yeast using an RGSH6 anti body for detection of the HvP_5 protein.

Functional complementation of Arabidopsis mia insertion mutant with HvP_5

Arabidopsis wildtype (WS-2) seeds and seeds homozygous for the mia1 T-DNA insertion mutant allele (Jakobsen et al. 2005) were incubated in 0.2% top agar for 3 nights at 4 °C. After sowing, the seed pots were placed in a controlled growth chamber with short-day conditions promoting vegetative growth (8 h light, 200 µmol m^{-2} sec^{-1}, 70 % humidity, 20 °C). After 3 weeks, the plants were transferred to long-day conditions to promote reproductive growth (16 h light, 200 µmol m^{-2} sec^{-1}, 70 % humidity, 20 °C).

Vectors for plant transformation were constructed by insertion of HvP_5 from KnpI/XbaI digested plasmid pAM2 into the binary plant transformation vector pPZP211 under the control

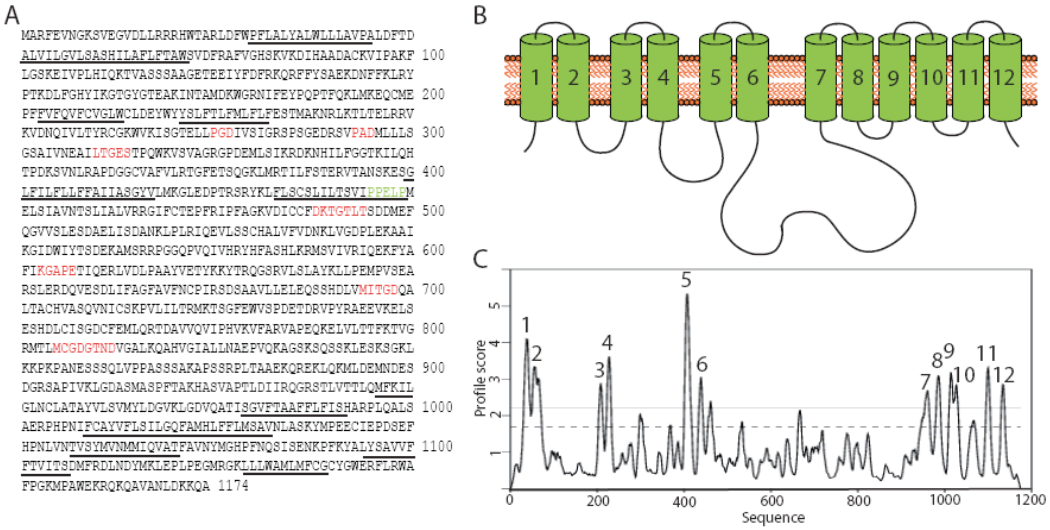


Figure 1. HvP_5 cDNA encodes a P_5 P-type ATPase of 1174 amino acids A) predicted to contain 12 membrane segments B) by a hydropathy plot; dashed line marks loose cut-off and straight line strict cut-off C). HvP_5 contains signature sequences present in all known P-Type ATPases (red) and signatures only found in P_5 ATPases (green). Putative transmembrane-spanning helices are underlined.

of the 35S promoter (pAM7). Arabidopsis mia12 seeds were transformed according to floral dip method (Clough and Bent 1998) with pAM7 and empty plasmid pPZP211. For kanamycin selection, plants were grown on half strength Murashige and Skoog plates containing 50mg/L kanamycin. Kanamycin selected plants were investigated for presence of *HvP_5* by PCR on genomic DNA using Oli35 5'ATGGCGCGGTTCGAG 3' (forward primer) positioned from ATG and oli36 5'AGTCCGA CAAAATTCTCCTAAACTAC3'. RNA was isolated with Qiagen RNA easy kit from seedlings that were tested positive for HvP_5 expression in PCR, and quantitative Real-time PCR was performed to investigate expression levels of HvP_5. For PCR conditions and cDNA preparation, see Methods: RNA isolation and Quantitative real-time PCR. The housekeeping gene actin was used for normalization in the real time PCR and amplified using gene specific actin primers: Rev. 5'TCTGTGAACGATTC CTGGAC 3' and Forw. 5'CTTCCCTCAGC ACATTCCAG3'.

For identification of fertilized ovules, siliques were bleached in 70% ethanol until chlorophyll had disappeared (1-3 hours). Number of fertilized ovules was counted under microscope.

Transient transformation and subcellular localization of HvP_5-GFP fusion protein

A chimeric fusion construct was generated to investigate the subcellular localization of the HvP_5 encoded protein. Full-length GFP was amplified using primers 5'-GGCAAGC CGTTCATGGTGAGC AAGGGCGAG-3' and 5'-AAACTGCAGTTACTTGTACAGCTCGTC CATG-3'. For introduction into TOPO entry Gateway vector pENTR.SD (Invitrogen), HvP_5 was PCR amplified with primers Oli45 5'CACCATGGCGCGGTTCGAG 3' and Oli38. The pENTR.SD clonase reaction (Invitrogen) with the HvP_5 fragment resulted in vector pAM8. A Gateway LR clonase II (Invitrogen) reaction between pAM8 and C-terminal vector pMDC45 (Curtis and Grossniklaus 2003) resulted in pAM10 (C-terminal GFP). As a control for ER localization we used the ER retention signal tagged GFP fusion (Runions et al. 2006).

Onion (*Allium cepa*) epidermal strips were

placed on agar containing MS salt mixture (1 x MS salt (Invitrogen, WC, USA) 2% agar, 3% sucrose, pH 5.8) and bombarded with either of the three vectors pAM10, GFP and ER-control using a PDS-1000/He biolistic particle delivery system (Bio-Rad, München, Germany). A total of 10 µg of each expression vector was coated onto 1 micron gold particles and transferred into cells using a hepta adaptor and a helium pressure of 1100 psi. Following bombardment, petri dishes containing bombarded epidermal strips were sealed with Micropore™ tape (Neuss, Germany) and placed in darkness at 22 °C for 18 hrs. Transformed cells expressing the HvP_5 GFP fusion protein were visualized using a Zeiss LSM Pascal 5 confocal laser scanning microscope equipped with a C-Apochromat 40x 1.2 water-immersion lens. The excitation wavelength e for GFP was 488 nm. A total of 50 cells for each construct were observed and images processed using the ImageJ software (NIH, USA).

