Annotation, phylogenetic analysis and functional characterization of barley [*Hordeum vulgare* L.] WRKY transcription factors in the interaction with powdery mildew fungus *Blumeria graminis*

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Table of Abbreviations

amiR	artificial microRNA
AVR	avirulence
Bgh	*Blumeria graminis* f.sp. *hordei*
BLAST	basic local alignment search tool
bp	base pair
CC	coiled-coil
cDNA	complementary DNA
ChIP	chromatin immunoprecipitation
cv.	cultivar
CWA	cell wall appositions
Da	Dalton
DNA	2'-deoxy-ribonucleic acid
dsRNA	double-stranded RNA
E. coli	*Escherichia coli*
EST	expressed sequence tag
et.al.	et altera
ETI	effector triggered immunity
f.sp	forma specialis
GFP	green fluorescent protein
GLP	germin-like proteins
GP	Golden Promise
GUS	β-glucuronidase
H	hour
HDA	histone deacetylase
HR	hypersensitive response
HSP	heat shock protein
JA	jasmonate
kb	kilobase (s)
kDa	kilodalton (s)
LRR	leucin-rich-repeat

MAMP	microbe-associated molecular patterns
MAPK	mitogen-activated protein kinase
miRNA	microRNA
ML	mildew resistance locus
MLA	mildew-resistance locus A
mRNA	messenger RNA
MTI	MAMP-triggered immunity
NBS	nucleotide-binding site
NLS	nuclear localisation signal
OD	optical density
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PCD	programmed cell death
PCR	polymerase chain reaction
PR	pathogenesis-related
qRT-PCR	quantitative real-time PCR
RNA	ribonucleic acid
RNAi	RNA interference
Ror	required for *mlo*-specific resistance
RT-PCR	reverse transcriptase-PCR
SA	salicylic acid
SAR	systemic acquired resistance
SDS	sodium dodecyl sulfate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
SE	standard error
siRNAs	small interfering RNAs
smRNAs	small RNAs
SPF1	sweet-potato factor 1
TBE	tris-borate-EDTA
TE	tris-EDTA
TTG2	transparent testa glabra2
VIGS	virus-induced gene silencing
WMD	web microRNA designer

Xoo	*Xanthomonas oryzae* pv. *oryzae*
R gene	resistance gene
X-Gluc	5-bromo-4-chloro-3-indoxyl-β-D-glucuronic acid

1. Introduction

To cope with variable environmental conditions, plants have evolved a great capacity to extensively reprogram their transcriptome in a highly dynamic and temporal manner through an integrated network of transcription factors. Among these transcription factors, WRKY factors are a group of regulatory proteins predominantly involved in stress responses (Pandey and Somssich, 2009). Together with other transcriptional regulators, WRKY proteins enable plants to better adapt to the changing environment and respond properly to internal and external stimuli.

1.1 Structure and evolution of WRKY transcription factors

1.1.1 Structure of WRKY transcription factors

The WRKY protein family is named after the most prominent feature of these proteins, the WRKY domain, a highly conserved motif spanning about 60 amino acids in all the family members (Eulgem *et al.*, 2000). Within this domain, there is an almost invariable heptapeptide signature WRKYGQK at the N-terminus and a novel zinc finger-like structure at the C-terminus. The WRKYGQK is the most dominant form of the signature followed by WRKYGKK and WRKYGEK (Eulgem *et al.*, 2000; Xie *et al.*, 2005b), however, there are at least 35 variants of this motif present in plant and non-plant species (Table 1.1). WRKY genes encode transcription factors and they are targeted to the nucleus as most of them contain a basic nuclear localization signal. WRKY proteins preferably bind to the consensus sequence TTGACC/T, the so-called W-box which is usually enriched in the promoter region of WRKY target genes such as stress responsive genes. Both the WRKY and zinc-finger motif are essential for proper DNA binding capacity of the protein (Maeo *et al.*, 2001).

Table 1. 1. List of WRKY signature variants in WRKY domains from plant and non-plant species (sequence from http://supfam.cs.bris.ac.uk/SUPERFAMILY/).

WRKY signature	Distribution of WRKY variants	Number of WRKY domains
WRKYGQK	All plant species	1761
WRKYGEK	All monocots,	47

	Some dicots (i.e, *Glycine max, Lotus japonicus*) Green algae (*Volvox carteri, Chlamydomonas reinhardtii*, Coccomyxa sp. C-169)	
WRKYGKK	All vascular plants	88
WKKYGQK	All monocots Some dicots (i.e, *Glycine max, Vitis vinefera*)	16
WQKYGQK	*Arabidopsis thaliana*, tomato (*Solanum lycopersicum*)	2
WSKYGQK	Tomato *(Solanum lycopersicum)*	1
WSKYGQM	Barley (*Hordeum vulgare*)	1
WTKYGQK	Barley (*Hordeum vulgare*)	1
WNKYGQK	Barley *(Hordeum vulgare)*	1
WKRKGQK	Rice (*Oryza sativa*)	1
WVKYGQK	Rice (*Oryza sativa*)	1
WRRYGLK	Rice (*Oryza sativa*)	1
WRKYEDK	Soybean (*Glycine max*)	1
WRKYGKR	Soybean (*Glycine max*)	1
WRKYGSK	Sorghum (*Sorghum bicolor*), *Medicago truncatula*, *Giardia lamblia*	3
WEKFGEK	Sorghum (*Sorghum bicolor*)	1
WRKYGQE	Wheat *(Triticum aestivum)*	2
WKKYGHK	*Giardia lamblia*	1
WRKCGLK	*Lotus japonicus*	1
WRKYGQN	*Lotus japonicus,* Moss (*Physcomitrella patens*)	3
WKKYGYK	*Lotus japonicus*	1
WKKYGED	*Lotus japonicus*	1
WLKYGQK	*Lotus japonicus*	1
WKKYEEK	*Medicago truncatula*	2
WKKYGEK	*Medicago truncatula,* *Asteraceae (Helianthus annuus; Lactuca sativa)*	7
WRKYGRK	*Medicago truncatula,*	3

	Black cottonwood (*Populus trichocarpa)*, Green alga (*Ostreococcus*)	
WKKYGNK	Moss (*Physcomitrella patens*)	5
WRKYGQT	*Brachypodium distachyon*	2
WKKYGPK	*Brachypodium distachyon*	1
WHKYGAK	*Micromonas* sp. RCC299	1
WRKYGHK	Green alga *(Ostreococcus)*	1
WRKYGNK	Green alga *(Ostreococcus)*	2
WKNNGNT	Alga fungi *(Phycomyces), Rhizopus*	12
WTKYDQR	Strawberry (*Fragaria vesca*)	1
WREYDQR	Strawberry (*Fragaria vesca*)	1
Total		1975

All WRKY proteins contain either one or two WRKY domains. Based on the number of WRKY domains and the structural features of the zinc-finger-like motif, WRKY protein family was originally divided into three groups. WRKY proteins with two WRKY domains are group I proteins while those with a single WRKY domain are group II or III. Group II WRKY proteins are further subdivided into five subgroups IIa, IIb, IIc, IId and IIe according to the presence of short conserved structural motifs. Group III differs from I and II in its variant C_2HC zinc finger motif $CX_7CX_{23}HXC$ (Eulgem *et al.*, 2000).

Some WRKY proteins exist as chimeric proteins combining NBS-LRR (nucleotide binding site - leucine rich repeat) proteins and WRKY domains (Deslandes *et al.*, 2002; Noutoshi *et al.*, 2005; Rushton *et al.*, 2010). AtWRKY52/RRS1 is such a protein that contains a group III WRKY domain C-terminal to a TIR-NBS-LRR (Toll/interleukin-1 receptor-nucleotide-binding site-leucine-riche repeat) domain and mediates R (resistance)-gene based resistance to the bacterial pathogen *Ralstonia solanacearum* (Deslandes *et al.*, 2003). In addition, AtWRKY16/TTR1 and AtWRKY19 are also NBS-LRR-WRKY proteins found in *Arabidopsis*. Other examples are GmWRKY176 from soybean (*Glycine max*), OsiWRKY41 (DAA05106) from indica rice (*Oryza sativa* indica) and ABF81432 from black cottonwood (*Populus trichocarpa*).

Till now, two reports on crystal structure of WRKY proteins are available (Duan *et al.*, 2007; Yamasaki *et al.*, 2005). The WRKY domain of AtWRKY4 consists of a four-stranded β-sheet, with the zinc coordinating Cys/His residues forming a zinc-binding pocket. The WRKYGQK residues correspond to the most N-terminal β-strand, which partly protrude from the protein surface and enable access to the major DNA groove during the interaction with the target DNA. This WRKYGQK-containing β -strand seems to make contact with an approximately 6-bp region, which is in consistent with the length of the consensus W-box (Yamasaki *et al.*, 2005; Yamasaki *et al.*, 2008). The crystal structure of the AtWRKY1 C-terminal WRKY domain is very similar with AtWRKY4 and contains an extral β-strands upstream of the WRKYGQK motif, thereby with DNA-binding residues located at the second and the third β-strands (Duan *et al.*, 2007).

1.1.2 Distribution and evolution of WRKY transcription factors

The first WRKY protein described was SPF1 (Sweet-Potato Factor 1) in sweet potato which was found to bind DNA upstream of genes coding for sporamin and beta-amylase (Ishiguro and Nakamura, 1994). Shortly after the first report in sweet potato, some discovery on WRKY proteins was made in other plant species including wild oat (*Avena fatua*), *Arabidopsis thaliana* and parsley (*Petroselinum crispum*) (de Pater *et al.*, 1996; Rushton *et al.*, 1996; Rushton *et al.*, 1995). Since then, knowledge about WRKY transcription factors has substantially accumulated (see review Eulgem and Somssich, 2007; Pandey and Somssich, 2009; Ross *et al.*, 2007; Rushton *et al.*, 2010). The WRKY gene family has been analyzed in a number of plant species including barley (*Hordeum vulgare*), bittersweet nightshade (*Solanum dulcamara*), chamomile (*Matricaria chamomilla*), *Citrus* spp, creosote bush (*Larrea tridentata*), cucumber (*Cucumus sativus*), grapevine (*Vitus aestivalis*), orchardgrass (*Dactylis glomerata*), potato (*Solanum tuberosum* and *S.chacoense*), rice (*Oryza sativa*), tobacco (*Nicotiana tabacum*), tree cotton (*Gossypium arboreum*), white weeping broom (*Retama raetam), tomato* (*Solanum lycopersicum*) and soybean *(Glycine max*) (Ross *et al.*, 2007; Rushton *et al.*, 2010; Ülker and Somssich, 2004).

The number of WRKY proteins is expanding from one in the unicellular green alga *Chlamydomonas reinhardtii* to nearly 200 in soybean (*Glycine max*) (Rushton *et al*., 2010). Therefore, it is believed that WRKY proteins had a lineage-specific expansion in plants during the evolutionary process. Compared to the green alga and moss, flowering plants have the largest WRKY family. Due to their wide presence in plants and their unique expansion in dicot and monocot plants, WRKY proteins were initially considered as plant-specific transcription factors. However, the discovery of proteins with WRKY domains in the protist *Giardia lamblia* and the slime mold *Dictyostelium discoideum* challenged this concept and implicated a much earlier origin of WRKY proteins (Pan *et al*., 2009; Ülker and Somssich, 2004). Up to now, proteins with putative WRKY domains have also been found in other non-plant organisms including the zygomycetes *Rhizopus oryzae*, *Phycomyces blakesleeanus*, *Mucor circinelloides* and the slime mold *Dictyostelium purpureum*.

Consecutive WRKY domain gain and loss led to an expansion of the WRKY family, and that a rapid amplification of the WRKY genes appeared to be earlier than the divergence of monocot and dicot plants (Wu *et al*., 2005). Despite some debate on the evolution of WRKY domains, it is now well accepted that group I WRKYs are the most ancient WRKY proteins evidenced from the unicellular green alga *Chlamydomonas reinhardtii*. There is evidence supporting a late evolution of group II, however, group III was also considered as the last evolved group due to its expansion in monocot plants (Mangelsen *et al*., 2008; Rushton *et al*., 2010; Ülker and Somssich., 2004; Zhang and Wang, 2005). It was revealed that some sequence-related homologous WRKY proteins have conserved functions between monocots and dicots (Mangelsen *et al*., 2008; Proietti *et al*., 2011).

1.2 Biological functions of WRKY transcription factors

Numerous studies have revealed the significance of WRKY transcription factors in multiple processes including development, hormone signalling and responses to biotic and abiotic stresses (Rushton *et al*., 2010). A single WRKY transcription factor might mediate transcriptional reprogramming associated with multiple signalling pathways. On the other hand, multiple WRKY proteins might act in a

single physiological process synergistically or antagonistically (Pandey and Somssich, 2009). The interconnected signalling network of WRKY factors possesses multiple inputs and outputs (Rushton *et al*., 2010)

1.2.1 WRKY in biotic stresses

Plants have evolved two layers of defense mechanisms to antagonize the invading pathogens (Jones and Dangl, 2006). These two interconnected branches are termed as MAMP-triggered immunity (MTI) and effector-triggered immunity (ETI) and they are initiated either relying on the recognition of conserved microbial molecules, so-called microbe-associated molecular patterns (MAMPs) or the recognition of pathogen-derived specific (a)virulence factors (effectors). The local and systemic defense responses activated by MTI or ETI often require the modulation from phytohormones such as jasmonate (JA) and salicylic acid (SA) (Bostock, 2005; Durrant and Dong, 2004; Pandey and Somssich, 2009). These responses upon pathogen invasion require massive transcriptional reprogramming, which was achieved by transcription factors including WRKY proteins (Eulgem, 2005; Naoumkina *et al*., 2008; Ryu *et al*., 2006; Wang *et al*., 2006). Such transcriptional reprogramming associated with plant defense leads to timely and balanced activation/repression of diverse targets in plant immune responses. Thus, WRKY factors are considered as central regulators in plant innate immune system (Eulgem and Somssich, 2007).