RNA isolation and quantitative real-time PCR

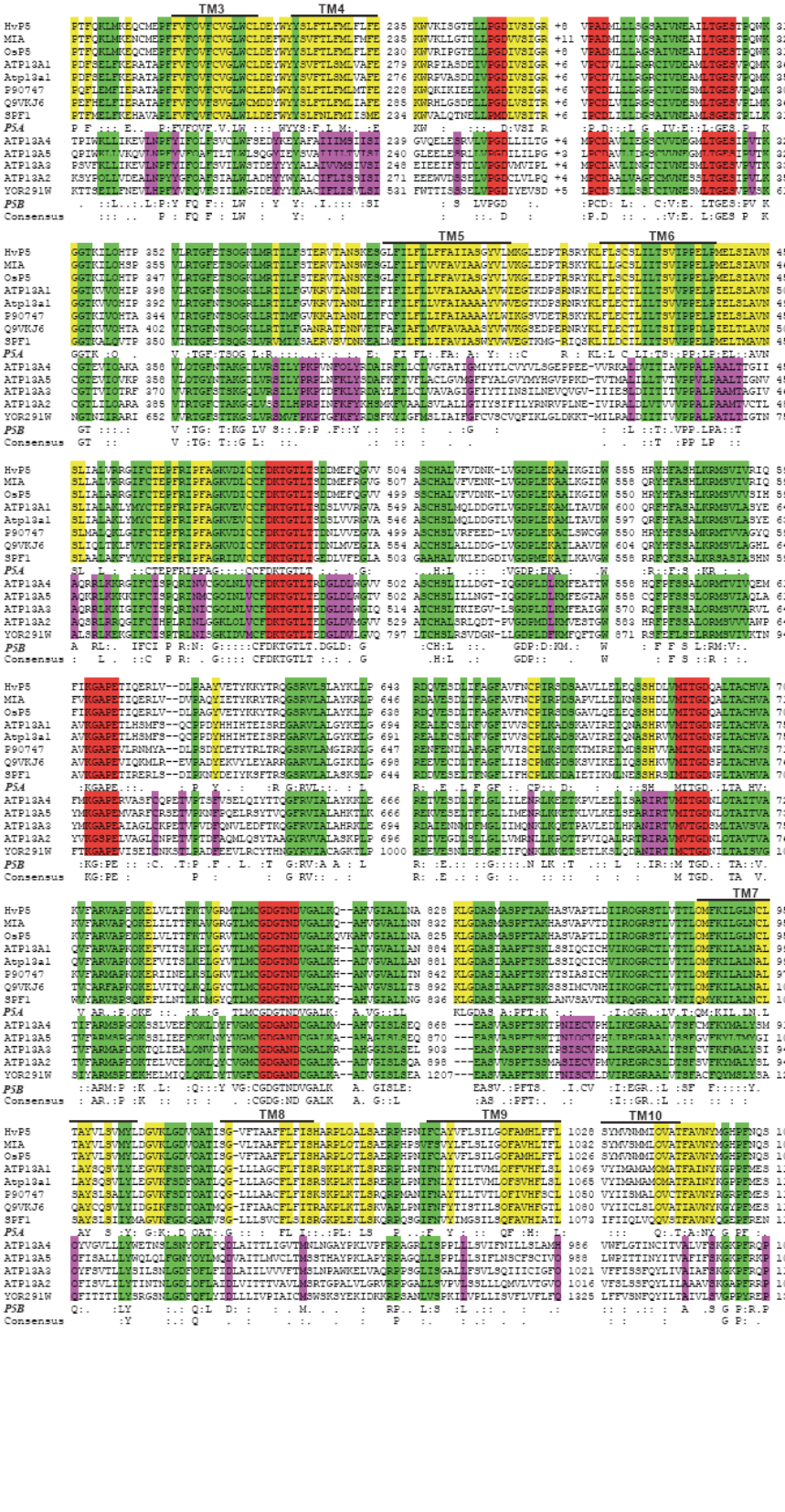
To investigate *HvP_5* expression pattern in different tissues of barley, non-grain tissues (stem, leaves, root) as well as grain tissues 14 days after pollination (DAP) (embryo and endosperm) were cut out. For each of the tissues, two independent biological samples were investigated. Total RNA was extracted using FastRNA Pro green kit (Qbiogene). For a time course investigation of *HvP_5* expression in the endosperm, tissue 14, 25 and 35 DAP were isolated.
Random hexamer primed (Fermentas) cDNA was generated from 1µg total RNA using SuperScript II First Strand Synthesis System (Invitrogen) in a 15 µl reaction. First-strand cDNA was diluted 10x and 1 µl was used in subsequent quantitative real-time PCR reactions, while a dilution series of 1 µl 5x, 25x, 125x and 625x was used to correct for primer efficiency. Gene specific primers for real-time PCR were generated by Primer Express software from ABI. *HvP_5* cDNA was amplified using gene specific primers that generate a PCR product of 50 bp: 5'GGCCAGTTTGCAATGCACT3' and 5'TGC TAAGTTGACAGCTGACATCAAG3'. Barley actin gene, a housekeeping gene, was used for normalization and amplified using gene specific primers 5' TCGCTCCACCTGAG AGGAAG 3' and 5'GCTAGGATGGACCCTCCGAT 3'. PCR conditions were: 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec, 60°C for 1 min. Resulting PCR product was investigated by dissociation analysis.

Results

Cloning and sequence analysis of HvP_5, a P_5 ATPase in barley

BLAST search identified several barley ESTs highly similar to the rice P_5 ATPase, OsP_5, and the sequence information was used for cloning of the barley P_5 ATPase. The cDNA encodes a polypeptide of 1174 amino acids with a predicted molecular mass of 131 kDa (Figure 1A). The encoded HvP_5 protein contains signature sequences present in all known P-Type ATPases, including the phosphorylation motif DKTGTLT, in which the aspartate (D) residue is phosphorylated during the reaction cycle (red, Figure 1A) (Toyoshima and Mizutani 2004).

Furthermore, HvP_5 contains a PPELP motif in the preceding putative transmembrane domain, corresponding to the PPxxP motif present in all P_5 ATPases (green, Figure 1A and B) (Catty et al. 1997; Axelsen and Palmgren 1998), proposed to be the ion-binding site in Ca^{2+} ATPases (Toyoshima et al. 2000). Hydropathy plots of the HvP_5 amino acid sequence (Figure 1C) predict HvP_5 to contain 12 membrane-spanning regions (underlined and numbered 1-12).
P_5 ATPases are exclusive to eukaryotes where they have been identified in all completely sequenced genomes (Møller et al. submitted). Based on a 265 amino acid core sequence (Axelsen and Palmgren 1998; Møller et al. submitted) as well as additional signature sequences for P_5 ATPases, HvP_5 was aligned to other P_5 P-type ATPases (Figure 2). All P_5 ATPases contain signature sequences for P-type ATPases like the amino acid motifs DKTGTLT, LTGES, KG(A/S)PE and M(I/V/C)TGD (Møller et al. 1996) (Figure 2, red). Furthermore, P_5 ATPases contain sequence motifs distinctive to this subfamily (Figure 2, green).
The analysis reveals that P_5 ATPases can be


TM3
TM4
TM5
TM6
TM7
TM8
TM9
TM10
HvP5
MIA
OsP5
ATP13A1
Atp13a1
P90747
Q9VKJ6
SPF1
P5A
ATP13A4
ATP13A5
ATP13A3
ATP13A2
YOR291W
P5B
Consensus

divided into two subgroups, P_{5A} and P_{5B} (Møller et al. submitted; Figure 2). HvP_5 appears to belong to the group of P_{5A} pumps that are characterized by a PP(E/D)LP motif in a putative ion binding region of the predicted trans-membrane segment 6 (TM6) (Figure 2, yellow), whereas P_{5B} pumps have a PP(A/V)LP motif at this position (Figure 2, purple).

Alignment of HvP_5 to other P_5 ATPases showed that HvP_5 is closest related to P_{5A} ATPases from the monocot rice (91% identity) and the dicot Arabidopsis (72% identity). When aligned to species more distantly related the identities to P_{5A} ATPases are reduced to 42% for the human ATP13A1 and 40% for *S. cerevisiae* Spf1p. Alignment of HvP_5 to P_{5B} ATPases (ATP13A2, 3, 4 and 5) revealed that pair-wise sequence similarity scores were significantly lowered ranging from 20% to 22%.

HvP_5 functionally complements a mutation in yeast spf1

Yeast P_5 ATPase mutant *spf1* is deficient in ubiquitin-dependent degradation of Hmg2p in the mevalonate biosynthetic pathway and therefore hypersensitive to lovastatin, an inhibitor of Hmg2p (Cronin et al. 2000). To test whether Spf1p and HvP_5 are functional homologues, we transformed *HvP5* under control of a galactose inducible promoter into the *spf1* mutant strain. Growth pattern observed
on lovastatin plates suggested that the putative P_5 ATPase encoded by HvP_5 functionally complements the *spf1* mutant (Figure 3A). Thus, the *spf1* strain transformed with HvP_5 restored growth to the level of the wild type, while the *spf1* strain transformed with an empty vector grew poorly. Replacement of Asp488 with Asn is expected to destroy the putative phosphorylation site of the pump leading to loss of function. When the *spf1* strain was transformed with the mutated HvP_5 (HvP_5*-D488N*), growth was reduced to the level of the *spf1* strain. Both HvP_5 and

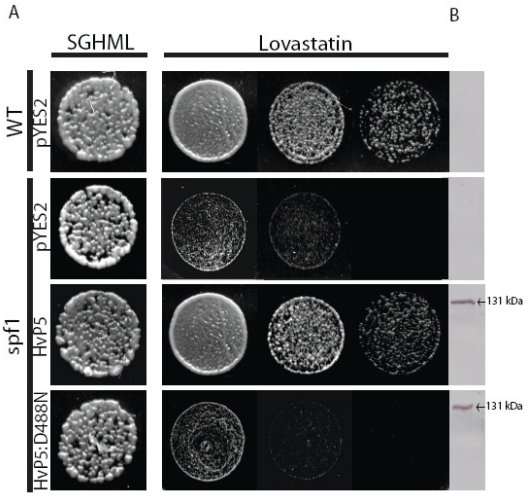


Figure 3. Functional complementation of the *spf1* mutant with HvP_5. A) The *spf1* mutant strain transformed with the HvP_5 restored growth to the level of the wildtype, while *spf1* strain transformed with empty vector pYES2 grew poorly. HvP_5 mutated in the putative phosphorylation site (HvP_5*:D488N*) could not functionally complement *spf1*. B) Western blot confirmed that both HvP_5 and HvP_5*:D488N* are expressed at similar levels in the yeast mutant strain.

HvP_5-D488N contain a RGSH6 tag for protein detection, and immunolabeling of protein blots confirmed that both ATPases are expressed at similar levels in the yeast mutant strain (Figure 3B).

HvP_5 functionally complements an Arabidopsis mia insertion mutant

Arabidopsis *mia* mutants have slightly reduced stature, markedly reduced sizes of their siliques, highly reduced fertility and reduced seed yield

Figure 2. Alignment of HvP_5 and other P_5 ATPases. All P_5 ATPases contain signature sequences for P-type ATPases (red). Furthermore, the P_5 ATPases contain sequence motifs distinct to this subfamily (green). The P_5 ATPases are divided into two sub groups with different ion binding domains in TM6; P_{5A} ATPases with motif PP(E/D)LP (yellow) and P_{5B} ATPases with motif PP(A/V)LP (purple).

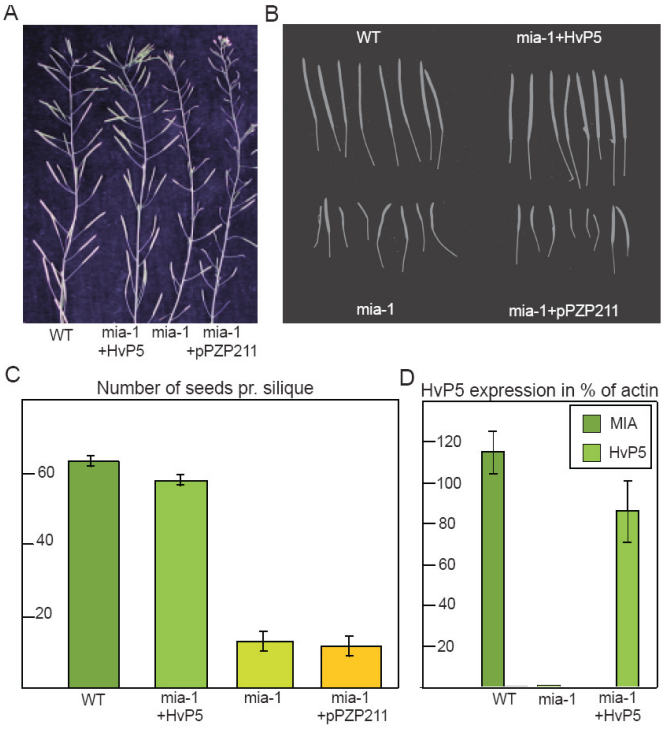


Figure 4. Functional complementation of the *mia* mutant with *HvP5*. A and B) The normal siliques of the wildtype resembles the siliques from *mia* mutants transformed with HvP_5, while the *mia* mutant and the mutant transformed with the empty vector (pPZP211) have markedly reduced sizes of their siliques. C) Expression of HvP_5 in the *mia* mutant restored the seed yield to wildtype levels, while the empty plasmid did not functionally complement the *mia* mutant. D) Quantitative real-time PCR revealed that HvP_5 is expressed almost at the same level in the *mia* mutant as MIA is expressed in the wildtype.

(Jakobsen et al. 2005). When a *mia-1* mutant was transformed with HvP_5, silique length and seed yield were restored to wildtype levels, while the empty plasmid did not have any impact on the phenotype of the *mia* mutant (Figure 4A-4C). The number of fertilized ovules was 92% compared to 94% in the wild type (n=30) demonstrating that fertility was restored in *mia-1* plants transformed with *HvP5*. Expression level of HvP_5 was determined in HvP_5 transformed Arabidopsis using quantitative real-time PCR analysis. Results revealed that the level of HvP_5 in the *mia* mutant was comparable to that of *MIA* in the wildtype (Fig. 4D).

Transiently expressed HvP_5 localizes to the ER of onion epidermal cells

Spf1p and MIA both localize to the ER of yeast cells and plant cells, respectively (Vashist et al. 2002; Cronin et al. 2002; Jakobsen et al. 2005). In order to study the subcellular localization of HvP_5 *in planta*, a gene fusion between HvP_5 and green fluorescent protein (GFP) under control of the cauliflower mosaic virus 35S promoter was introduced into onion epidermal cells by particle bombardment. Epidermal leaf cells expressing HvP_5:GFP fusion protein exhibited dense fluorescent nuclei (insert in Figure 5A) and also

a cortical ER-like network (Figure 5A). The network aligned the cortical cytoplasm and did not extend into trans-vacuolar cytoplasmic strands. A control construct for ER-localization, HDEL-GFP, had a similar fluorescence pattern (Figure 5C). In contrast, cells expressing GFP alone showed diffuse fluorescence throughout the cytoplasm and nucleus of transformed cells (Figure 5E).

HvP_5 is highly expressed in the endosperm

Quantitative real-time RT-PCR results showed that *HvP_5* was expressed in all tissues of barley, however at a lower level in stems and roots (Figure 6A). Among tissues tested, the highest expression was found in the endosperm of developing grains (Figure 6A). During endosperm maturation expression of *HvP_5* increased, while expression ceased toward the end of grain maturation (Figure 6B).

Discussion

In the present study we expanded our understanding of the function of P_5 ATPases in plants from the model Arabidopsis to cereals. We identified a P_5 ATPase in barley, one of the major cereals, and subsequently cloned the P_5 ATPase, which we named HvP_5. We found that i) *HvP_5* can functionally complement mutations in yeast and Arabidopsis homologues, ii) HvP_5 is localized to the ER, and iii) that HvP_5 is highly expressed in the endosperm, with an increase in expression during peak of endosperm biosynthetic activities.