Gain- or loss-of-function studies have demonstrated that WRKY proteins are critical regulators of plant immune responses either positively or negatively in a sophisticated defense response network (Deslandes *et al*., 2002; Journot-Catalino *et al*., 2006; Kim *et al*., 2008; Li *et al*., 2006; Murray *et al*., 2007; Shen *et al*., 2007; Zheng *et al*., 2007). The R - gene type protein AtWRKY52 confers strong resistance towards the bacterial pathogen *Ralstonia solanacearum* (Deslandes *et al*., 2002). This R-gene mediated resistance was achieved through its nuclear interaction with the bacterial effector PopP2 (Deslandes *et al*., 2003). Interestingly, a single amino acid insertion in the WRKY domain led to conditional activation of defense responses and a loss in the DNA-binding capability (Noutoshi *et al*., 2005). In addition, AtWRKY52 provides dual resistance against fungal and bacterial

pathogens by synergistically acting with the R protein RPS4. AtWRKY70 was described as a shared component in SA and JA-dependent defense pathways and played crucial role in the cross-talk of SA and JA signalling (Li *et al*., 2004; Li *et al*., 2006; Ren *et al*., 2008). Moreover, AtWRKY70 was indispensable for the transduction of R gene-mediated resistance and receptor-like protein (RLP)-mediate immunity (Knoth *et al*., 2007; Zhang *et al*., 2010). It was also suggested to positively modulate systemic acquired resistance (SAR) (Wang *et al*., 2006). Other positive regulators of resistance in *Arabidopsis* include AtWRKY3, -4 and -33, which play a role in resistance against necrotrophic pathogens *Botrytis cinerea* and *Alternaria brassicicola* (Lai *et al*., 2008; Zheng *et al*., 2006). In addition, AtWRKY8 was characterized recently as a positive regulator of basal defense to *B.cinerea* but a negative regulator to *Pseudomonas syringae* (Chen *et al*., 2010a). Most information about functions of WRKY transcription factors came from the model dicot plant *Arabidopsis*, but their importance in pathogen defense was also demonstrated in monocots like rice and barley. In rice, majorities of the OsWRKY genes are responsible to pathogen challenge, abiotic stresses and phytohormone treatment (Ramamoorthy *et al*., 2008; Ryu *et al*., 2006). Overexpression studies have demonstrated several WRKYs (OsWRKY3, -13, -31, -45, -53, -71 and -89) to be associated with rice resistance towards *Magnaporthe grisea* and/or *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) (Chujo *et al*., 2007; Liu *et al*., 2005; Liu *et al*., 2007; Qiu *et al*., 2007; Qiu *et al*., 2008a; Qiu and Yu, 2009; Shimono *et al*., 2007; Tao *et al*., 2009; Wang *et al*., 2007; Zhang *et al*., 2008). For instance, overexpression of OsWRKY13 enhances resistance to the bacterial blight *Xoo* and the rice blast *M. oryzae* by activating the SA synthesis and suppressing the JA pathway (Qiu *et al*., 2007; 2008a). Similarly, OsWRKY71 was shown to be inducible by SA and overexpression of OsWRKY71 enhanced the rice resistance to *Xoo* through the indirect activation of OsPR1b and OsNPR1 (Liu *et al*., 2007). OsWRKY45 plays pivotal role in BTH-induced resistance to rice blast fungus through the SA pathway (Shimono *et al*., 2007; Shimono *et al*., 2011). *OsWRKY45-1* (japonica-derived *WRKY45)* and *OsWRKY45-2* (indica-derived *WRKY45)* overexpression resulted in enhanced resistance to the rice fungal pathogen *M. oryzae*, however, they have opposite effects on the resistance to *Xanthomonas oryzae* pv. *oryzae* (*Xoo*). The introduction of japonica-derived

WRKY45 (*WRKY45-1*) enhanced susceptibility to *Xoo* while overexpression of indica-derived *WRKY45* (*WRKY45-2*) resulted in resistance to *Xoo* (Tao *et al.*, 2009). Overexpression of OsWRKY53 protected the transgenic rice plants from the blast fungus *M. grisea* and stimulated the expression of PR proteins and peroxidase enzymes (Chujo *et al.*, 2007).

Depending on the pathogen, WRKY proteins may have contrasting effects on the resistance to diverse pathogens (e.g. the aforementioned AtWRKY8), which is also known for SA- and JA-mediated defense responses. But a number of WRKY transcription factors act, as far as known till now, only as negative regulators in plant defense responses. In *Arabidopsis*, the sequence-related AtWRKY18, -40 and -60 function in a partially redundant way in negatively regulating resistance to bacterial pathogen *Pseudomonas syringae* (Xu *et al.*, 2006). The *wrky18wrky40* double mutant exhibited enhanced resistance towards the biotrophic fungus *Golovinomyces orontii* but enhanced susceptibility to the necrotrophic fungus *Botrytis cinerea* (Shen *et al.*, 2007; Xu *et al.*, 2006). This mutant executes exaggerated expression of some defense related genes upon pathogen attack. WRKY40-complementation of the *wrky18wrky40* double mutants was able to partially restore susceptibility (Pandey *et al.*, 2010).Therefore, *AtWRKY18/40* are assumed to act in a feedback repression system that controls basal defense. Similarly, the barley orthologs *HvWRKY1* and *HvWRKY2* were shown to act as negative regulators of MTI (Eckey *et al.*, 2004; Shen *et al.*, 2007). The ETI to barley powdery mildew (*Blumeria graminis* f.sp. *hordei*) is dependent on the recognition of the fungal effector AVR10 by the resistance protein MLA (mildew-resistance locus A) resulting in a hypersensitive response (HR) to the biotrophic fungus. Interestingly, activated MLA10 translocates from plant cytoplasm into nucleus and interacts with *HvWRKY1* and *-2,* leading to the derepression of MTI. Hence, *HvWRKY1* and *-2* function as a linker between MTI and ETI. Other negative regulators in *Arabidopsis* include AtWRKY7, -11, -17, -23, -25, -27, -38, -48, -53 , -58 and -62 (Grunewald *et al.*, 2008; Journot-Catalino *et al.*, 2006; Kim *et al.*, 2006; Kim *et al.*, 2008; Mao *et al.*, 2007; Mukhtar *et al.*, 2008; Wang *et al.*, 2006; Xing *et al.*, 2008). AtWRKY38 and -62 negatively regulate the basal resistance to *P. syringae* and their expressions were modulated by PKS5, a SNF1-related kinase (Kim *et al.*, 2008; Xie *et al.*, 2010). Likewise, AtWRKY7, -11 and -17

play a negative role in the defense responses to *P. syringae* (Journot-Catalino *et al*., 2006; Kim *et al*., 2006). Several rice WRKY genes including OsWRKY62 and OsWRKY76 were described as negative regulators in immune responses (Peng *et al*., 2008; 2010; Seo *et al*., 2011). The XA21 protein confers resistance to most strains of the bacteria *Xoo* in rice (Song *et al*., 1995). Transgenic rice plants overexpressing OsWRKY62 or OsWRKY76 are compromised in basal defense and XA21-mediated resistance to *Xoo* (Peng *et al*., 2008; Seo *et al*., 2011).
Recently, expanding reports from other plant species confirmed the importance of WRKY proteins in the regulation of biotic stress responses (Giacomelli *et al*., 2010; Guo *et al*., 2011; Ishihama *et al*., 2011; Li *et al*., 2010a; Marchive *et al*., 2007; Molan and El-Komy, 2010; Mzid *et al*., 2007; Oh *et al*., 2008; Ramiro *et al*., 2010; Ren *et al*., 2010b; Skibbe *et al*., 2008; Van Eck *et al*., 2010). Ovexpression of grapevine *VvWRKY1* and *VvWRKY2* in tobacco plants reduced susceptibility to various fungi (Li *et al*., 2010a; Marchive *et al*., 2007; Mzid *et al*., 2007). CaWRKY1 from pepper (*Capsicum annuum*) appears to function negatively in the defense based on results from its overexpression and gene silencing (Oh *et al*., 2008). Expression profile studies confirmed the significance of WRKY factors for pathogen resistances in sunflower and coffee respectively (Giacomelli *et al*., 2010; Ramiro *et al*., 2010). In tobacco, WRKY4 and WRKY8 were recently demonstrated as positive regulators in pathogen defense (Ishihama *et al*., 2011; Ren *et al*., 2010b). An elegant set of experiments in the native tobacco *Nicotiana attenuate* showed that two WRKY genes, *NaWRKY3* and *NaWRKY6*, coordinate responses to herbivory (Skibbe *et al*., 2008). *NaWRKY3* is required for *NaWRKY6* elicitation by fatty acid–amino conjugates in *Manduca sexta* larval oral secretions, and gene silencing made plants highly vulnerable to herbivores. Similarly, silencing of *TaWRKY53* in wheat through virus-induced gene silencing (VIGS) resulted in susceptible phenotype to aphid infestation (van Eck *et al*., 2010). In the recently sequenced genomes, such as poplar (*Populus spp.*), sorghum (*Sorghum bicolour*), papaya (*Carica papaya*) and moss (*Physcomitrella patens*), the presence of a large number of WRKY proteins was observed (Pandey and Somssich, 2009). However, their functions in plant immunity are yet to be characterized.

1.2.2 WRKY in abiotic stresses

Though lagging behind the studies in biotic stresses, unravelling the roles of WRKY transcription factors in abiotic stress responses has recently become an active emerging field. First evidence for the involvement of WRKY protein in abiotic stress responses came from expression profile studies (Jiang and Deyholos, 2006; Qiu *et al.*, 2004; Ramamoorthy *et al.*, 2008; Sanchez-Ballesta *et al.*, 2003; Seki *et al.*, 2002; Zou *et al.*, 2007). WRKY transcription factors are differentially regulated by abiotic stresses including drought (Mare *et al.*, 2004; Ramamoorthy *et al.*,2008; Rizhsky *et al.*, 2002; Seki *et al.*, 2002), cold (Huang and Duman, 2002; Lee *et al.*, 2005; Qiu *et al.*, 2004; Zou *et al.*, 2010), heat (Li *et al.*, 2010b; Qiu *et al.*, 2004; Wu *et al.*, 2009), salt (Jiang and Deyholos, 2006; Jiang and Deyholos, 2009; Qiu *et al.*, 2004; Wei *et al.*, 2008), nutrient deficiency (Devaiah *et al.*, 2007; Kasajima *et al.*, 2010;) and UV radiation (Wang *et al.*, 2007). Recent functional analyses have provided direct evidences for their roles in abiotic stress tolerance. For example, overexpression of *OsWRKY45* in *Arabidopsis* resulted in enhanced salt and drought tolerance (Qiu and Yu, 2009). Similarly, overexpression of *OsWRKY11* under heat shock inducible HSP101 promoter conferred tolerance to heat and drought (Wu *et al.*, 2009). Further examples illustrate that WRKY factors are crucial in reprogramming plants when they are under drought or dehydration stress. The barley *HvWRKY38* (also called *HvWRKY1*) was inducible by drought and cold (Mare *et al.*, 2004). Its ectopic overexpression in turf and forage grass (*Paspalumnotatum Flugge*) enhanced drought tolerance (Xiong *et al.*, 2010). Overexpression of *AtWRKY39* increased thermotolerance whereas mutation of *AtWRKY39* caused susceptibility to heat stress (Li *et al.*, 2010b). Moreover, the *AtWRKY39*-mediated thermotolerance appeared to be co-regulated by SA and JA. The *AtWRKY25* was also reported to be involved in the heat stress responses (Li *et al.*, 2009). Recently, the important role of *AtWRKY63* in ABA response and drought stress was uncovered (Ren *et al.*, 2010a). The AtWRKY63 mutant *abo3* showed enhanced sensitivity to ABA treatment and reduced drought tolerance. A good example to elucidate the signalling pathways for WRKY-regulated abiotic stresses is from the study on the resurrection plant *Boea hygrometrica* (Wang *et al.*, 2009). Galactinol synthase (*BhGolS1*) is a key regulator mediating drought tolerance and the *BhGolS1* gene

is inducible by dehydration and ABA. The *BhGolS1* promoter contains four W boxes and chromatin immunoprecipitation (ChIP) revealed its *in vivo* binding with the dehydration and ABA-inducible *BhWRKY1* (Wang *et al.*, 2009). This finding provides a link between a dehydration-inducible WRKY factor and a downstream target gene that plays a vital role in drought tolerance whereas in most cases the native downstream target genes are largely unknown.

1.2.3 WRKY in developmental processes

Compared with the studies of WRKY genes in stress responses, fewer reports are available on their roles in development processes such as trichome development, seed germination and senescence. Several evidence suggest that members of WRKY proteins are involved in trichome development (Guillaumie *et al.*, 2010; Ishida *et al.* 2007; Johnson *et al.* 2002; Wang *et al.*, 2010),embryo formation (Alexandrova and Conger 2002; Lagace and Matton 2004), seed germination (Jiang and Yu, 2009; Zou *et al.*, 2008), senescence (Hinderhofer and Zentgraf 2001; Miao *et al.*, 2010; Robatzek and Somssich 2001; Robatzek and Somssich 2002; Zentgraf *et al.*, 2010; Zhou *et al.*, 2011), dormancy (Pnueli *et al.* 2002) and metabolic pathways (Sun *et al.* 2003).

In *Arabidopsis*, *AtWRKY44*, also known as Transparent Testa Glabra2 (TTG2), plays a role in trichome development and tannin synthesis in the seed (Johnson *et al.*, 2002). Another study provides evidence that it is controlling lethality in interploidy crosses of *Arabidopsis* (Dilkes *et al.*, 2008). Recently, Wang *et al.*, (2010) reported the role of WRKY proteins in controlling the secondary cell wall formation and lignifications in dicot plants *Medicago truncatula* and *Arabidopsis*. Mutation of *AtWRKY12* or the *Medicago* WRKY gene *Mtstp1* initiated pith secondary wall formation and substantially increased the stem biomass. This discovery of negative regulators of secondary wall formation in pith shed lights on the possibility of significantly increasing the biomass in bioenergy crops. In rice, OsWRKY78 was suggested to be a positive regulator in stem elongation and seed development evidenced from semi-drawf and small kernel phenotype in RNAi and T-DNA insertion lines (Zhang *et al.*, 2011). Other examples of development-related WRKYs include MINISEED3 (*AtWRKY10*) in seed development, *VvWRKY2* in

regulation of lignifications and *AtWRKY75* in root development (Devaiah *et al.*, 2007; Guillaumie *et al.*, 2010; Luo *et al.*, 2005).

In seed germination, the GA-inducible α-amylase enzymes play central roles in germination and post-germination. One early study revealed that wild oat WRKY proteins (ABF1 and ABF2) could bind to the W-box of the GA-regulated α-Amy2 promoter (Rushton *et al.*, 1995), thus linking WRKY proteins with seed germination. Through transient expression studies, some activators and repressors of GA signalling in rice aleurone cells were identified (Xie *et al.*, 2006; Xie *et al.*, 2007; Zhang *et al.*, 2004; Zou *et al.*, 2008). For instance, rice *OsWRKY51* and *OsWRKY71* were found to encode repressors of α-amylase whereas *OsWRKY72* and *OsWRKY77* appeared to be activators. However, genetic evidence is required to validate their real involvement in seed germination. First direct evidence from *Arabidopsis* indicated that *AtWRKY2* acted as a mediator in the ABA-dependent seed germination and postgermination growth arrest (Jiang and Yu, 2009). Similarly, the sequence-related AtWRKY18, -40 and -60 were recently characterized as negative regulators of ABA-signalling during seed germination and postgermination growth, with AtWRKY40 playing a central role (Shang *et al.*, 2010). In *Arabidopsis*, ectopic overexpression of *OsWRKY72* caused retarded seed germination, enhanced sensitivity to ABA and altered expression of auxin-responsive genes (Song *et al.*, 2010).

Senescence in plants is a controlled process involving the timely activation of metabolic pathways through transcription factors including WRKY proteins. The WRKY factors are reported to be the second largest group of transcription factors of the senescence transcriptome (Guo *et al.*, 2004). One well-studied example is *AtWRKY53*, which showed a specific expression at the onset of leaf senescence (Hinderhofer and Zentgraf, 2001). It appeared to directly interact with the MEKK1, an upstream components in MAPK cascade (Miao *et al.*, 2007). Moreover, epigenetic programming was also implicated in the mechanism whereby *AtWRKY53* regulates senescence (Ay *et al.*, 2009). Recently, degradation of *AtWRKY53* by E3 ubiquitin ligase UPL5 was found essential in executing the leaf senescence at the right time frame (Miao *et al.*, 2010). *AtWRKY6* and *AtWRKY22* are also involved in senescence (Robatzek and Somssich 2001; Robatzek and Somssich 2002; Zhou *et al.*, 2010).