HvP_5 functionally complements a deletion in yeast *Spf1*. The *spf1* mutant displays a range of phenotypes related to alterations in protein processing, and shows defects in; i) glycosylation and cell wall biosynthesis (Suzuki and Shimma 1999), ii) transport of cargo proteins to Golgi (Ng et al. 2000; Vashist et al. 2002), iii) control of protein insertion orientation (Tipper and Harley 2002), iv) HMGR protein degradation (Cronin et al. 2000) and v) sensitivity to UPR activators (Rand and Grant 2006; Suzuki 2001; Suzuki and Shimma 1999; Vashist et al. 2002). Additionally, HvP_5 functionally complements a mutation in the P_5 ATPase MIA from Arabidopsis. MIA is most prominent in tissues with high secretory

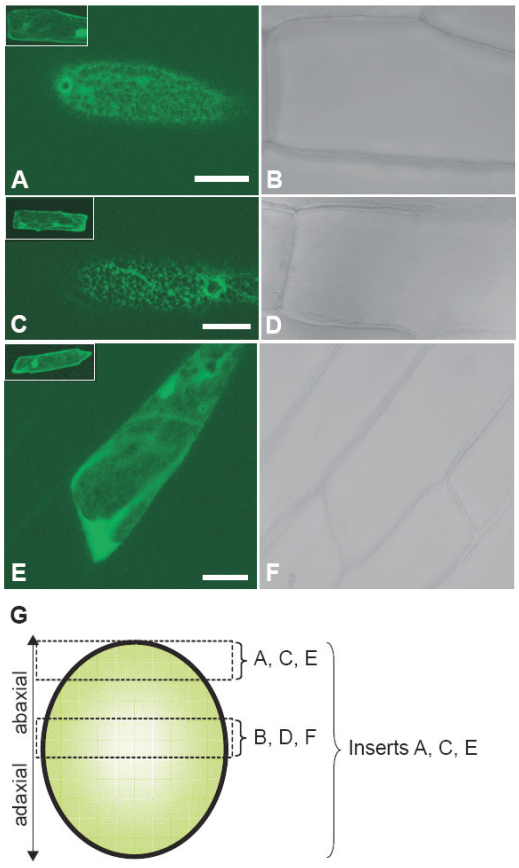


Figure 5. HvP_5 localizes to ER-like structures in the cortical cytoplasm and to the nucleus of onion epidermal cells. HvP_5-GFP (A-B), GFP-HDEL (C-D) and GFP (E-F) were individually transiently expressed. G shows a diagrammatic representation of a cross-sectioned epidermal cell of the confocal planes used in A-F. HvP_5 and GFP-HDEL shows distinct fluorescent cortical networks, while GFP alone exhibits characteristic diffuse fluorescence throughout the cytoplasm and nucleus. A, C and E are stacked images of the upper 10 microns of transformed fluorescent cells. Inserts are maximum intensity images of Z-projections of all confocal planes of the same individual cells. B, D and F are bright field images visualizing the morphology of the cells. Scale bars = 50 microns.

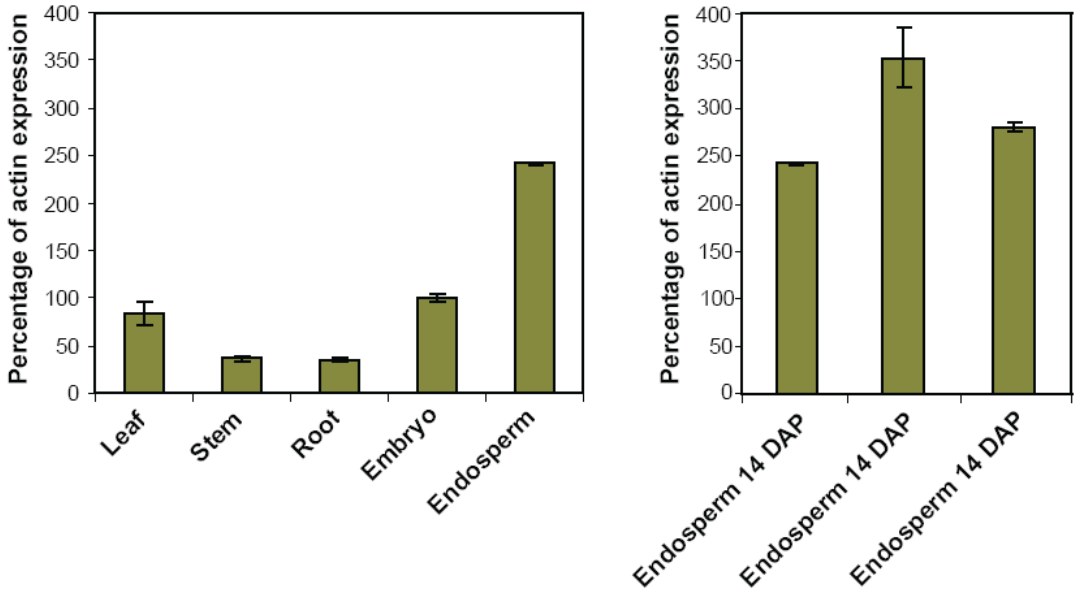


Figure 6. Expression of *HvP*$_5$ in different tissues of barley at different developmental stages. A) *HvP*$_5$ was expressed in all tissues of barley, however at a lower level in stem and root. In developing barley grains, high level of expression were especially found in the endosperm. B) *HvP*$_5$ expression is increased during endosperm maturation and storage phase (14 Dap and 25 DAP), while expression ceased at desiccation stage (35 DAP).

activity like pollen grains and tapetal cells, and the mutant exhibits major changes in expression of genes involved in protein secretion (Jakobsen et al. 2005).

Functional complementation experiments illustrate that the function of P_5 ATPases is conserved across distant evolutionary lineages within eukaryotes. Conserved function of P_5 ATPases across distant evolutionary lineages suggests that the transporters serve universal and important functions. This is also supported by the fact that P_5 ATPases, although absent from prokaryotes, are present in all eukaryotes examined so far (Møller et al. submitted).

HvP_5 is localized to ER, which constitutes the starting point for the secretory pathway directing proteins from ER, through Golgi apparatus to their final destinations (Battey et al. 1999). *S. cerevisiae* P_{5A} ATPase Spf1p localizes to ER (Cronin et al. 2002; Vashist et al. 2002) and *cis*-Golgi (Suzuki 2001). ER localization was also found for the Spf1p homologue in *Saccharomyces pombe*, Cta4p (Okorokova-Façanha et al. 2002). Likewise, MIA was detected in ER of developing pollen grains and tapetal cells as well as in small, probably ER-derived vesicles (Jakobsen et al. 2005). Taken together, complementation and localization studies support that HvP_5 might be involved with protein processing and/or trafficking from the ER to the Golgi in barley.

Our study of tissue specific expression pattern in the developing barley grain showed that HvP_5 is expressed in all tissues of the barley plant. Interestingly, expression in the endosperm of developing barley grains is significantly higher than all other tissues. In the present context synthesis and secretion of storage proteins is in particular relevant. In the grain, the secretory pathway synthesizes storage proteins on rough ER membranes with a co-translational transport into the lumen of ER. Storage proteins then pass via the Golgi apparatus to vesicles that

fuse with the protein storage vacuoles (Costa et al. 2004). In wheat, vesicles can directly bud off from ER and migrate to the protein storage vacuoles (Arcalis et al. 2004). Functionality of the secretory pathway relies on efficient transfer of cargo molecules from their site of synthesis in ER to vacuolar compartments (Hanton et al. 2006). The endosperm is therefore structurally and functionally adapted to ensure efficient translocation of storage proteins into storage vacuoles.

Activity of the secretory pathway and delivery of storage proteins to protein storage vacuoles varies during grain development (Vensel et al. 2005). Grain development is divided into three phases: a cell division phase, a storage phase and a final desiccation phase (Goldberg et al. 1989; Wobus et al. 2005). Storage phase involves active biosynthesis of proteins and metabolites followed by efficient secretion to storage compartments. In desiccation phase there is an overall shift from active biosynthesis of storage proteins towards maintenance and defence-related compounds (McIntosh et al. 2007; Vensel et al. 2005).

In barley endosperm expression of HvP_5 increases during seed development from early storage (14 days after pollination; DAP) and reaches its maximum at the stage of intense storage protein, starch and cell wall biosynthesis in late storage phase (25 DAP), while expression decreases toward the end of grain maturation (35 DAP). Expression profile thus coincides with that of storage proteins.

In summary, our report represents the first characterization of P_5 ATPases in a monocot plant where it is highly expressed in grain endosperm. Even though the biochemical function of P_5 ATPases remains unknown in any system, studies in yeast and plants show their requirement for protein processing and vesicle trafficking from the ER. In cereal grains this endomembrane system is essential for storage protein biosynthesis and deposition in intracellular compartments. It remains to be shown whether HvP_5 sustains vesicle trafficking of storage proteins in the barley endosperm.

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6

Conclusions and Perspectives

In this thesis, expression modulations of the isolated *HvNAC6* sequence provide a significant phenotype on barley penetration resistance towards *Bgh*. This highlights the power of reverse genetics for the conversion of sequence information into biologically meaningfulness. A stable knock-out line in the *Arabidopsis HvNAC6* homologue *ATAF1* phenocopied the transient *HvNAC6* gene silencing effect on reduced penetration resistance towards *Bgh*, providing robustness to the obtained results. Furthermore, acknowledging the observed cross-species regulatory conservancy underlying this line of pre-invasive plant defence, questions related to the occurrence of this phenotype were targeted by top-down and bottom-up approaches for an understanding of the regulatory perturbations potentially determining the phenotypic outcome of such expression modulations. From these analyses, it became evident that *ataf1-1* mutant plants alter the expression of ABA-responsive genes, particularly upon *Bgh* inoculation. The *ataf1-1* mutant seeds furthermore displayed ABA hypersensitivity with respect to germination and seedling development and additionally showed increased vegetative growth; characteristic of ABA accelerated signalling (Barrero *et al.*, 2005). Hence, it could be speculated that the close *HvNAC6* homologue in *Arabidopsis*, *ATAF1*, is a negative regulator of ABA signalling. Interestingly, the link between the large-scale ABA-responsive transcript changes, ABA hypersensitivity of *ataf1-1* mutant plants, and the reduced penetration resistance observed upon *Bgh* penetration attempts in *ataf1-1*, was presumably resolved by the fact that exogenously applied ABA negatively regulates the formation of papillae, thereby compromising effective penetration resistance in *Arabidopsis* towards *Bgh*. The ABA-induced reduction of penetration resistance was even more pronounced in *ataf1-1* mutant plants. In conclusion, this suggests that ATAF1 negatively regulates ABA signalling for efficient penetration resistance towards *Bgh*. When *ATAF1* is knocked out, *Bgh* penetration attempts succeeds more often due to de-repressed ABA signalling. Hence, it is furthermore suggested that *Bgh* corrupts ABA signalling in the non-host, and possibly barley host plants (Edwards, 1983), for increased entry to epidermal cells.

The long term overall objective of plant molecular biology is to enhance our understanding of plant life; its orchestration of responses upon exogenous and endogenous stimuli. In this context, it is relevant to ask how we can use the information gathered from the model plant *Arabidopsis thaliana*, used in this study, for improved crop protection? *Arabidopsis* has already proved an essential weapon in the armoury of modern plant scientists for their study of crop plants or model systems (Livingston, III *et al.*, 2007;Murray *et al.*, 2002;Sandmann *et al.*, 2006). It has made fundamental contributions to our understanding of gene regulation, both genetic and epigenetic, in development and environmental adaptation (Cui *et al.*, 2007;Henderson and Jacobsen, 2007), and it has contributed to the identification of the genes activated in response to biotic and abiotic stress, potentially interesting for improved stress tolerance in crop plants (Anderson *et al.*, 2004;Govrin and Levine, 2002;Kariola *et al.*, 2006). An important lesson underscored from my studies is that the dicot *Arabidopsis* serves as a strong reference for studies of defence regulations on large-genome monocot cereals. The study presented in this thesis highlights a potential conservancy of ABA signalling

modulation in response to the host and non-host pathogen *Bgh*, of barley and *Arabidopsis*, respectively. Hence, mechanistic, genetic, regulatory and hormone signalling conservancy potentially underlies this economically important broad range type of resistance in both the reference weed, *Arabidopsis*, and a large-genome monocotyledonous cereal.

Environmental constraints, including factors like salt, drought, extreme temperatures, and attack by microbial pathogens and insect pests severely limit crop productivity. Breeding for traits that confer tolerance towards these major abiotic and biotic stresses may provide means to increased yield. To this end, considerations of the strengths and weaknesses of *ATAF1* and *HvNAC6* studies in the context of crop-protection application would be useful. However, it should be noted, that unlike many laboratory environments, plants are constantly exposed to more than one kind of stress at a time in the field. This renders the situation more complex, both in terms of identifying and interpreting observed phenotypical and molecular changes induced by reactions to combinations of stress, and topics related to induced susceptibility and resistance could arise (Lyngkjaer and Carver, 1999;Olesen *et al.*, 2003). Furthermore, often the balance between overall cost and benefit is fine-tuned; modulations of parameters benefiting one trait may hamper or impair another (Anderson *et al.*, 2004). For instance, allocation costs occur if large quantities of fitness-limiting resources are allocated to resistance (Baldwin, 1998;Heil and Baldwin, 2002). Thus, induction of defences is always a trade-off with some alternative target for energy, such as growth and reproduction (Baldwin, 1998). Silenced *ATAF1* activity exemplifies this compromise. The *ataf1-1* mutant attenuates plant capacity for pathogen resistance, properly by interfering with hormonal equilibrium under both control and stressed conditions, hereby allocating components needed for growth. In this respect, it could also be speculated that wild-type plants have ATAF1, and possibly *Hv*NAC6, as capacitors guarding the use of plant resources. Upon *Bgh* inoculation, their negative effects on ABA signalling delay the ABA signal and biosynthesis, and thus, improves the timely assembly of cell wall fortifications. Hence, these gene products should be seen as switches deciding when and to what extent ABA mediated signalling should be expanding. However, as mentioned above, in situations where plants are under threat from different environmental factors, improving tolerance or resistance to some stresses may render the plant less fit or hypersusceptible to other stresses. This raises the question of how moderate abiotic stresses, such as mild drought, salinity, and freezing, commonly encountered in crops during the growing season influence the capability of the same plants to induce pathogen resistance, and *vice versa*.

Apart from the mutual drawbacks (*e.g.*, less drought tolerance) and benefits (*e.g.*, improved penetration resistance) of ATAF1-dependent attenuation of ABA signalling in wild-type plants, another illustrative example gathered from my studies is speculation related to a possible NAC-association of the pleiotropic effects, like increased senescence, observed in *mlo* mutant alleles (Consonni *et al.*, 2006;Wolter *et al.*, 1993). In this case, it is interesting that *HvNAC6* is more abundantly expressed in an *mlo* mutant allele compared to wild-type *MLO* plants upon *Bgh* attack, as this expression pattern correlates with increased penetration

resistance. This complements my studies on basal and non-host resistance. Interestingly, a wheat *OsNAC6* homologue shows increased senescence associated phenotype, calling for speculations of whether NAC proteins of the ATAF subfamily are regulatory proteins fine-tuning and optimizing responses to environmental stimuli. In such a scenario, it could be speculated that *Hv*NAC6 is the regulatory node of convergence that, when induced, enables effective penetration resistance, for the price of potential massive acceleration of senescence associated cell death at later developmental stages. This compromise highlights the topic – and complexity - of future research on crop breeding for improved adaptations to environmental constraints; *integrated functional genomics*, aiming at targeting knowledge of natural variations in crops for improved understanding of optimal growth environment and subsequent improved yield. In terms of breeding for durable resistance to fungal pathogens, data presented by Zierold *et al.* (2005) suggests that it might be difficult to obtain this by marker-assisted breeding or by engineering of many differentially expressed candidate genes apart from *MLO*, unless it is possible to set up a hierarchical system of gene impact or epistasis. In such a system, components of signal transduction, like transcription factors and protein kinases, may have a higher impact on final outcome of barley-*Bgh* interaction, than for instance PR proteins that might be part of a highly redundant set of terminal effectors (Zierold *et al.*, 2005). Indeed, modulating the expression of several NAC genes have proved their worth as successful regulators of both abiotic and biotic stress tolerances, *i.e.* withstanding environmental stresses compared to wild-type plants (Delessert *et al.*, 2005;Hu *et al.*, 2006;Lu *et al.*, 2007). In this respect, and most importantly, elucidation of whether the effects observed on stress tolerance under laboratory conditions hold true in the field should be tested. If verified, ectopic expression of *HvNAC6*, among other NACs, could prove beneficial in fields of low water stress and high *Bgh* epidemic occurrences.