1.3 Transcriptional regulatory network of WRKY

1.3.1 WRKY signalling transduction

Expression of many WRKY proteins is induced by the aforementioned diverse stresses. But WRKY transcription factors are also thought to be regulated or activated on the protein level. Some shared components have been identified or postulated upstream of WRKY proteins, for instance, the receptors for microbial-derived molecular signatures and the mitogen-activated protein kinases (Andreasson *et al.*, 2005; Asai *et al.*, 2002; Fiil *et al.*, 2009; Hofmann *et al.*, 2008; Kim *et al.*, 2004; Koo *et al.*, 2009; Liu *et al.*, 2004). In *Arabidopsis*, WRKY22 and WRKY29 transcription factors act downstream of the flagellin receptor FLS2, a leucin-rich-repeat (LRR) receptor kinase (Asai *et al.*, 2002).
AtWRKY33 was shown to form nuclear complexes with the MAP kinase MPK4. MAMP perception led to the dissociation of the protein complex and release of AtWRKY33, which activated the expression of the camalexin synthesis gene PAD3. (Qiu *et al.*, 2008b). WRKY38 and WRKY62 were shown to act downstream of cytosolic NPR1 in the regulation of jasmonate-responsive gene expression (Mao *et al.*, 2007; Xie *et al.*, 2010). In addition, phosphorylation appeared to be a very important step in the activation of WRKY protein. The MAP kinase kinase kinase (MEKK1) was found to bind directly to the *AtWRKY53* promoter and meanwhile phosphorylate AtWRKY53 protein to take a shortcut in signalling (Miao *et al.*, 2007).
Very recently, *Arabidopsis* WRKY33 was shown to be a direct phosphorylation target of MPK3/MPK6 following the infection of *B. cinerea* (Mao *et al.*, 2011). In tobacco, overexpression of the MAP kinase SIPK triggers cell death through the phosphorylation of WRKY1 (Menke *et al.*, 2005). Moreover, phosphorylation of the *Nicotiana benthamiana* WRKY8 by MAPK has an important role in the defense response through activation of downstream genes (Ishihama *et al.*, 2010).
Induced WRKY expression is often extremely rapid and transient, and seems not to require *do novo* synthesis of regulatory factors (Eulgem *et al.*, 1999; Hara *et al.*, 2000; Lippok *et al.*, 2007; Rushton *et al.*, 1996). Therefore, many WRKY genes are generally considered as early and intermediate stress responsive genes. This

fast responsive behaviour indicates a role for the WRKY proteins in regulating subsequently activated downstream response genes, which eventually leads to protective reactions in plants. As suggested by the binding preference of WRKY proteins for W boxes, genes containing these promoter elements are possible targets of WRKY proteins. They represent a number of stress-related genes (i.e, *PR* genes) and *WRKY* genes themselves (Eulgem *et al*., 2000; Yu *et al*., 2001). A large body of evidences have confirmed the direct targeting of these genes by WRKY proteins. For example, AtWRKY40 was demonstrated to have direct *in vivo* interaction with promoter regions of the regulatory gene *EDS1*, the AP2-type transcription factor gene *RRTF1* and *JAZ8*, a member of the JA-signaling repressor gene family (Pandey *et al*., 2010). *AtWRKY6* was found to positively influence the senescence- and pathogen defense-associated *PR1* promoter activity (Rabatzek and Somssich, 2002). In addition, it specifically activates the promoter of a receptor-like protein kinase SIRK likely through direct W-boxes interactions but represses its own promoter activity (Rabatzek and Somssich, 2002).

1.3.2 Mechanisms of WRKY function

WRKY proteins can function as transcriptional activator or repressor. In *Nicotiana benthamiana*, ectopic expression of *WRKY8* was found to activate defense-related genes, such as 3-hydroxy-3-methylglutaryl CoA reductase 2 and NADP-malic enzyme (Ishihama *et al*., 2010). The tobacco *NtWRKY6* acts as an activator in the induction of *PR1a* gene expression by SA and bacterial elicitor (van Verk, *et al*., 2008). Heterologous expression of *OsWRKY6* in *Arabidopsis* was shown to activate the expression of defense related genes (Hwang *et al*., 2011). Some WRKY members may possess both capacities. For example, *OsWRKY71* and *OsWRKY77* have been shown to act as activators in ABA signalling but as repressors in GA signalling (Xie *et al*., 2005). The similar feature was found for AtWRKY6 and AtWRKY53, which activate other promoters but repress their own promoters (Miao *et al*., 2008; Robatzek and Somssich, 2002).

An elegant model was proposed for the derepression of MTI in barley-*Blumeria graminis* interaction (Shen *et al*., 2007). In this proposed model, *HvWRKY1* and

HvWRKY2 act as repressors in the basal defense. Following the recognition of fungal-derived effector AVR10 by the resistance protein MLA10 in the cytosol, the MLA10 protein was activated and translocated into plant nucleus and physically interacted with *HvWRKY1/2* repressor. This was thought to remove the repression from *HvWRKY1/2* and activate the promoters of unidentified downstream defense genes. Another example is the activation of *PcPR1* by *PcWRKY1* in parsley (Turck *et al*., 2004; Ülker and Somssich, 2004). It was found that W-box elements are generally occupied by WRKY factors. Upon recognition of an elicitor (MAMP) by the cognate receptor, the MAPK cascade is activated and translocates a protein kinase into the nucleus where it directly interacts with bound WRKY proteins. Thus, these WRKY factors are replaced and released from the bound W-boxes resulting in the activation of PcPR10.

Other possible mechanisms of WRKY functions include the operation through small RNAs (smRNAs) and histone modifications (Kim *et al*., 2008; Pandey and Somssich, 2009). Small RNAs, including microRNAs (miRNAs) and small interfering RNAs (siRNAs), have been shown to play fundamental roles in the modulation of gene expression. Some WRKY transcription factors are predicted to be targets of certain miRNAs (Pandey and Somssich, 2009). On the other hand, WRKY proteins might regulate smRNA synthesis. Nevertheless, it is a novel field awaiting further advances. Histone modifications through histone deacetylases are often associated with transcriptional repression by reducing the access of DNA for transcription factors (Zhou *et al*., 2005). The WRKY genes *AtWRKY38*, *-53*, *-62* and -*70* have been implicated in processes involving histone modifications in the fine-tuning of plant senescence and immunity (Ay *et al*., 2009; Kim *et al*., 2008; Liu *et al*., 2004). The AtWRKY38 and AtWRKY62 function additively as negative regulators of basal defense and interact with histone deacetylase 19 (HDA19). HDA19 function positively in the basal defense and can abolish the transactivation activity of AtWRKY38 and AtWRKY62.

Due to the enrichment of W-box elements in the promoter region of WRKY genes, WRKY proteins might physically interact with their own promoter or the promoter of other WRKY genes. This auto-regulation or cross-regulation is a common feature for WRKY action. AtWRKY53 was described to involve in both auto-regulation and cross-regulation (Miao *et al*., 2008). Likewise, the promoter of

parsley *PcWRKY1* was found to be bound with WRKY proteins and activation of *PcWRKY1* promoter was achieved through cross-regulation by other WRKY factors that activate transcription (Turck *et al*., 2004).

1.4 Basal defense in barley-*Bgh* interaction

1.4.1 Barley-*Bgh* interaction

The obligate fungal biotroph, *Blumeria graminis* f.sp. *hordei* (DC) Speer (*Bgh*), is the causal agent of powdery mildew on barley (*Hordeum vulgare*. L.). Indicated by the name, this fungus infects only barley but not other cereals. The genetics and physiology of barley-*Bgh* interaction has been studied since one century ago (Biffen, 1907). Upon landing of a conidial spore on the host surface, the fungus builds the first germ tube for attachment on the leaf surface and water uptake, while a secondary germ tube is built for penetration of the host cuticle and cell wall (Thordal-Christensen *et al*., 1999). By means of hydrolytical and mechanical power, a small amount of germinated conidia spores might break the cell wall barrier and produce a functional haustorium while others fail to penetrate (Pryce-Jones *et al*., 1999). After successful penetration of the host cell, the fungus has the ability to reprogram the host cell in the sense that it becomes a nutrient sink and supports fungal proliferation (Schulze-Lefert and Panstruga, 2003). One good example is the green island effect on powdery mildew infected leaves (Schulze-Lefert and Vogel, 2000), though the molecular basis of this redefinition of the infected site as a nutrient sink is not fully understood.

The outcome of a fungal penetration attempt on a compatible host relies on the fungal virulence and the defense state of the attacked cell. Any fungal penetration is only successful when it antagonizes the host defense machinery which is evolved in diverse ways. Early defense prevents penetration and is mainly achieved by the formation of cell wall appositions (CWAs). This mechanical and chemical barrier is constituted of 1,3-glucans (callose), silicon, lignin-like material, and various cell wall proteins. The second line of defense inhibits nutrient uptake of haustoria and it is mainly achieved via hypersensitive response (HR) which is featured by a programmed cell death (PCD) of the attacked and/or the neighboring cells. In addition, HR is associated with accumulation of lignin-like material,

release of phytoalexins and other defense compounds which are toxic to the fungi (Oldach *et al.*, 2001; von Röpenack *et al.*, 1998).

Though the molecular mechanism of HR is largely unknown, it is accepted that an HR reaction is typically triggered by effector-activated resistance (R) proteins (Jones and Dangl, 2006). The number of dominant or semi-dominant race-specific R proteins in barley was estimated to be around 85 (Jørgensen, 1994). Recognition of *B.graminis* f. sp. *hordei* is mediated by several loci distributed throughout the barley genome which are designated as Ml (Mildew resistance locus) (Jørgensen, 1994). As the most prominent locus, Mla (Mildew resistance locus A) is located on the short arm of chromosome 1H, with approximately 30 alleles that mediate race-specific resistance (Jørgensen, 1994; Wei *et al.*, 1999). Over the last decades, genetic studies in breeding material have identified a large number of functional resistance genes at the Mla locus in breeding material. Distinct from the genetic structure of Mla with multiple alleles at a single locus, putative AVR genes are scattered throughout the *B.graminis* f. sp. *hordei* genome, with the cloned AVR10 belonging to a diverse family encoding proteins lacking secretion signals (Ridout *et al.* 2006; Skamnioti *et al.* 2008). Alleles of Mla encode cytoplasmic- and membrane-localized coiled-coil (CC), nucleotide binding site (NBS), leucine-rich repeat (LRR) proteins (Halterman and Wise 2004; Seeholzer *et al.* 2010; Shen *et al.* 2003) that translocate into the nucleus after recognition of a cognate AVR effector from *B. graminis* f. sp. *hordei*. Nuclear localization of AVR is required to mediate the hypersensitive response (Shen *et al.* 2007). This may be dependent on the direct interaction between appropriate MLA and AVR proteins (Seeholzer *et al.*, 2010). Following recognition, the CC domain of MLA interacts with the transcription factors WRKY1 and WRKY2 (WRKY1/2) (Shen *et al.*, 2007).

1.4.2 Germin-like proteins (GLP) in plant immunity

Members of germin-like protein (*GLP*) genes were originally isolated from germinating seeds and were regarded as specific marker for the onset of germination (Dunwell *et al.*, 2008; Lane *et al.*, 1993; Thompson and Lane, 1980). They belong to the cupin superfamily proteins which exhibit diverse functions (Dunwell and Gane, 1998). GLPs have been identified from a number of plant

species including *Arabidopsis (*Carter *et al*., 1998; Membré *et al*., 1997; Membré *et al*., 2000), soybean (Klink *et al*. 2007), grapevine *(*Cramer *et al*., 2007; Ficke *et al*., 2002; Godfrey *et al*., 2007), conifers (Mathieu *et al*., 2006), *Medicago* (Doll *et al*., 2003; Soares *et al*., 2009) and peanut (Chen *et al*., 2010b). The majority of germin and GLP studies are focused on Gramineae species such as wheat, barley and maize (Breen and Bellgard, 2010; Dunwell *et al*., 2008; Lane, 2002).

Apart from the role of GLPs in germination and early development (De Los Reyes and McGrath, 2003; Federico *et al*., 2006), they are also implicated in abiotic stress responses such as salt, drought and aluminium stress (Cramer *et al*., 2007; Houde and Diallo 2008; Ke *et al*., 2009). Accumulating evidence suggests that GLPs are essential players in plant immune system (Breen and Bellgard, 2010; Lane, 2002). Some GLP genes showed induced expression in response to pathogen, herbivores as well as the chemical treatments like salicylic acid, hydrogen peroxide (H_2O_2), or ethylene (Dumas *et al*., 1995; Federico *et al*., 2006; Godfrey *et al*., 2007; Lou and Baldwin, 2006; Schweizer *et al*., 1999; Wei *et al*., 1998; Zhang *et al*., 1995; Zhou *et al*., 1998; Zimmermann *et al*., 2006).

The direct involvement of GLP in plant defense has been demonstrated in many cases. For instance, overexpression of a wheat germin in sunflower (*Helianthus annuus*) enhanced resistance to pathogens (Hu *et al*., 2003). Silencing of a GLP in native tobacco *Nicotiana attenuata* increased the performance of native herbivore (Lou and Baldwin, 2006). In rice, a cluster of GLPs on chromosome 8 was identified to function as the QTL (quantitative trait locus) responsible for broad-spectrum level resistance (Manosalva *et al*., 2009). In barley, transient overexpression of certain barley GLP subfamilies resulted in enhanced resistance to the powdery mildew fungus, and silencing of *GER4* resulted in enhanced susceptibility to the pathogen (Himmelbach *et al*., 2010; Zimmermann *et al*., 2006). In both rice and barley, the GER4 subfamily was identified to contribute most to disease resistance. Recently, a germin-like protein was identified as a transcriptional target of the MLA transcriptional regulon based on quantitative time-course expression profile (Moscou *et al*., 2011). This reflects an overlapping of basal defense process and R gene-mediated signalling.

Germins and GLPs are targeted to cell surface and have oxalate-oxidase (OXOX) activity (Lane *et al*., 1993; Lane, 2000) or superoxide dismutase (SOD) activity

(Christensen *et al.*, 2004; Godfrey *et al.*, 2007; Zimmermann *et al.*, 2006). Both enzyme activities are linked to the generation of hydrogen peroxide (H_2O_2), which has possible roles in a range of defense reactions, including cell wall reinforcement, cell death, and induction of *PR* gene expression (Alvarez *et al.*, 1998; Bolwell and Wojtaszek, 1997; Chen *et al.*, 1993; Lamb and Dixon, 1997; Olson and Varner, 1993; Thordal-Christensen *et al.*, 1997; Wei *et al.*, 1998).

In barley, eight GLP genes (*HvGER4 a-h*) are clustered in the *GER4* locus and the promoter contains multiple WRKY factor binding sites (W-boxes) (Himmelbach *et al.*, 2010). Mutational analysis of W-boxes in *GER4c* promoter-β-glucuronidase fusions revealed the enhancing effects of W-boxes in the pathogen-induced promoter activity. Enrichment of W-box elements was also observed in the promoter of *OsRGLP2* and *TaGLP3* (Mahmood *et al.*, 2010), implicating a potential transcriptional regulation of *GLP* promoters by WRKY proteins.