Future perspectives for the elucidation of ATAF1 and *Hv*NAC6 functionalities should focus on post-trancriptional events. By expression modulation we suggest that *Hv*NAC6 and ATAF1 are important future candidates for research on the regulation underlying broad-spectrum basal resistance to fungal pathogens. However, several studies have identified only temporal and quantitative changes, as opposed to major qualitative changes, in transcript profiles when comparing susceptible and resistant barley responses towards *Bgh* inoculation (Caldo *et al.*, 2004;Caldo *et al.*, 2006;Trujillo *et al.*, 2004;Zierold *et al.*, 2005). This leads to speculation for post-transcriptional changes in search for major differences between susceptible and resistant plants. Indeed, ubiquitination has been reported to influence on the basal host resistance of barley towards *Bgh* (Dong *et al.*, 2006). Interestingly, results obtained using transient gene silencing on barley showed that ubiquitin was dependent for the accumulation of papilla formations in nominally susceptible hosts, but not affected the non-host- or *mlo*-mediated penetration resistance (Dong et al., 2006). This raises the question of whether the qualitative differences of basal resistance and non-host resistance are to be found at the proteome level, and not on the transcriptome level. Acknowledging the large-scale proteomics tools available, future research on the post-transcriptional modifications of signalling components of regulatory cascades mediating the timely assembly of cell wall

appositions towards potential fungal intruders should improve our understanding of this important line of defence.

Finally, substantial progress has been accomplished on the characterization of plant responses to their environment in recent years. This is mostly due to the reverse genetic tools established and deployed on the basis of the ever increasing amounts of biological sequence information being generated (Hsing *et al.*, 2007;Rhee *et al.*, 2006). The aim of current plant molecular biology is not limited to the simple functional characterization of individual genes and their expression patterns upon environmental challenges, as presented in this thesis. The vast amounts of data sets generated from transcriptomics, proteomics, and metabolomics ideally encompass the link between genes and phenotypes in a biological system (Holtorf *et al.*, 2002;Yang *et al.*, 2005). When adequately standardized, all these data serve as essential components of large-scale modelling in systems-oriented biology, aiming at the elucidation of gene functions and the description of complex biological systems (Yang *et al.*, 2005). Integration of all the above mentioned large-scale *-omics* approaches will enhance our understanding of the complex signalling and metabolic networks underlying plant growth, development and interaction with the environment. Ultimately, this will enable predictable metabolic engineering of plant processes that are important to crop yield, nutrition and defence (Chen and Harmon, 2006).

7

Appendices

Appendix I

Barley-*Bgh* transient over-expression studies. Compatible interaction (P-01/A6).
Total number of observations: 1970

Leaf\Treat	Control		*HvSNAP34*		*HvNAC4*		*HvNAC6*		*HvNAC1*	
	Papillae	Total	Papillae	Total	Papillae	Total	Papillae	Total	Papillae	Total
1	2	21	6	19	10	24	0	1	4	9
2	5	22	8	12	1	4	2	3	5	21
3	10	21	1	3	9	30	3	5	8	34
4	13	21	21	50	5	14	7	17	2	10
5	6	13	11	21	1	4	0	0	8	32
6	12	18	3	11	4	12	0	0	4	16
7	10	40	25	50	0	3	1	3	0	1
8	9	25	12	52	2	13	8	14	12	34
1	2	6	10	12	0	0	18	35	12	27
2	0	0	7	8	1	1	0	0	9	19
3	6	13	4	6	11	19	42	73	12	16
4	4	12	21	41	3	6	10	17	10	30
5	12	20	15	28	3	4	14	26	13	35
6	5	12	0	0	4	6	37	49	19	29
7	5	10	2	4	0	0	0	0	6	10
8	5	9	46	59	1	2	0	2	0	2
1	9	24	15	22	9	30	16	23	2	7
2	1	2	4	4	5	11	18	28	1	4
3	8	14	4	4	16	27	8	12	13	32
4	16	34	11	16	1	2	5	5	17	24
5	6	8	3	5	19	42	11	21	4	15
6	2	5	16	17	7	19	21	37	2	12
7	0	0	11	21	13	36	3	4	1	7
8	3	3	4	4	16	34	4	8	3	5
Total	**151**	**353**	**260**	**469**	**131**	**343**	**228**	**383**	**167**	**431**

Appendix II

Barley-*Bgh* transient gene silencing. Compatible interaction (P-01/A6)
Total number of observations: 1852

Leaf \ Treat	Control		*HvSNAP34*		*HvNAC4*		*HvNAC6*		*HvNAC1*	
	Papillae	Total	Papillae	Total	Papillae	Total	Papillae	Total	Papillae	Total
1	0	2	3	4	1	6	0	0	2	2
2	0	1	2	4	0	0	3	6	2	8
3	1	2	0	0	1	1	1	2	0	0
4	3	4	0	0	0	1	0	0	0	0
5	3	2	1	1	1	2	0	3	1	2
6	1	2	0	3	5	7	0	0	1	2
7	0	2	2	2	1	3	0	0	1	3
8	2	0	0	0	0	0	0	0	1	2
9	0	0	0	0	0	0	0	0	0	0
1	1	1	1	5	1	1	0	0	0	2
2	1	5	0	0	0	4	0	1	0	1
3	2	13	0	0	2	6	4	12	4	22
4	3	4	0	0	0	2	0	1	0	1
5	4	6	0	0	0	3	0	0	0	0
6	0	9	0	3	0	0	0	1	1	7
7	2	5	1	3	5	33	0	7	3	21
8	1	3	1	1	1	8	1	11	1	8
9	0	0	0	3	3	9	1	8	1	11
1	11	41	0	1	0	0	0	1	3	13
2	0	1	1	4	0	0	0	6	1	2
3	0	0	1	9	3	12	4	14	3	11
4	0	0	2	3	0	1	1	7	0	1
5	2	3	3	20	0	0	0	9	3	20
6	8	20	3	18	2	11	3	14	0	0
7	1	1	0	15	0	0	0	2	2	7
8	0	0	2	7	0	0	1	16	2	3
9	2	7	7	32	0	0	6	16	3	8
1	16	66	5	22	2	31	13	56	1	12
2	5	18	6	33	14	38	5	13	4	11
3	2	14	2	16	0	2	0	4	3	13
4	16	47	2	7	0	1	1	6	8	47
5	4	12	0	6	0	0	0	1	7	36
6	2	5	3	12	0	0	2	5	3	11
7	11	29	6	28	7	37	7	19	5	25
8	1	8	0	11	20	38	1	12	4	23
9	10	39	5	14	1	4	3	7	4	31
Total	**115**	**366**	**59**	**287**	**70**	**261**	**57**	**260**	**74**	**366**

Appendix III

Arabidopsis-Bgh (Col-0 vs. *ataf1-1*)
Total number of observations: 3990

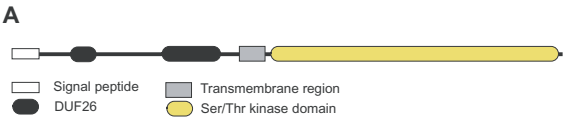
Leaf \ Treat	**Col-0**			***ataf1-1***		
	Papillae	HR (+sec. muc. growth)	Total	Papillae	HR(+sec. myc. growth)	Total
1	27	2 (1)	30	190	23 (4)	217
2	237	10	247	148	26 (3)	177
3	82	1 (4)	87	296	47	343
4	169	29	198	185	9 (2)	196
5	495	52 (1)	548	274	61	335
6	179	31 (1)	211	262	60 (2)	324
7	125	33	158	369	61	430
8	279	15	294	171	24	195
Total	**1593**	**173 (7)**	**1773**	**1895**	**311 (11)**	**2217**

Appendix IV

Arabidopsis-Bgh +/- ABA
Total number of observations: 2972

Leaf \ Treat	**Col-0 Buffer**		**Col-0 ABA**		***ataf1-1* Buffer**		***ataf1-1* ABA**	
	Penetration	Total	Penetration	Total	Penetration	Total	Penetration	Total
1	13	130	21	111	25	118	39	189
2	14	112	22	112	14	131	147	340
3	15	121	1	3	43	199	116	381
4	9	114	21	52	78	378	100	303
Total	**51**	**477**	**65**	**278**	**160**	**826**	**402**	**1213**

Appendix V



B

```
   1 GAACCTCACTCTGGCAAAAGAGAAACAAGAACCTGTTGTAGTTAGCTACTCTTGTACTCG
  61 TGTTAGCTTGCCATGGCGACCATCGGCGTCGTCCTCCTCCTGCTCCTCCTACCCACACCC
                M  A  T  I  G  V  V  L  L  L  L  L  L  P  T  P     16
 121 ACACCCTTTCTGGTGGCCGCCAATTTCTGCGACAACGTCAAGGTAGCCGCTGCCGCCCTT
     T  P  F  L  V  A  A  N  F  C  D  N  V  K  V  A  A  A  A  L     36
 181 TCCAAGAACGTCACCTCCTCCCCGGAAAACTTCGCCACCGCCACCTTTGGCCAGCCCCCC
     S  K  N  V  T  S  S  P  E  N  F  A  T  A  T  F  G  Q  P  P     56
 241 GACGTCGTCTACGCGCTCGCGCTCTGCCGCGCCGGTGACGTCGTCGACGGCTCCACCTGT
     D  V  V  Y  A  L  A  L  C  R  A  G  D  V  V  D  G  S  T  C     76
 301 AGTGACTGCATCGCTAACTGGTTCGCTGCAGTAAACCAGACGGAGTGCCACAAGGTCCGC
     S  D  C  I  A  N  W  F  A  A  V  N  Q  T  E  C  H  K  V  R     96
 361 TCCAGCTACGGCGACTGTATTGTCGTCTACAGCGCCGGCGACGACACCTACCGCCACCC
     S  S  Y  G  D  C  I  V  V  Y  S  A  G  D  D  N  L  P  P  P    116
 421 TCAAATGTCATGGGAGGATCCTCCGGTGAGAACACGCTGCCCTTCGAGCAGTGGGACATC
     S  N  V  M  G  G  S  S  G  E  N  T  L  P  F  E  Q  W  D  I    136
 481 ACGAACGTCACCACCGGCGACGTCCAACTCATCGTCGGCCTCACCCGCGAGTTGCTGGTC
     T  N  V  T  T  G  D  V  Q  L  I  V  G  L  T  R  E  L  L  V    156
 541 GAGACAGCCGAGACGGCGGCCAGCACGACGCCGAGTCGGTACGCCACTGGCGTCATAGAC
     E  T  A  E  T  A  A  S  T  T  P  S  R  Y  A  T  G  V  I  D    176
 601 ATGGACAGAGTGACGACCTACCCGAAGGTGTACTCCCAGGCGCATTGCATGCCGGACCTG
     M  D  R  V  T  T  Y  P  K  V  Y  S  Q  A  H  C  M  P  D  L    196
 661 TCCGCCGATAACTGCTTGGCATGCCTGCGGCGTCTCCTCGGGATGGTCAACTCCACCATG
     S  A  D  N  C  L  A  C  L  R  R  L  L  G  M  V  N  S  T  M    216
 721 TCCCTCCGCACGGGTGGACAAATGGGCATCACGCAATGCTATTTCAGGTACGAAGCTTAT
     S  L  R  T  G  G  Q  M  G  I  T  Q  C  Y  F  R  Y  E  A  Y    236
 781 CGGTTCTATGGCGCCCAACCCATGCTGATTGTGCCATTGCCGACACCGGCGCTGGCAACC
     R  F  Y  G  A  Q  P  M  L  I  V  P  L  P  T  P  A  L  A  T    256
 841 AAACACAGGAGCATGTTGTGGGTAATTCTCGCAGTTGTAGTTCCTCTTTCGGCAGCAGCA
     K  H  R  S  M  L  W  V  I  L  A  V  V  V  P  L  S  A  A  A    276
 901 TTTGTCTTCTTCGTCTGTTACTCTCGTCGGCTCAGGAGCCAAAGAAAAGGATCAAGACGT
     F  V  F  F  V  C  Y  S  R  R  L  R  S  Q  R  K  G  S  R  R    296
 961 GATTGGAATTTAAAGGGAGATCTAGcTTGGCAAGGGAAAAATTCGGAGTTCTCTTTGTTT
     D  W  N  L  K  G  D  L  V  W  Q  G  K  N  S  E  F  S  L  F    316
1021 GACTTTCACCAGCTAGTGGAGGCCACAGACAGTTTTTCAGAAGAAAACAAACTTGGACAG
     D  F  H  Q  L  V  E  A  T  D  S  F  S  E  E  N  K  L  G  Q    336
1081 GGTGGCTTTGGCGCTGTCTACAAGGGCGaACTTCCCGAGGGATTGGAGGTAGCAGTCAAG
     G  G  F  G  A  V  Y  K  G  E  L  P  E  G  L  E  V  A  V  K    356
1141 AGACTGTCTTCGCATTCTGGACAAGGTTTCATGGAGTTCAAAAATGAAGTCCAACTAATA
     R  L  S  S  H  S  G  Q  G  F  M  E  F  K  N  E  V  Q  L  I    376
1201 GCCAAACTCCAGCACACAAATTTGGTTAGGCTCTTGGGATGTTGCTCTCAAGATGAGGAG
     A  K  L  Q  H  T  N  L  V  R  L  L  G  C  C  S  Q  D  E  E    396
1261 AACATACTGGTTTATGAATACTTGCCAAACAGAAGTCTGGATTTCTTCATCTCTGACGTG
     N  I  L  V  Y  E  Y  L  P  N  R  S  L  D  F  F  I  S  D  V    416
1321 AATAAAAGAGCTTTAATGGATTGGTCAACACATGTAGCAATAATTGAAGGAGTAGCACAT
     N  K  R  A  L  M  D  W  S  T  H  V  A  I  I  E  G  V  A  H    436
1381 GGACTTCTTTACCTACATAAGCACTCTCGTCTGCTTGTCATACACCGAGACCTTAAACCA
     G  L  L  Y  L  H  K  H  S  R  L  L  V  I  H  R  D  L  K  P    456
1441 AGCAATATTCTCTTGGACTACGAACTGAATCCAAAAATTTCAGATTTTGGGCTAGCAAAA
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1501 ATATTGAGCTCAAATGACACTGAAGGAAATACCACAAGACGAGTAGTTGGCACATCTGGC
     I  L  S  S  N  D  T  E  G  N  T  T  R  R  V  V  G  T  S  G    496
1561 TACATGGCACCAGAGTATGCTTCAAAGGGCGTATTCTCAATTAAATCCGATGTCTTCAGT
     Y  M  A  P  E  Y  A  S  K  G  V  F  S  I  K  S  D  V  F  S    516
1621 TTTGGTGTTGTCATTTTTGAGATCCTTAGCGGAAAACAGAATTCTGGTAACGAACAATAT
     F  G  V  V  I  F  E  I  L  S  G  K  Q  N  S  G  N  E  Q  Y    536
1681 GGAGGTTTCCTCAATCTGCTTGGACATGCATGGCAATTATGGGAAGAAGGAAAGTGGGCT
     G  G  F  L  N  L  L  G  H  A  W  Q  L  W  E  E  G  K  W  A    556
1741 GATCTCATTGCTGCACCATTGCTACCCGGAAGTCACTCAGCAAAGATGATGAGATACTTT
     D  L  I  A  A  P  L  L  P  G  S  H  S  A  K  M  M  R  Y  F    576
1801 AATATAGCATTATTGTGTGTACAAGAGAATGCAACTGATAGACCAACCATGGGCGATATT
     N  I  A  L  L  C  V  Q  E  N  A  T  D  R  P  T  M  G  D  I    596
1861 GTTGCAATGCTAAGCAATGATGCTATGATATTGGCTGAGCCTAAGCAGCCAGCATATATT
     V  A  M  L  S  N  D  A  M  I  L  A  E  P  K  Q  P  A  Y  I    616
1921 AATGTAAGGGTGGGAAATGAAGAAGCGTCCACAGCTCTTGAGGCGTGCAATATTAAAGAT
     N  V  R  V  G  N  E  E  A  S  T  A  L  E  A  C  N  I  K  D    636
1981 ATGACCATATCTGCGACAATTCATGGATAGTCTTATCCCTGGTGCCATGTTTTAAAATTT
     M  T  I  S  A  T  I  H  G  *                                   645
2041 CCAGTCGTAATATTCACAACAAAATGTTGTATAATGCGTACCGTCAAATGGTGTTCAAAT
2101 GTGAGGTTATGTGCCCTAAAGAGTATCAAAAGAAAGCTTCCGCGGTAGCTGGAGTAAACA
2161 ATCAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
```
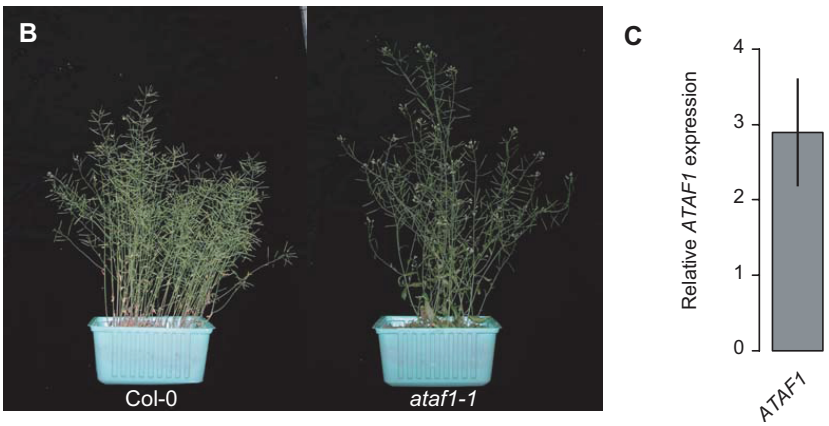