1.5 Objectives of this study

The main objective of the current study was to identify putative WRKY transcription factors in barley and characterize the functions of the previously identified *HvWRKY1* and *-2* with a particular focus in the interaction of barley with *Blumeria graminis* f.sp. *hordei* (*Bgh*). In order to provide an overview of the WRKY family in barley, whole-genome search was performed to identify putative WRKY transcription factors based on genomic sequence and transcript databases.

The specific aims were:

1). To identify, annotate members of the barley *WRKY* gene family and analyze their phylogenetic relationship.

2). To analyse the gene structure and function of *HvWRKY1* and *HvWRKY2.*

3). To identify target genes of *HvWRKY1* and *HvWRKY2* which were suggested to be negative regulators of barley basal defense.

4). To characterize the disease resistance phenotype of HvWRKY2 overexpression barley lines and compare the defense-related gene expression.

5). To identify candidate genes for further studies and genetic approaches which aim at improving broad-spectrum and durable resistance of barley.

2. Materials and Methods

2.1 Plant growth condition and pathogen infection

Blumeria graminis f.sp. *hordei (Bgh)* race A6 was maintained in a climate cabinet and propagated on young seedlings of the susceptible barley cultivar 'Golden Promise' at 20°C/18°C (day/night) with 60% relative humidity and a photoperiod of 16 h with 240 µmol m^2 s^{-1} photon flux density.

Evaluation of powdery mildew resistance was performed on detached leaves. Plants from the cultivars Golden Promise, Ingrid, BCIngrid *mlo-5*(I22) and Sultan 5 (Mla12) were grown in spore-free Percival growth chamber under a photoperiod of 16 h with 240 µmol m^{-2} s^{-1} photon flux density. Seven days after sowing, the primary leaf was cut and placed in 0.5% water agar medium containing 40 mg/L benzimidazole in a square (10 × 10 cm) petri dish with the adaxial side of the leaf facing upwards. Each petri dish accommodated about 10 leaf segments.

To use freshly produced conidia for inoculation, old conidia spores from the heavily infected Golden Promise seedlings were removed by gentle shaking of the plants 2 days prior to inoculation. A settling tower was used for inoculations. During inoculation, petri dishes containing the leaf segments were placed inside the tower and conidia from *Bgh* colonized seedlings were blown and allowed to settle for 10 minutes. The density of inoculum was monitored by haemocytometer and was adjusted to 10-15 conidia per mm^2 for macroscopical observation. Five days after incubation, the accessions were scored by counting the number of powdery mildew pustules per 2 cm^2 of leaf segment, using a magnifying glass (10x). For evaluation of resistance on single cell level with microscopy, the inoculation density was adjusted around 150 conidia spores per mm^2. In the promoter studies, a much higher inoculation density (over 200 conidia spores per mm^2) was used to activate the *HvGER4c* promoter.

2.2 Hygromycin-based selection of transgenic plants

Selection of transgenic plants was established and optimized for barley based on the method of Wang and Waterhouse (1997). Leaf segments 2 cm in length from transgenic and non-transgenic barley plants were cut and immediately placed in MS medium containing 200 mg/L of hygromycin, 0.5mg/L 6-BA (6-benzylaminopurine) and 8g/L agar. With the cut ends embedded inside of the

media, leaf segments were kept at 24 ^oC under a long-day photoperiod (16h/8h light dark regime) for one week until bleaching occurred on the wild type plants.

2.3 Isolation of DNA and RNA

DNA was isolated by CTAB method according to a modified protocol of Doyle and Doyle (1987). Briefly, plant material was ground into fine powder in liquid N_2 and transferred into 2.0 ml microcentrifuge tube. 700 µL hot (pre-warmed at 65°C) CTAB Extraction Buffer was added and the tubes were indubated for 25 minutes. 700 µL of Chloroform:Isoamyl-Alcohol (24:1,CIA) was added and mixed by inversion for 5 minutes. The samples were centrifuged at 10000 rpm for 15 minutes under room temperature. Supernatant was transferred to a new Eppendorf tube containing 600µl of CIA, mixed by inversion for 5 minutes and centrifuged at 10000 rpm for 15 minutes (RT). The supernatant was thoroughly mixed with 500 µL of isopropanol and placed on ice for 15 minutes. Supernatant was discarded and pellet was washed with 70% ethanol/10mM NH_4OAc. Finally, dry pellet was resuspended in 100 µL ddH_2O. The DNA concentration was measured by NanoDrop N1000 (peqLab Biotechnologie GmbH, Erlangen).

CTAB Extraction Buffer

2%	CTAB
20mM	EDTA
100mM	tris-Cl, pH 8.0
1.4M	NaCl
0.2%	mercaptoethanol (add prior to use)

Wash Buffer

70%	ethanol
10mM	NH_4OAc

Extraction of total RNA was performed by phenol-chloroform extraction method. Barley leaves from mock treated or powdery mildew infected samples were harvested at the indicated time points and immediately frozen in liquid nitrogen. Leaf samples were crushed into fine powder in liquid nitrogen using mortar and pestle. 1 mL RNA Extraction Buffer was added to the sample and vortexed vigorously. 200 µL chloroform was added and vortexed again. Thereafter, samples

were vortexed for 15 seconds and centrifuged at 13500 rpm for 15 min at 4°C. The supernatant was transferred into a clean Eppendorf tube with 850 uL chloroform and vortexed briefly. Then all the samples were centrifuged at 13500 rpm for 15 min at 4°C. The supernatant was transferred to a new tube with 1 mL 5 M LiCl and mixed by brief vortex. After overnight incubation at -20°C for precipitation of RNA, pellet was spinned down at 13500 rpm for 20 min at 4°C. The supernatant was carefully discarded and the pellet was washed with 70% ethanol by short vortex followed with centrifugation at 13500 rpm for 10 min at 4°C. The washing step was repeated once and the liquid was removed completely. Then, the pellet was air dried under clean bench for 15 min and dissolved in 50 µL H_2O_{DEPC}. RNA concentration was measured by Nanodrop ND-1000 Spectrophotometer (peqLab Biotechnologie GmbH, Erlangen) and the RNA integrity was examined on denaturing 1.5% agarose-gel containing 5% formaldehyde. Trace DNA was removed using 1 µL DNaseI per µg sample RNA prior to cDNA synthesis.

RNA Extraction Buffer

38%	phenol
0.8 M	guanidin thiocyanat
0.4M	ammonium thiocyanat
0.1M	sodium acetate, pH 5
5%	glycerol

2.4 Expression analysis

For gene expression analysis, Golden Promise and pUbi::WRKY2 plants were inoculated with *Blumeria graminis* f.sp *hordei* A6 or mock treated and harvested at 0, 4 and 12 hours. Total RNA was extracted as described in section 2.3. One µg of RNA was reverse-transcribed using Fermentas reverse transcriptase kit (Fermentas, Sankt Leon-Rot) according to the manufacturer's instruction. The cDNA was diluted 5-fold (estimated equivalent concentration 10 ng/µL) and used for expression analysis with semi-quantitative PCR and quantitative real-time PCR. In the quantitative real-time PCR, the expression level of *IGS*, *synaptotagmin*, *HvPR2* and *HvPR5* was determined using the $2^{-\Delta Ct}$ method (Schmittgen and Livak, 2008). Amplifications were performed with 20 µl SYBR green JumpStart Taq ReadyMix (Sigma–Aldrich, Munich) with 350 nM oligonucleotides and an Mx3000P thermal cycler with a standard protocol (Stratagene, La Jolla, CA). Briefly, the

amplification was performed with an initial denaturation step at 95°C for 8 min followed with 40 cycles (94°C for 30 s, 60°C for 30 s, 72°C for 30 s, and 82°C for 15 s). Three fluorescent reading were monitored once at 72°C and twice at 82°C during each cycle. Melting curves were determined at the end of cycling to ensure specific amplification. Ct (cycles of threshold) values were determined and processed with the Mx3000P V2 software. For comparison of expression level, Ct values were generated by deducting the raw Ct values of the candidate genes from the respective raw Ct values of the reference gene barley ubiquitin (Accession Nr., M60175).

In the semi-quantitative RT-PCR, the amplification was performed in 25 µL reaction with 20 ng cDNA as template. The barley ubiquitin gene was used as internal control for equal cDNA usage in PCR reactions. Amplification was achieved by incubation in a DNA thermal cycler for 28-32 cycles, each consisting of 30 s of denaturation at 94 °C, 30 s of annealing at 60 °C, and 30 s of extension at 72 °C.

Semi-quantitative RT-PCR (25 µL)

2 µL	cDNA (10ng/ul)
2.5 µL	10×PCR Buffer
2.5 µL	2 mM dNTPs-Mix
1.5 µL	25 mM $MgCl_2$
1.0 µL	forward primer (10 pM)
1.0 µL	reverse primer (10 pM)
0.15 µL	Taq polymerase (5 U/µl)
14.35 µL	MilliQ-H_2O

2.5 Molecular cloning and plasmids constructions

Primer design was mainly performed with the online tool Primer 3 (http://frodo.wi.mit.edu/primer3/). Restriction sites were introduced on 5' ends of the primers to facilitate cloning when necessary. In this case, 2-4 bp extra protection nucleotides were added at the ends to improve digestion efficiency of PCR products. All primers used in this study were ordered from Eurofins MWG Operon and listed in Appendix 3. The freeware pDRAW32 (http://www.acaclone.com/) was used for vector information management and *in silico* cloning. During cloning, the digestion was performed with restriction

enzymes from Fermentas (Fermentas, Sankt Leon-Rot). Selection of suitable reaction buffer system for double digestion was assisted by the online tool DoubleDigest™ (http://www.fermentas.com/en/tools/doubledigest).
Standard transformation procedure was followed using 60 seconds heat shock of *E.coli* strain DH5α at 42 ^{o}C. Positive colonies grown on antibiotic selection plates were verified by colony PCR. The colonies were further confirmed by restriction digestion and sequencing (LGC Genomics, Berlin).
Vector constructs were generated following standard cloning procedure. Maps for all the vectors generated in this study were included in Appendix 4. The wild-type and truncated *HvGER4c* promoter GUS fusion constructs were provided by Dr. Patrick Schweizer (IPK, Gartesleben). Plasmid pGY1-*mCherry* (4213 bp) was generated from p123mCherry and pGY1-*GFP*. In principle, GFP in pGY1-*GFP* was replaced by *mCherry* released from p123mCherry after *Nco*I/*Eag*I digestion. For cloning of pUbi::*AtWRKY40*, *AtWRKY40* CDS was amplified by AtW40Bam_F and AtW40Hind_R from *pDONR-AtWRKY40*, which was kindly provided by Dr. Imre E. Somssich (Max Planck Institute for Plant Breeding Research, Köln). As the stop codon was absent in the original cDNA clone, the codon TAA was added in the reverse primer AtW40Hind_R. Ligation of BamHI/HindIII digested *AtWRKY40* PCR product and the backbone *pUbi-AB* yielded *pUbi::AtWRKY40*.
HvWRKY1 promoter was amplified using primer pHvW1Bam_F1/ pHvW1EcoR_R (954 bp) and pHvW1Bam_F2/ pHvW1EcoR_R (1940 bp) from barley (cv. Golden Promise) genomic DNA. PCR products were digested with *Bam*HI/*EcoR*I and ligated with linearized pGusi-AM (5.3 kb). *HvWRKY2* promoter (2876 bp) was amplified by pHvW2Bam_F and pHvW2Hind_R.
To clone artificial microRNA for *HvWRKY2* silencing, a 21-bp-long sequence (TTCAGACGTAGTCACCGACTA) was selected by WMD (Web MicroRNA designer, http://wmd.weigelworld.org/cgi-bin/mirnatools.pl) for specific targeting of *HvWRKY2*. Four primers including HvW2-394ImiR-s, HvW2-394IImiR-a, HvW2-394IIImiR*s and HvW2-394IVmiR*a were used to run PCR using pNW55-osaMIR528 as template (kindly provided by Prof. Detlef Weigel). Three PCR products were fused by the primer pair amiRPCR4_F and amiRPCR4_R. The final PCR product was digested with *Eag*I/*Spe*I and ligated at the compatible ends of

pGY1-GFP produced by *Eag*I/*Xba*I digestion. The resulting plasmid was named pGY1-GFP-amiRWRKY2.

2.6 Isolation of plasmid DNA

The recombinant bacterial were cultured with LB-medium including 100 mg/L ampicillin. The mini-preparation of plasmid DNA was performed using PureYield™ Plasmid Miniprep System (Promega) from 4 mL overnight bacterial culture following the manufacturer's instructions (Technical Bulletin #TB374). For midi-preparation, PureYield™ Plasmid Maxiprep System (Promega) was used for plasmid isolation from 75 mL overnight culture. As the last step, all plasmids were eluted in ddH_2O instead of TE buffer to facilitate further analysis. Plasmids concentration and purity were examined with NanoDrop N1000 spectrophotometer (NanoDrop Technologies, Wilmington, USA).

2.7 Particle bombardment

A home-made helium biolistic system was used for particle bombardment-mediated transient transformation of barley epidermal cell as described previously (Schultheiss *et al.* 2002; Schweizer *et al.*, 2000). For each shot, 312 µg of 1.1-µm tungsten particles were coated with *pUbi-AtWRKY40* (1 µg) together with *35S::GFP* (0.5 µg) as a transformation control. In the control bombardment, the empty vector *pUbi-AB* (1 ug) was used together with the GFP construct. 24 h after the bombardment, leaf segments were inoculated with *Bgh* race A6. Inoculation density was adjusted ca. 150 conidia mm^{-2}. The interaction outcome (penetration efficiency, PE) was analysed 48 h after inoculation by fluorescence microscopy. Transformed GFP expressing cells and the presence of haustorium were identified under blue light excitation. Surface structure of the powdery mildew fungus was detected using fluorescence staining with 0.3% calcofluor (w/v in water) for 30 s. Transformed GFP cells were categorized into three groups as penetrated cells that contained a haustorium, cells that were attacked by a *Bgh* appressorium but did not generate a haustorium, and cells that were not infected by fungus. Cells with more than one haustorium or that contained haustoria but less than fungi attacked were recorded as only one penetrated cell. The penetration efficiency (PE), referring to the haustorium index (%) in the transformed GFP cells was obtained based on a set of a minimum of three experiments each consisting of at least 100

interaction sites. PE was calculated for each experiment as the number of penetrated cells (presence of a functional haustorium) divided by the total number of attacked cells multiplied by 100.

In the promoter study, particle bombardment was performed using the same biolistic system. For normalization of GUS expression, a plasmid containing the GFP reporter under the control of maize ubiquitin promoter was co-bombarded (pUbi-GFP). In the bombardment, a mass ratio of 2:1:1 for pHvGER4c::GUS (or its truncated forms), 35S::WRKY (HvWRKY1 or HvWRKY2) and pUbi::GFP and 2 ug total DNA was adopted. In the control bombardment, same amount of the empty vector pGY1 was used instead of the WRKY constructs. Bombarded leaves were transferred to 0.5% agar plates supplemented with 40 mg/L Benzimidazole and incubated at 18°C for 48 h before adequate inoculation with *B. graminis* spores.

2.8 GUS assay

Bombarded leaf segments were inoculated with powdery mildew (*B.graminis* f.sp *hordei* A6) 48 h after transformation. The number of GFP expressing cells was first counted under fluorescence microscope (Zeiss Axioplan Imaging 2) for each shot. Thereafter, the leaf segments were stained histochemically for GUS expression. Leaf segments were placed in 2 mL Eppendorf tubes and GUS staining solution was added until the leaf tissue was immersed. Short vacuum infiltration was performed till the leaves were completely water-soaked. After 24 h incubation in GUS staining solution at 37°C in dark, the solution was removed and GUS-stained leaves were cleared in clearance solution with shaking. The clearance solution was changed once after 48 hours of incubation. Subsequently, the number of GUS cells per bombardment was counted under macroscopy. The obtained numbers of GUS-stained cells were normalized together to the number of GFP expressing cells from cobombarded pUbi-GFP. Eventually, the normalized number of GUS cells per bombardment was taken as a measure for the HvGER4c promoter activity as previously described (Himmelbach *et al.*, 2010). Average values were based on raw data from at least three independent bombardment experiments.

GUS staining solution

0.1 M	Na_2HPO_4/NaH_2PO_4, pH 7.0
1.4 mM	potassium hexacyanoferrat (II)
1.4 mM	potassium hexacyanoferrat (III),

0.5 mg ml^{-1}	5-bromo-4-chloro-3-indoxyl-b-D-glucuronic acid, cyclohexylammonium salt (X-Gluc; Duchefa, Netherlands)
0.1% (v/v)	triton X-100

Clerance solution (1L)

250 ml	chloroform
750ml	technical ethanol
1.5g	trichloroacetic acid (TCA)

2.9 Purification of recombinant protein

To produce recombinant HvWRKY2 protein, the full-length coding sequence of *HvWRKY2* was PCR amplified using HvWRKY2Sal_F and HvWRKY2Hind_R. The fragment was fused to the C-terminal of thioredoxin-6xHis-S-tag in the expression vector pET32a(+) (Novagen) and resulted in pET32a-HvWRKY2. Subsequently, the construct was electro-transformed into *E. coli* strain BL21 (DE3) pLysS (Stratagene, La Jolla, USA) using Bio-Rad *E.coli* Pulser Apparatus at 2.5 kV with 0.2 cm cuvettes.

The bacterial clones containing 6xHis-HvWRKY2 were first verified for the rate of protein production and the solubility of the protein using a small scale protein induction. Large scale (1L) protein production was performed in Luria-Bertani (LB) medium overnight under shaking at 37°C. After inoculation of fresh medium with the overnight culture, bacteria were allowed to grow until mid log phase (OD600 of 0.5-0.8) before isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM and further incubated for 4 hours for protein induction. The bacteria pellets were harvested by centrifugation at 4000 rpm for 20 min at 4°C and dissolved in 30 ml lysis buffer for 30 minutes. The bacterial cells were disrupted by sonication eight cycles with 20 seconds intervals. As the recombinant is present in the inclusion body, the sonicated lysate was centrifuged at 9500 rpm for 30 min at 4°C and the pellet was dissolved in Buffer B and incubated under shaking for 1 hour at room temperature. Afterwards, the cell debris was removed from the lysate solution by centrifugation for 30 min (12000 rpm). The supernatant was collected and stored at 4°C. To prepare the column for purifying the 6× His-tagged fusion protein, 1 ml of Ni-NTA resin (Qiagen, Hilden, Germany) was pipetted into the column that was clamped onto a stand. The resin was allowed to

settle down and once settled the valve was opened to drain off the residual liquids in the column. Thereafter, the supernatant containing soluble protein was loaded onto a Ni-NTA prepared column and washed three times with 4 ml washing buffer and thereafter, the column was eluted three times with elution buffer. Finally, proteins were desalted and concentrated using an ultra-filtrate column (VIVASPIN 6 ml concentrator) with a molecular weight cut-off (MWCO) at 10 kDa (Vivascience, Lincoln, UK) and stored at -80°C. Protein concentration was estimated by Bradford assay. Different concentrations of bovine serum albumin (BSA) were prepared and used to create a standard curve. Purity and integrity of HvWRKY2 recombinant protein was determined by separating protein aliquots using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). After electrophoresis, gel was fixed by fixation solution (one part Acetic Acid, 3 parts Isopropanol and 6 parts Water) for 30 min. Eventually, the gel was visualized with colloidal Coomassie blue (Roth, Karlsruhe, Germany). Staining solution was added to the gel and shaking at room temperature overnight. To minimize the background staining, destaining was performed using destaining solution for 30 minutes.

LB (Lauria Bertani) Broth

1 %	tryptone peptone
0.5 %	yeast extract
0.5 %	NaCl
1%	agar

lysis buffer (pH 8.0)

50 mM	NaH_2PO_4
300 mM	sodium chloride
10%	glycine,
1 mg/ml	lysozyme,
0.5 mM	PMSF

Buffer B

10 mM	Tris-HCl (pH 8.0)
8 M	urea
100 mM	NaH_2PO_4

washing buffer

8 M urea,

25 mM imidazole

100 mM NaH_2PO_4

10 mM Tris-HCl, pH 6.3

Elution Buffer

8 M urea

500 mM imidazole,

100 mM NaH_2PO_4

10 mM Tris-HCl, pH 4.5

Staining Solution

20 % (v/v) coomassie blue stock

20 % (v/v) methanol

60 % (v/v) H_2O

Destaining Solution

40% (v/v) methanol

10% (v/v) glacial acid

50% (v/v) H_2O

2.10 Electrophoretic mobility shift assay (EMSA)

To investigate the binding of HvWRK2 and the HvGER4c promoter, we employed Electro mobility shift assays (EMSA) with HvGER4c promoter fragments containing the consensus W-box *cis*-elements. The fragment consisting of a single W-box (W-box 1, W-box2, W-box5 or W-box 6) in the HvGER4c promoter region was picked to design probes. Accordingly, all the mutated probes (mW-box1, mW-box2, mW-box5 and mW-box6) harboring a point mutation in the core sequence of consensus W-box from TGAC to TGAA were designed as control. At 5'-end of the 36 bp fragments, two additional nucleotides AA were added to facilitate the labeling with DIG-11-dUTP. The sense and anti-sense primers corresponding to each probe were synthesized by Eurofins MWG Operon. Double stranded DNA

was generated using the complementary single stranded DNA. The DIG-labeling was performed in a 5'-overhang fill-in reaction using T4 DNA polymerase.

W-box1 aaGTTGGGCGGTTCCACTTGACTATGTCCATTACTCATuu
mW-box1 aaGTTGGGCGGTTCCACTTGAaTATGTCCATTACTCATuu
W-box 2 aaAAGATTTGAATATGGCTGACCACATCTCAAAAGTTTuu
mW-box 2 aaAAGATTTGAATATGGCTGAaCACATCTCAAAAGTTTuu
W-box 5 aaCATTTGGAGAGAAATTTGACCAGGCAACTGTATATC uu
mW-box 5 aaCATTTGGAGAGAAATTTGAaCAGGCAACTGTATATCuu
W-box 6 aaCTAGTCATGAACCTTAGTCAGGACTAGATTGTTAGAuu
mW-box 6 aaCTAGTCATGAACCTTAtTCAGGACTAGATTGTTAGAuu
Amy32b aaGCCCGGATTGACTTGACCATCATCTGuu

The non-labeled double strand oligonucleotides were used as competitors. Another probe made from the promoter fragment of Amy32b was used as a positive control based on its demonstrated binding affinity to HvWRKY1 protein in a previous study (Marè *et al.*, 2004). The assays for all binding reactions contained the following components: probe, poly (dI-dC), 10 mM Tris–HCl (pH 7.6), 50 mM KCl, 0.5 mM EDTA, 50 uM $ZnCl_2$, and 10% glycerol. Recombinant His-HvWRKY2 protein (2 ug) was added with or without the presence of competitor (50-fold molar excess) and incubated at room temperature for 30 min. Reactions were resolved by electrophoresis on 5% polyacrylamide gel in 0.5x TBE (45 mM Tris, 45 mM boric acid, and 1 mM EDTA) buffer for 2 h. Signals were detected with DIG detection system.

2.11 Database search and sequence annotation

Protein sequences of the assigned WRKY genes for *Arabidopsis thaliana* were retrieved from NCBI using the accession numbers listed online (http://www.mpiz-koeln.mpg.de/english/research/pmi-dpt/somssich/WRKY_Superfamily/Arabidopsis_WRKY_Superfamily/index.html).
For protein sequences of rice WRKY family, they are downloaded from the WRKY Wide Web (http://systemsbiology.usm.edu/PhytoTech/WRKY07012011/Species.html) using the list of OsjWRKY from *Oryza sativa* ssp*. japonica*. OsWRKY33, OsWRKY38 and OsWRKY41 are obtained from *O. sativa* ssp*. Indica*.

To obtain the barley WRKY protein sequences, a blastp search was performed using the WRKYGQK signature as query sequences against non-redundant proteins dataset for barley (Organism: *Hordeum vulgare*, date: May 30, 2011). The search resulted in 100 hits, among which 9 sequences did not contain WRKY signature and was removed. The remaining 91 sequences were used for alignment in Clustal W and 32 sequences were found to be duplicates. As a result, 59 non-redundant HvWRKY protein sequences were obtained. To prevent confusion, I adopted the nomenclature for from HvWRKY1 to HvWRKY46 as described previously (Mangelsen, *et al.*, 2008). HvWRKY35 and HvWRKY41 were combined as HvWRKY35. Two WRKY domains were found for HvWRKY25 and therefore assigned as group I WRKY. Whenever possible, the accession numbers for complete WRKY protein sequences were used. To identify more putative WRKY members in barley, the translated nucleotides corresponding to WRKYGQK were used in blastn search against the barley genome draft assembly (http://webblast.ipk-gatersleben.de/barley/index.php). In addition, tblastn search against the barley genome was performed using protein sequences of rice WRKY representatives from different groups (OsWRKY3, -6, -7, -13, -15, -45,-62, -66, -71 -78,-88 and -97) as queries. The best matching contigs were downloaded and subjected to gene prediction with online Genescan tools using maize as a model. Gene prediction was confirmed by FGENESH using the model of monocots. Some contigs failed in the prediction using monocot as model but contained WRKY domains using *Arabidopsis* as model (HvWRKY66, -88, -90, -91, -93, and -95). Altogether, 41 additional peptides containing at least one putative WRKY domain were identified. Taken together, 100 putative WRKY proteins were obtained and used for further analysis.

WRKY protein sequences from the other species are retrieved from Superfamily 1.75 (http://supfam.cs.bris.ac.uk/SUPERFAMILY). Redundant sequences or sequences lacking a WRKY domain were removed after alignment.

2.12 Data alignments and phylogenetic analysis

Sequence data processing was performed as shown in Figure 2.1. For the group I WRKY proteins which have two conserved WRKY domains, they were manually spliced from the middle into N-terminal and C-terminal fragments, each containing the consensus WRKY domains, and was regarded as independent sequences.

Protein sequences from *Arabidopsis thaliana*, *Oryza sativa*, *Hordeum vulgare* and other species were aligned using MUSCLE programm which is integrated in MEGA5 software (Tamura *et al*., 2011). The alignment was then manually adjusted to reduce opened internal gaps. WRKY domains were kept starting 4 aa in front of WRKY signature and ending 2 aa after the zinc-finger motif (position of the last C or H in the motif C_2HC or C_2H_2). The final sequences contain a conserved region spanning about 60 aa plus gaps with a region of 64 sites. Phylogenetic reconstruction was performed by MEGA5 (Tamura *et al*., 2011). In the analysis for 844 WRKY domains, about 90 aa crossing the conserved regions were included in multiple alignment.

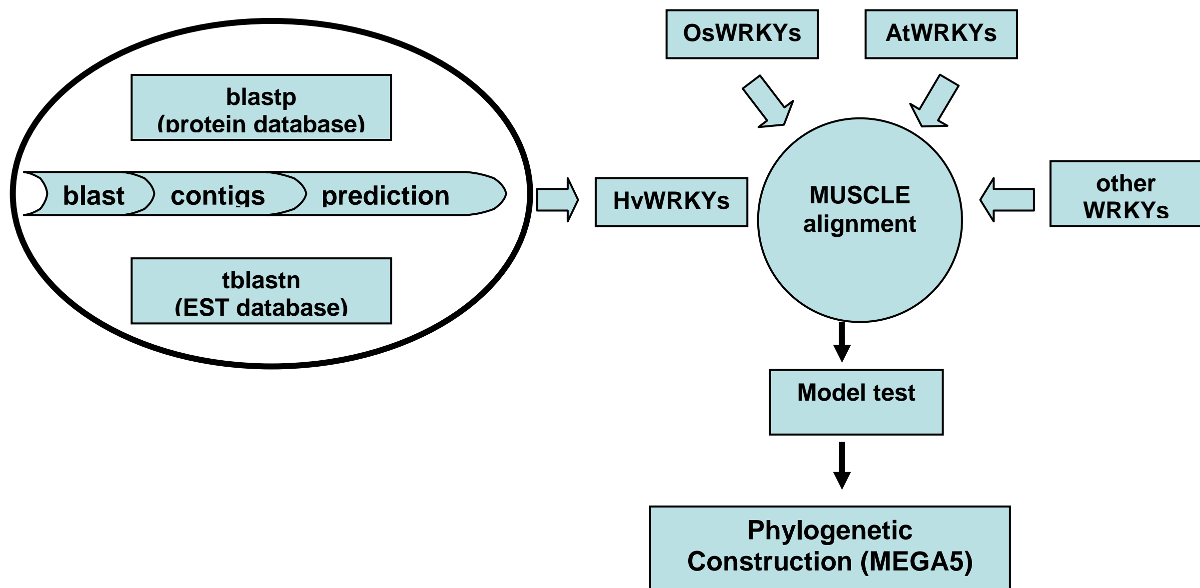

Figure 2. 1 Overview of the pipeline used in the sequence data processing. Arrows represent the direction followed in the phylogenetic analysis of barley WRKY transcription factors.

3. Results

3.1 Phylogenetic analysis of barley WRKY transcription factors

3.1.1 Identification and annotation of WRKY proteins in barley genome

Previously, the first phylogenetic analysis of barley WRKY gene family was performed by Mangelsen and coworkers (2008). The identified 45 putative barley WRKY genes were estimated to cover 50% of the whole gene family. Four of them had been described to play crucial roles in barley development, abiotic stresses and biotic stresses (Eckey *et al.*, 2004; Mare *et al.*, 2004; Shen *et al.*, 2007; Sun *et al.*, 2003; Xie *et al.*, 2007). *HvWRKY1* and *HvWRKY2* played a role in negatively regulating barley responses to powdery mildew (*Blumeria graminis*) infections (Shen *et al.*, 2007). *HvWRKY38*, also known as *HvWRKY1*, was found to be involved in drought and cold responses as well as the regulation of α-amylase during seed germination (Mare et al., 2004; Xie *et al.*, 2007; Xiong *et al.*, 2010). SUSIBA2, annotated as *HvWRKY46*, is involved in sugar signalling and regulation of starch biosynthesis (Sun *et al.*, 2003). Despite the significant importance in stress responses and development, barley WRKY transcription factors were less studied and this field is lagging much behind the model plants *Arabidopsis* and rice. A well-resolved phylogenetic analysis of the barley WRKY family would facilitate the intra- and interspecies comparisons and help to functionally characterize further WRKYs with important roles in pathogen resistance, as there is much reference information available from *Arabidopsis* and rice. To fill in this gap, I screened the available EST data in GenBank and take advantage of the newly available barley genome information for discovery of novel putative barley WRKYs. I used WRKYGQK as query to BLASTP search against the GeneBank protein data set for *Hordeum vulgare*. 59 non-redundant putative WRKY-encoding proteins were identified. The recent release of over 24,000 full-length barley cDNAs allowed me to obtain 42 full-length sequences for the barley WRKY genes (Matsumoto *et al*., 2011). The rest 17 WRKY sequences are not complete. Among them, two domains were found for *HvWRKY25* and therefore re-assigned as Group I WRKY. *HvWRKY35* and *HvWRKY41* were merged to *HvWRKY35* because they match the same genomic contig.