Appendix V. *Hv*RLK1 structure and sequence. (**A**) Diagramatic structure of the *HvRLK1* gene product predicted from InterProScan. The structure is based on the amino-acid sequence. (**B**) The sequence of *HvRLK1* cDNA and the deduced primary structure. The amino acids involved in the different structural motifs are as follows; putative signal peptide underlined; transmembrane region grey-shaded; Arg-based ER-retention signal open-boxed; bipartite DUF26 domain black-boxed. The predicted Ser/Thr kinase domain spanning the C-terminal approx. 350 amino acids are left unchanged.

Appendix VI



Appendix VI. *ATAF1* negatively regulates drought tolerance and is induced by ABA. (**A**) Eight weeks old *Arabidopsis* plants were left without water for two weeks. (**B**) After a further week without water. (**C**) *ATAF1* is induced by ABA. Three hours after spraying 6-weeks old *Arabidopsis* plants with 100 μM ABA or buffer (0.2% MeOH), five rossettes from each of treatment were harvested. Grey bar represents *ATAF1* relative expression in ABA treated plants compared to buffer treated plants. Error bar indicates standard deviation from two biological replicates.

Appendix VII

E-mail confirmations from editors

Dear Professor David B. Collinge, 15-06-07

We are pleased to inform you that your manuscript, "The HvNAC6 Transcription Factor: A Positive Regulator of Penetration Resistance in Barley and Arabidopsis", has been accepted for publication in Plant Molecular Biology.

Any queries concerning your manuscript should now be addressed to Pauline.Lichtveld@springer.com

If you would like to have your accepted article published with open access in our Open Choice program, please access the following URL: http://www.springer.com/openchoice.

Please remember to quote the manuscript number, PLAN2418R1, whenever inquiring about your manuscript.

With best regards,
Christiane Gatz
Associate Editor

Dear Prof. D.B. Collinge, 27-06-07<

We have received the reports from our advisors on your manuscript, "How can we exploit functional genomics to understand the nature of plant defences? Barley as a case study.", which you submitted to the European Journal of Plant Pathology.

Based on the advice received, I feel that your manuscript could be reconsidered for publication should you be prepared to incorporate revisions. When preparing your revised manuscript, you are asked to carefully consider the reviewer comments which are attached, and submit a list of responses to the comments.

In order to submit your revised manuscript, please access the following web site:

http://ejpp.edmgr.com/

Your username is: ******
Your password is: ******

We look forward to receiving your revised manuscript within
eight weeks.

With kind regards,
Ellen Klink
JEO Administrator

8

Abbreviations

ABA	Abscisic acid
AGT	Appressorial germ tube
At	*Arabidopsis thaliana*
Bgh	*Blumeria graminis* f.sp. *hordei*
Bp	Base pair
CaMV	Cauliflower mosaic virus
EST	Expressed sequence tag
ET	Ethylen
ER	Endoplasmatic reticulum
GFP	Green fluorescence protein
GUS	β-Glucuronidase
Hai	Hours after inoculation
HR	Hypersensitive response
Hrs	Hours
Hv	*Hordeum vulgare*
H_2O_2	Hydrogen peroxide
JA	Jasmonic acid
MIAME	Minimum information about microarray experiment
miRNA	micro RNA
mRNA	messenger RNA
NAC	NAM; ATAF1,2; CUC2
NO	Nitric oxide
ORF	Open reading frame
Os	*Oryzae sativa*
PAMP	Pathogen associated molecular pattern
PGT	Primary germ tube
PLM	Probe-level model
PRR	Pathogen-recognition receptors
QTL	Quantitative trait locus
RACE	Rapid amplification of cDNA ends
RNAi	Ribonucleic acid interference (RNA interference)
SA	Salicylic acid
SEM	Standard error of the mean
Sl	*Solanum lycopersicum*
SNARE	Soluble N-ethylmaleimide-sensitive-factor associated protein receptor
Ta	*Triticum aestivum*
T-DNA	Ti (or tumor inducing) DNA
TLCV	Tomato leaf curl virus
TILLING	Targeting induced local lesions in genomes
UTR	Untranslated region
WT	Wild-type
X-GAL	β-D-galactopyranoside

9

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