To identify more putative barley WRKY genes, I performed blastn search using the nucleotides corresponding to WRKYGQK as queries against barley genome database. In parallel, TBLASTN search was performed against the genome database of barley using representative WRKY domains from rice as query (OsWRKY3, -6, -7, -13, -15, -45,-62, -66, -71 -78,-88 and -97). The best matching genomic sequence contigs were downloaded and the exon prediction was done with Genescan using the model of maize (*Zea mays*). Gene prediction was confirmed by FGENESH using the model of monocots. Some genomic contigs failed in the exon prediction using maize as model but contained WRKY sequences using the gene model of *Arabidopsis*. As a result, 41 additional WRKY proteins were predicted from HvWRKY62 to HvWRKY102 and 25 of them were predicted to be full-length (Table 3.1). Altogether, 100 barley WRKYs were predicted with 67 full length sequences.

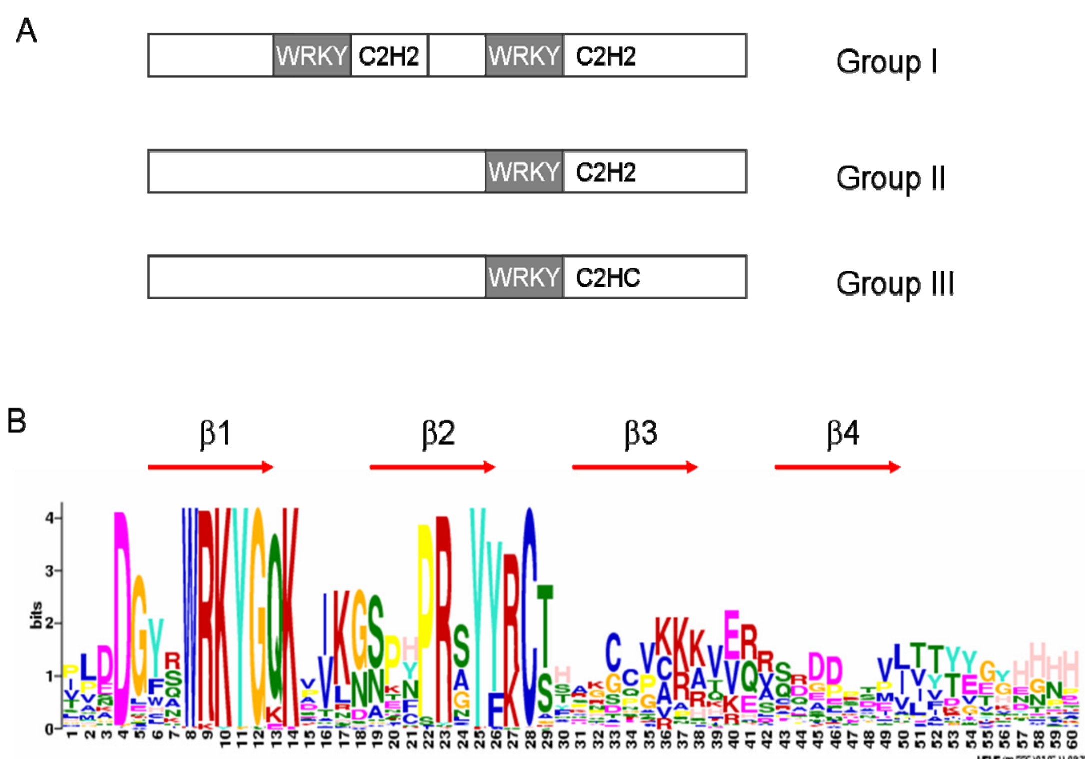

Figure 3. 1 Organization of WRKY domains and WRKY signatures. **A**, Schematic structure of WRKY domains in WRKY proteins group I, II and III. Zinc-finger motif and the WRKY signatures in each group are displayed. **B,** Sequence LOGO view of the consensus WRKY domain sequences based on all the WRKY domains from barley WRKY family. MEME tool (Bailey and Elkan, 1994) was used to generate the plot. The height of the letter (amino acid) at each position represents the degree of conservation. The four β-strands are shown in red at the top of the plot.

EST data are available for eight of the predicted barley WRKY genes *HvWRKY63*, *HvWRKY64*, *HvWRKY65*, *HvWRKY74*, *HvWRKY77*, *HvWRKY81*, *HvWRKY87* and *HvWRKY94*. For HvWRKY50, the NCBI database wrongly assigned it to a non-WRKY protein. In addition, HvWRKY19 has two variants named HvWRKY19-1 and HvWRKY19-2. The cumulative information of the 100 members from barley WRKY gene family is shown in Table 3.1. Using all-to-all blast, the orthologous genes in *Arabidopsis* and rice for each barley WRKY member were identified based on the similarity of full-length sequences. The best hit from *AtWRKYs* and *OsWRKY*s is included in Table 3.1. Organization of WRKY domains and WRKY signatures for barley WRKY proteins are shown in Figure 3.1.

Table 3. 1. List of barley WRKY genes in comparison with *Arabidopsis thaliana* and rice (*Oryza sativa*). The group names, accession number of cDNA and proteins (if available), genomic contigs and the best hit in AtWRKY and OsWRKY are included. For the predicted WRKY proteins, the gene model of *Zea mays* was used for prediction by Genescan if not indicated. na, not available; H, hypothetical protein deduced from the predicted CDS; At, exon prediction was based on the model of *Arabidopsis thaliana*.

Name	Group	cDNA Accession.	Genomic Contig	Protein Accession	Complete CDS	Best hit in AtWRKY	Best hit in OsWRKY
HvWRKY1/38	IIa	AJ536667	contig_1006384	CAD60651	YES	40	71
HvWRKY2	IIa	AJ853838	contig_2162573	CAH68818	YES	40	28
HvWRKY3	IIa	AK359706	Contig_342474	BAJ90915	YES	40	76
HvWRKY4	III	AK371133	contig_121742	BAK02331	YES	70	47
HvWRKY5	IIc	AK358362	contig_50952	BAJ89576	YES	50	77
HvWRKY6	I	EF488106	contig_1014506 contig_48252	ABR87003	YES	4	82
HvWRKY7	IId	DQ840406	contig_407077 contig_300787 contig_1622612 contig_370278 contig_1139064 contig_249381 contig_144351	ABI13373	YES	17	68

HvWRKY8	IId	AK374747	contig_2159503	BAK05943	YES	74	83
HvWRKY9	IId	DQ840408	contig_47880	ABI13375	NO	21	87
HvWRKY10	IId	DQ840409	contig_47808	ABI13376	YES	17	51
HvWRKY11	IId	AK370043	contig_5720	BAK01244	YES	15	6
HvWRKY12	IIc	AK354853	contig_42619 contig_1011363	BAJ86072	YES	75	73
HvWRKY13	IIc	DQ840412	contig_2162994	ABI13379	NO	24	23
HvWRKY14	IIc	DQ840413	contig_1014483 contig_2184465	ABI13380	NO	13	79
HvWRKY15	IIc	AK370299	contig_2165862	BAK01500	YES	48	3
HvWRKY16	IIc	DQ840415	nd	ABI13382	NO	50	67
HvWRKY17	IIc	AK357196	contig_47911	BAJ88410	YES	50	67
HvWRKY18	IIc	DQ840417	contig_45869	ABI13384	NO	51	26
HvWRKY19-1	IIc	BY846302 BE412464	contig_257635	na	YES,	50	7
HvWRKY19-2	IIc	AK363783	contig_257635	na	YES	50	7
HvWRKY20	IIc	AK363451	contig_1006739	BAJ94655	YES	50	7
HvWRKY21	III	AK356339	contig_50431	BAJ87557	YES	53	15
HvWRKY22	III	AK377066	contig_1035021	BAK08260	YES	41	74
HvWRKY23	IIa	DQ863131	contig_352217	ABI13413	NO	40	28

HvWRKY24	I	DQ863108	contig_53603	ABI13390	NO	41	15
HvWRKY25	I	DQ863109	nd	ABI13391	NO	41	15
HvWRKY26	III	AK369804	contig_2231621	BAK01005	YES	53	19
HvWRKY27	III	DQ863111	contig_110298,	ABI13393	NO	53	19
HvWRKY28	III	AK355533	contig_43617 contig_225483	BAJ86752	NO	41	74
HvWRKY29	III	AK362234	contig_50375	BAJ93438	YES	53	19
HvWRKY30	III	AK361795	contig_17373	BAJ92999	YES	41	69
HvWRKY31	III	DQ863115	contig_10262	ABI13397	NO	70	48
HvWRKY32	III	AK360029	contig_9580 contig_2164723 contig_2165093	BAJ91238	NO	70	45
HvWRKY33	III	AK363340	contig_43596	BAJ94544	YES	46	55
HvWRKY34	III	AK365657	contig_94785	BAJ96860	NO	46	46
HvWRKY35	I	AK365469	contig_79457	BAJ96672	YES	3	96
HvWRKY36	IIc	AK362686	contig_39931 contig_1038033	BAJ93890	YES	13	101
HvWRKY37	IIb	AK368042	contig_126234	BAJ99245	YES	61	97
HvWRKY39	IIe	AK367643	contig_140874	BAJ98846	YES	65	13

HvWRKY40	I	DQ863123+ FD518408	contig_123835	na	YES	34	102
HvWRKY42	I	AK362158	contig_254252	BAJ93362	YES	2	80
HvWRKY43	I	AK376482	contig_59998	BAK07677	YES	33	70
HvWRKY44	IIe	DQ863129	contig_246056	ABI13411	NO	27	39
HvWRKY45	IIe	AK356823	contig_1032364	BAJ88038	YES	65	14
HvWRKY46	1	AY323206 AK369730	contig_120368 contig_195112	BAK00931	YES	20	78
HvWRKY47	III	DQ900687	contig_5918 contig_39219 contig_384 contig_1122796	ABL11228	YES	53	93
HvWRKY48	IIb	AK248555	contig_1015996	na	NO	61	97
HvWRKY49	I	AK250089	contig_353367	na	NO	4	84
HvWRKY50	IIc	AK361512	contig_353208	na	YES	50	10
HvWRKY51	I	AK357671	contig_62027	BAJ88885	YES	33	24
HvWRKY52	I	AK376765	contig_52325 contig_2156719 contig_38638	BAK07959	YES	2	35
HvWRKY53	IIc	AK358052	contig_54575	BAJ89266	YES	71	16

HvWRKY54	IIa	AK368514	contig_1014802 contig_2222013	BAJ99717	NO	40	76
HvWRKY55	IIe	AK368513	contig_352685 contig_124987	BAJ99716	YES	27	12
HvWRKY56	IIe	AK368994 AK372278	contig_49022	BAK00197 BAK03476	YES	14	66
HvWRKY57	IIb	AK357452	contig_53770	BAJ88666	YES	6	1
HvWRKY58	I	AK363803	contig_123099	BAJ95006	NO	58	4
HvWRKY59	IIb	AK363247	contig_2168077	BAJ94451	YES	9	32
HvWRKY60	IIc	AK367216	contig_58302	BAJ98419	YES	23	8
HvWRKY61	IId	AK375802	contig_142440 contig_123665	BAK06997	YES	74	83
HvWRKY62	IIc	na	contig_8315	na	YES, H	71	11
HvWRKY63	IId	na	contig_2165457	na	YES, H	17	68
HvWRKY64	III	BM372327	contig_1016776	na	YES, H	63	22
HvWRKY65	IIa	EX577594	contig_318509	na	YES,H	18	62
HvWRKY66	I	na	contig_254811	na	YES, H, At	2	30
HvWRKY67	III	na	contig_1038013	na	NO, H	54	48
HvWRKY68	IIe	na	contig_39744	na	YES, H	35	88

HvWRKY69	IIc	na	contig_327948	na	NO,H	35	66
HvWRKY70	IIc	na	contig_2160593	na	YES,H	12	34
HvWRKY71	IIc	na	contig_93119	na	NO,H	13	17
HvWRKY72	IId	na	contig_51501	na	YES,H	17	68
HvWRKY73	III	na	contig_50885	na	NO,H	70	45
HvWRKY74	III	GH209861	contig_251171	na	NO,H	41	86
HvWRKY75	IIc	na	contig_116342	na	YES,H	48	29
HvWRKY76	III	na	contig_52137	na	YES,H	54	50
HvWRKY77	IIc	EX585854	contig_50538	na	YES,H	12	36
HvWRKY78	I	EX596185	contig_2161300 contig_249258	na	NO,H	4	85
HvWRKY79	IIc	GH227897	contig_1023762	na	YES,H	71	49
HvWRKY80	IIe	na	contig_120171	na	YES,H	22	92
HvWRKY81	IIc	BM370096	contig_52837	na	YES,H	71	60
HvWRKY82	III	EX572151	contig_153208	na	NO,H	70	47
HvWRKY83	III	na	contig_6987	na	NO,H	53	75
HvWRKY84	IIe	na	contig_2168101	na	YES,H	22	92
HvWRKY85	III	na	contig_2231621	na	NO,H	53	19
HvWRKY86	III	na	contig_60821	na	YES,H	30	21
HvWRKY87	IIe	BQ763606	contig_42181	na	YES,H	22	21

		BY872404					
HvWRKY88	III	na	contig_2161133	na	YES,H,At	70	44
HvWRKY89	III	na	Contig_9731	na	NO,H	62	48
HvWRKY90	III	na	contig_202409	na	YES,H,At	41	18
HvWRKY91	III	na	contig_1064624	na	YES,H,At	30	69
HvWRKY92	III	na	contig_1008793	na	YES,H	70	91
HvWRKY93	III	na	contig_75387	na	YES,H,At	38	90
HvWRKY94	III	na	contig_1006364	na	YES,H	38	64
HvWRKY95	IIb	na	contig_4298	na	YES,H,At	6	97
HvWRKY96	IIb	na	contig_2185084	na	NO,H	31	43
HvWRKY97	III	na	Contig_121639	na	NO,H	41	91
HvWRKY98	III	na	contig_2161480	na	NO,H	38	90
HvWRKY99	III	na	contig_1034909	na	YES,H	38	90
HvWRKY100	III	na	contig_45438	na	NO,H	55	75
HvWRKY101	IIc	na	contig_19074	na	NO,H	45	73
HvWRKY102	IIc	na	contig_48863	na	NO,H	75	23

3.1.2 Distribution of WRKY members in each subgroup

The WRKY protein family can be classified into seven subgroups based on the sequence characteristic and structure. To classify the members of barley WRKYs into the predefined groups, multiple alignments were performed with 72 *AtWRKYs*, 96 *OsWRKYs* and 100 *HvWRKYs*. In combination with the local all-to-all blast results, the barley WRKY proteins were placed into the corresponding groups. The number of each WRKY subgroup is listed in Table 3. 2.

Table 3. 2. Number of each WRKY subgroup in *Arabidopsis*, rice and barley

WRKY Group	AtWRKY	OsWRKY	HvWRKY
I	14	12	14
IIa	3	4	6
IIb	8	7	6
IIc	18	20	24
IId	7	9	8
IIe	8	11	9
III	14	33	33
Total	72	96	100

As shown in Figure 3. 2, the total number of WRKYs in the monocot crop rice and barley is higher than that of *Arabidopsis*, a dicot model plant. From the distribution pattern of the WRKY members in each subgroup, the number of WRKY members in group III differs a lot between the dicot plant *Arabidopsis* and the two monocot plants rice and barley whereas the other subgroups have similar sizes among the three plant species. In rice and barley, group III WRKY account for 34% and 33% in the whole family respectively. However, the percentage of WRKY III members in *Arabidopsis* WRKY family is only 19%. The difference in group III indicate an expansion of group III WRKY members in barley and rice compared to *Arabidopsis*.

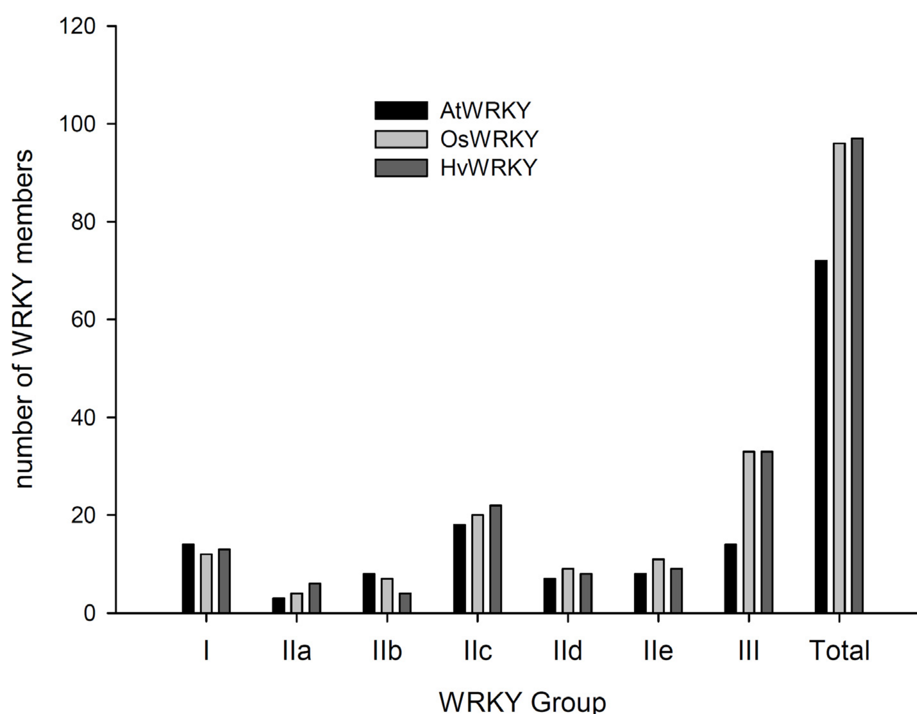

Figure 3. 2. Distribution of WRKY members in each subgroup from *Arabidopsis*, rice and barley.

3.1.3 Phylogenetic analysis of barley WRKY transcription factors

To resolve the phylogenetic relationships of the barley WRKY family, an unrooted phylogenetic tree was generated using the conserved WRKY domain spanning about 60 amino acids (section 2.12). Representative *Arabidopsis* WRKY members from each subgroup were selected to facilitate the group assignments. For group I WRKYs which contain two conserved WRKY domains, they were manually split to I-N terminus and I-C terminus and treated as independent sequences. Multiple alignment was performed with MUSCLE algorithm which is integrated in MEGA5 (Edgar, 2004; Tamura *et al.*, 2011). Sequences causing gaps (HvWRKY74, HvWRKY76, HvWRkY89, HvWRKY93, HvWRKY94, HvWRKY95, HvWRKY96, HvWRKY98, HvWRKY99, HvWRKY100, HvWRKY101, and HvWRKY102) or too short sequences (HvWRKY25C) were eliminated in the further analysis. In total, 119 WRKY domains were included in the final alignment for phylogenetic reconstruction (Appendix 2.1). As a result, most of the barley WRKYs could be clearly classified into corresponding pre-defined subgroups I, II and III (Figure 3. 3; Appendix 2.2).

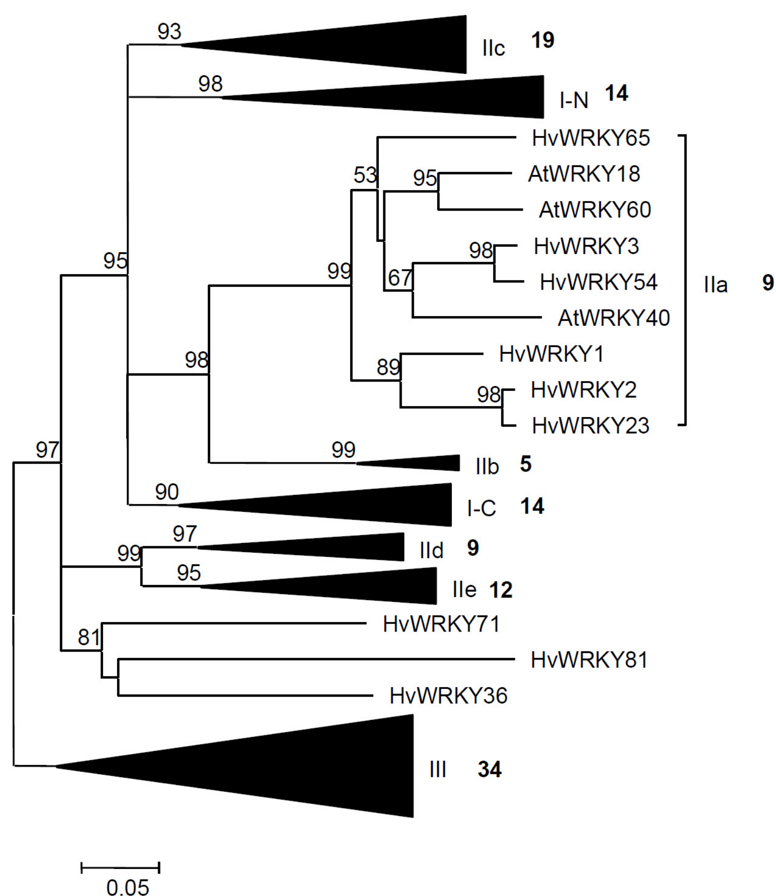

Figure 3. 3. Phylogenetic tree of barley WRKY transcription factors.
The unrooted phylogenetic tree of 119 WRKY domains comprising the identified barley WRKYs and some representative WRKYs from *Arabidopsis* was constructed using MEGA5 (Tamura *et al.*, 2011.). The resolved clades were compressed and represented by black triangles.The labels and bolded Arabic numerals at the black triangles indicate the respective WRKY groups and number of WRKY members included in the group. The labels I-N and I-C indicate N-terminal and C-terminal domains from group I WRKY members, respectively. The evolutionary history was inferred using the Neighbor-Joining method based on the conserved WRKY domain spanning about 60 amino acids. The confidence probability in percentage estimated from bootstrap test (1000 replicates) higher than 50 are included. The multiple alignment and complete phylogenetic tree shown in extended way is shown in Appendix 2.2. Bar lengths indicate the number of amino acid differences per site.

As suggested by the modified classification system for WRKY family (Zhang and Wang, 2005), the group IIa and IIb were actually one group whereas group IId and IIe should be combined. My phylogenetic reconstruction supports their suggestion. However, three barley WRKYs namely HvWRKY36, HvWRKY71 and HvWRKY81 could not be clearly assigned into any of the subgroups. Based on their full-length sequence similarity, their best hits in AtWRKYs were AtWRKY13 and AtWRKY71,

both of which are group IIc members. Therefore, these three HvWRKYs are nonetheless classified as group IIc members in this study. In agreement with the previous report, HvWRKY36 could not be assigned into the subgroups unambiguously solely based on phylogenetic analysis of the conserved domain (Mangelsen *et al.*, 2008).

As mentioned above, there are differences in the total number of WRKY proteins in *Arabidopsis* and monocot plants rice and barley. This difference is mainly attributed to the size of group III subfamily between these species (Figure 3. 2). I speculated that the monocot plants might have highly distinct and specific WRKY members in group III. Therefore, I constructed a second phylogenetic tree including all the identified WRKYs to compare the differences between the three plant species. Altogether, 291 WRKY domains were used in the phylogenetic reconstruction These WRKY proteins divided into three groups (Figure 3. 4). In all the subclades of group I and II, there is co-presence of WRKY members from the three plant species. However, the subclades of group III revealed differences between monocots and dicots. In the three subclades within group III, there is one branch comprising AtWRKYs, one with WRKY members solely from rice and barley and one with members from both dicot and monocot. The first branch in group III therefore appears to be dicot-specific. The second branch is considered to be monocot-specific group and might result from gene expansion after the diversification of monocots and dicots. They may have particular functions in monocots development and adaptation.

I compared the sequences in this monocots-specific branch with the other members in group III and observed that some of these members contain a WRKYGEK signature instead of the canonical WRKYGQK in the highly conserved domain. Interestingly, this WRKYGEK motif was not observed in any WRKY proteins of the dicot model *Arabidopsis*. To verify the specificity of these WRKYGEK members, I retrieved WRKY domain sequences from 22 plant species including 2 green algae, 1 moss (non-vascular plant), 1 spikemoss (ancient vascular plant), 11 dicot plants and 7 monocot plants. These plant species were selected due to the completion or nearly completion of their genome sequencing. After multiple alignments and sequence comparison of the identified 1940 WRKY domains, I found that WRKYGEK motif is present in WRKYs of the single-celled

green algae *Chlamydomonas reinhardtii* and multi-cellular green algae *Volvox carteri.* This indicates an ancient origin of WRKYGEK signature. However, the WRKYGEK members were absent in moss (*Physcomitrella patens)* which is considered as close relative for vascular plants, and in spikemoss *(Selaginella moellendorffii),* a primitive vascular plant.

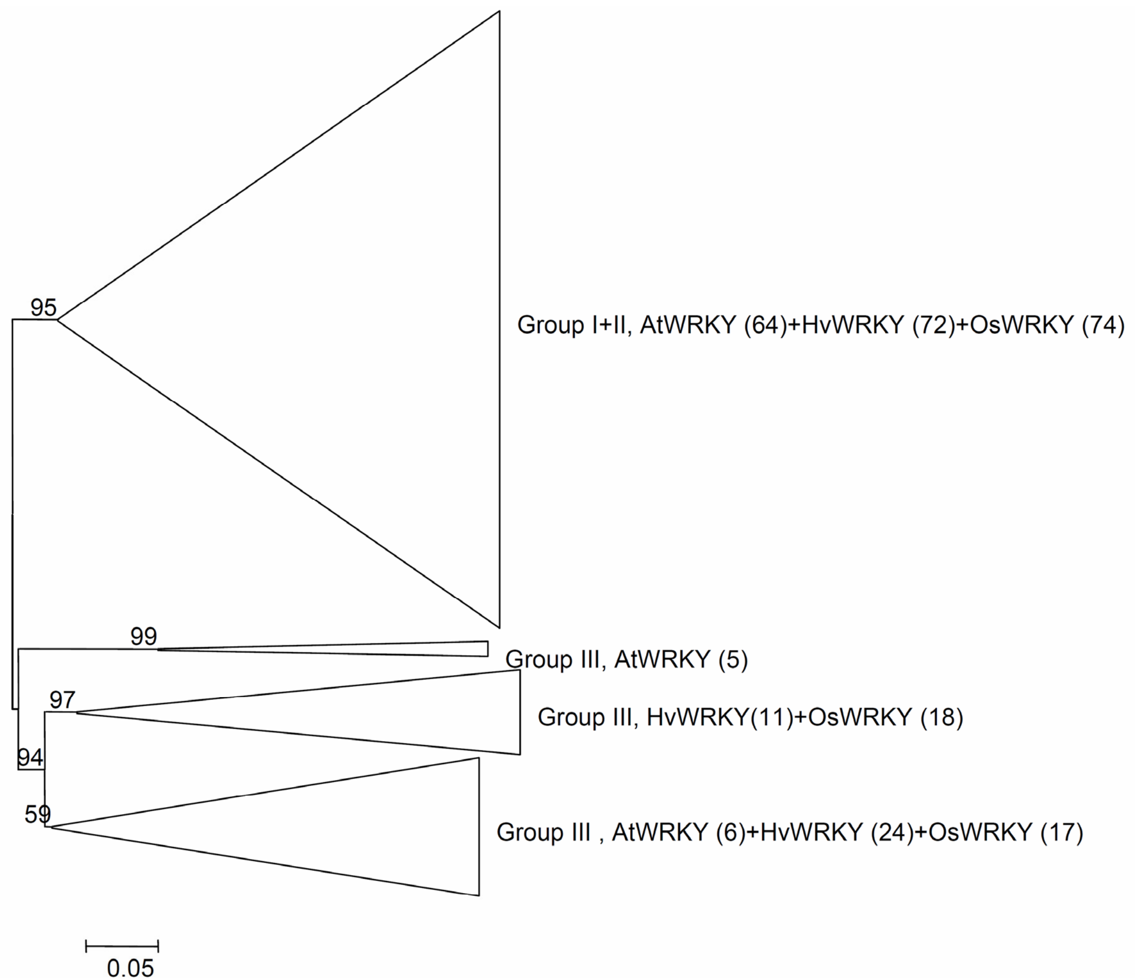

Figure 3. 4. Monocot-specific and dicot-specific subclades in group III WRKYs. Phylogram of group III WRKY domains from *Arabidopsis*, rice and barley is shown in the tree. The unrooted phylogenetic tree of 291 WRKY domains comprising all the WRKYs from *Arabidopsis*, barley and rice was constructed using MEGA5 (Tamura, *et al.*, 2011). The evolutionary history was inferred using the Neighbor-Joining method spanning about 60 conserved amino acids. The resolved clades were compressed and represented by black triangles. The labels indicate the number of respective WRKY groups from the corresponding species. The confidence probability in percentage estimated from bootstrap test (1000 replicates) higher than 50 are shown. The evolutionary distances were computed using the p-distance method. Bar length indicates the number of amino acid differences per site.

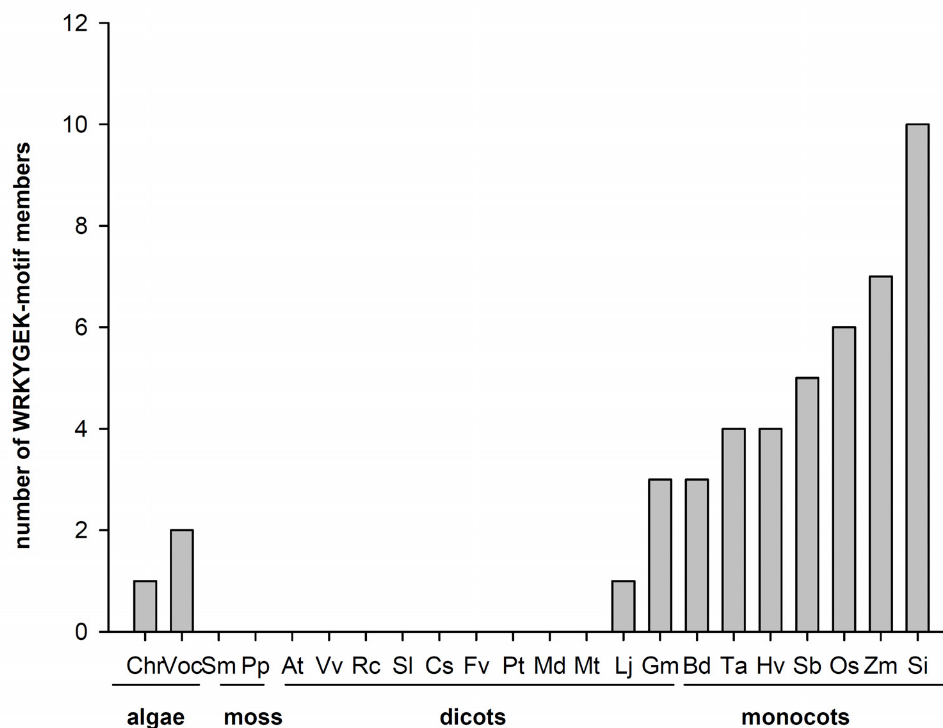

Figure 3. 5. Expansion of WRKYGEK members in monocot plants.
Genome-wide data mining was performed from the available datasets for 22 plant species including algae, mosses, 11 dicot plants and seven monocot plants. Shown is the distribution of WRKYGEK-containing WRKYs in these plant species . Chr, *Chlamydomonas reinhardtii*; Voc, *Volvox carteri*; Sm, *Selaginella moellendorffii*; Pp, *Physcomitrella patens*; At, *Arabidopsis thaliana*; Vv, Vitis vinefera (wine grape); Rc, *Ricinus communis (*castor bean**)***; Sl, Solanum lycopersicum(tomato); Cs, Cucumis sativus (cucumber); Fv, Fragaria vesca (*Alpine strawberry);Pt, *Populus trichocarpa(*Black cottonwood);Md, *Malus x domestica (apple); Mt, Medicago truncatula (*Barrel medic); Lj, *Lotus japonicus; Gm, Glycine max (soybean); Bd, Brachypodium distachyon; Ta, Triticum aestivum (wheat); Hv, Hordeum vulgare (barley); Sb, Sorghum bicolor (sorghum);Os, Oryzae sativa (rice); Zm, Zea mays (maize);*Si, *Setaria italica.*

In addition, the WRKYGEK members were absent in 9 out of 11 selected dicot species (Figure 3. 5). The two exceptions from dicots are soybean (*Glycine max*) and *Lotus japonicus*, two members in the family Fabaceae, with three and one WRKYs having the WRKYGEK motif respectively. Interestingly, all the seven selected monocot plants contain at least three WRKYGEK-type WRKY members. Phylogenetic analysis using all the WRKYGEK-containing WRKYs from monocots and dicots support its monocot-specific expansion. The WRKYGEK members from monocots stand separately as an independent branch in group III. However, the WRKYGEK-containing WRKY members from dicots and the ancient green algae

are classified in distant divisions (Figure 3. 6). Therefore, it is more likely to be a gain of these specific branches in monocots than a loss of WRKYGEK members in dicot plants during evolution. Though not functionally verified, the expanded WRKYGEK members in monocot plants might be of specific significance in regulating the processes of development, adaptation to environmental changes and .pathogen resistance.

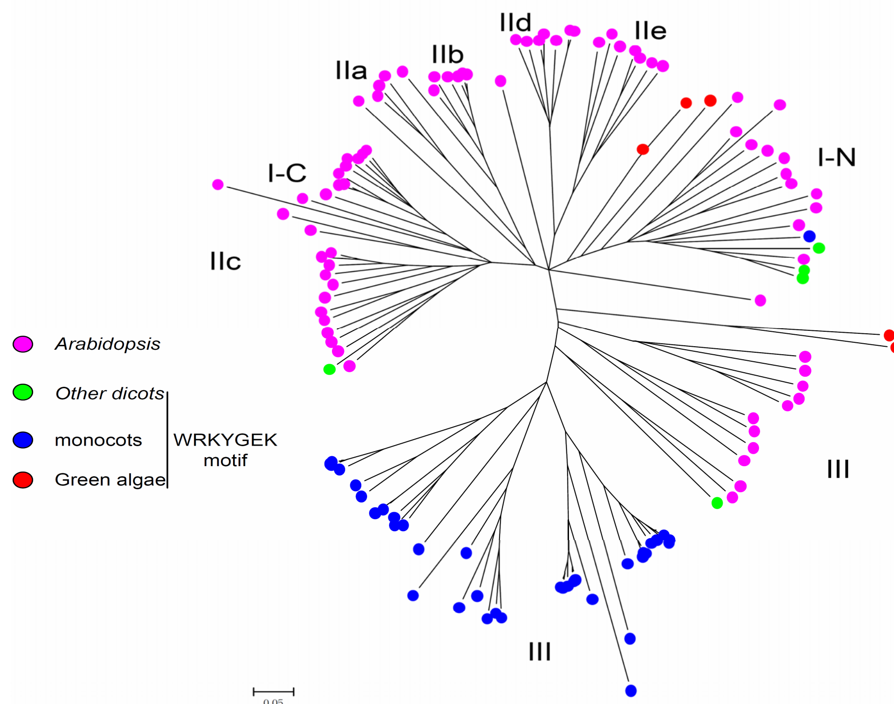

Figure 3. 6. Expansion of WRKYGEK members in monocots. All the WRKYGEK motif-containing WRKY proteins from monocots (*Brachypodium distachyon; Triticum aestivum;, Hordeum vulgare; Sorghum bicolor; Oryzae sativa; Zea mays* and *Setaria italica)* and dicots (*Glycine max*, *Lotus japonicus* and *Helianthus annuus*) were included in the phylogenetic reconstruction together with the whole family of WRKY proteins in *Arabidopsis*.The tree was inferred using Neighbor-Joining method in MEGA5 (Tamura *et al.*, 2011). The WRKY subfamilies are indicated; I-NT and I-CT represent the N-terminal and C-terminal domains of Group I members, respectively. Bar length represent the number of amino acid differences per site.

The evolution and plant specific expansion of WRKY protein family is interesting but still under active debate. As the only WRKY present in the single-celled green algae *Chlamydomonas reinhardtii* belongs to group I, it is well accepted that group I WRKYs are the ancestors of the WRKY family (Rushton *et al.*, 2010). However, there are contrasting viewpoints on the evolving order of group II and III. Due to

the expansion of group III WRKY in monocot plants (i.e, rice), it was suggested that group III WRKYs are the most advanced and last evolved group in higher plants (Zhang and Wang, 2005). To address this question, I constructed a phylogenetic tree including sequences from 9 plant species (Figure 3. 7, Figure 3. 8). In the tree, 844 WRKY members were included and they clearly classified into seven subgroups (Figure 3. 7). The members from moss (*Physcomitrella patens)* and spikemoss (*Selaginella moellendorffii)* were marked red and dark violet in the tree (Figure 3. 8). WRKY members from these two ancient species were distributed in most of the subgroups. However, IIe and IIa WRKY members do not appear in *Physcomitrella patens,* which is a non-vascular plant (Wang *et al.*, 2010b). In the lycophyte *Selaginella moellendorffii,* group IIe WRKY member appears but IIa members are not present. *Selaginella* is considered an ancient vascular plant (Banks *et al.*, 2011), the absence of group IIa in *Selaginella* genome indicated that it is the last group evolved in higher plants.

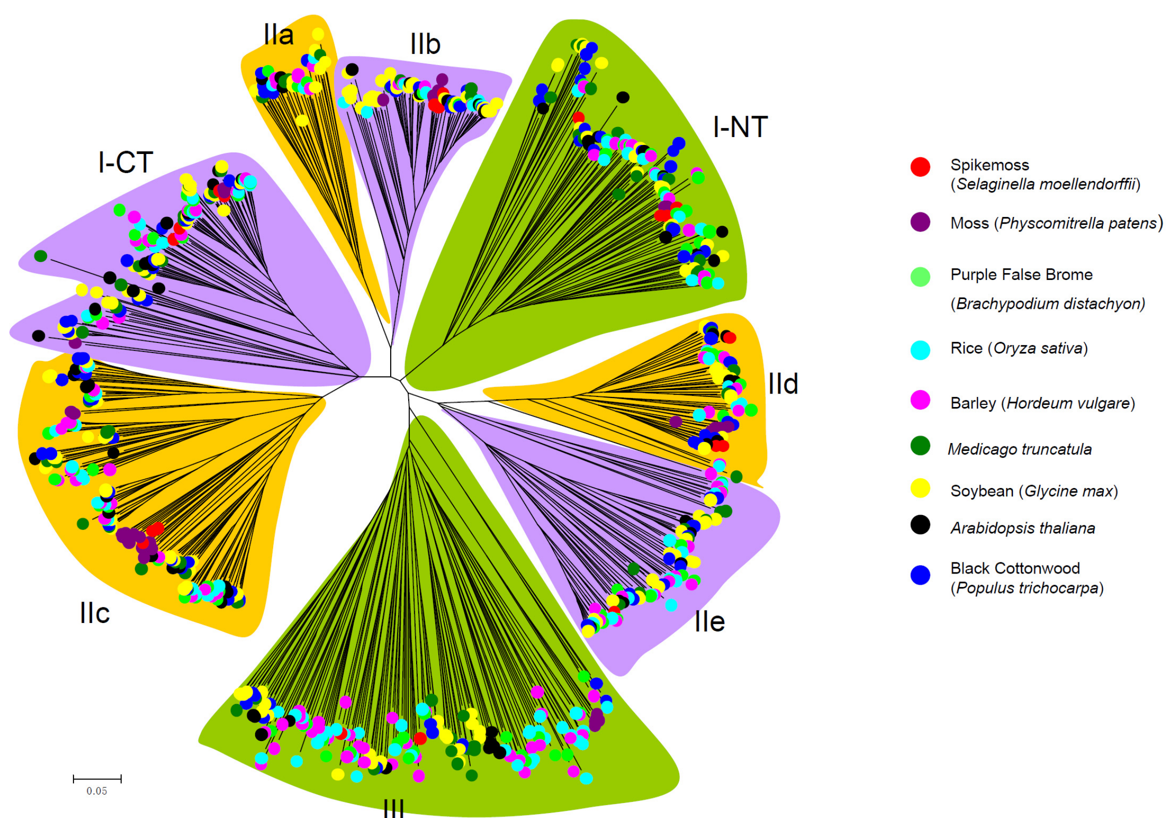

Figure 3. 7. Phylogenetic tree of the WRKY family in plants. 844 WRKY domains from 9 plant species are included to infer the evolutionary history of WRKY gene family using the Neighbor-Joining method. The conserved region spanning about 90 amino acids are included in the multiple alignment. The WRKY subfamilies are

indicated; I-NT and I-CT represent the N-terminal and C-terminal domains of group I members, respectively. The tree supports the pre-defined subgroups of group I, group II (IIa+IIb, IIc, IId+IIe) and group III. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the p-distance method. Bar length represent the number of amino acid differences per site. Phylogenetic analyses were conducted in MEGA5 (Tamura *et al.*, 2011).

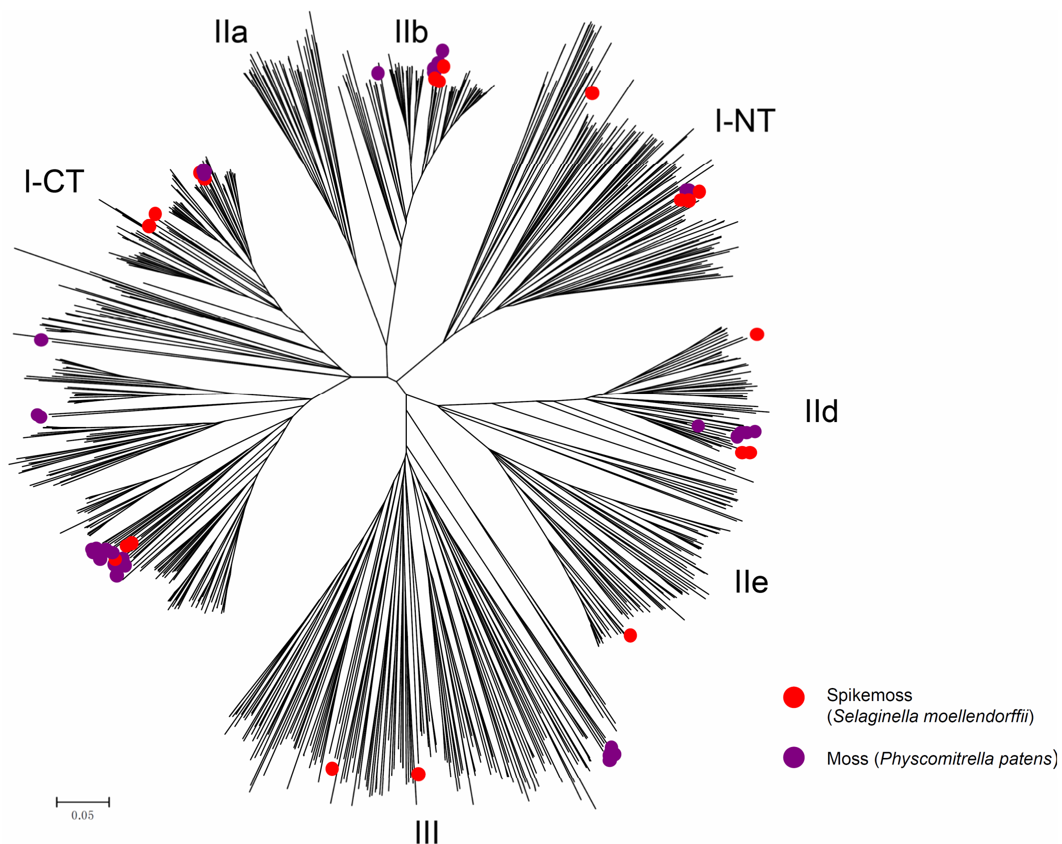

Figure 3. 8. Subgroup IIa is the last group to evolve in plants. The phylogenetic tree from Figure 3. 7 is presented in a simplified manner. Spikemoss (*Selaginella moellendorffii*) and moss (*Physcomitrella patens*) are indicated as red and dark violet, respectively. The tree support the absence of group IIa WRKY members in both ancient species which appears to be the close living relatives of higher plants or primitive vascular plants (Banks *et al.*, 2011; Rensing *et al.*, 2008).

3.2 Expression studies of HvWRKY1 and HvWRKY2

3.2.1 Expression profile of barley WRKY during *Bgh* infection

In our group, *HvWRKY1* was previously identified as a differentially regulated gene after inoculation with the powdery mildew fungus *Blumeria graminis* f.sp*. hordei* (*Bgh*) (Eckey *et al.*, 2004, Figure 3.10). In addition, several barley WRKY genes were identified as *Bgh*-responsive genes such as *HvWRKY2* (Leib, 2005).

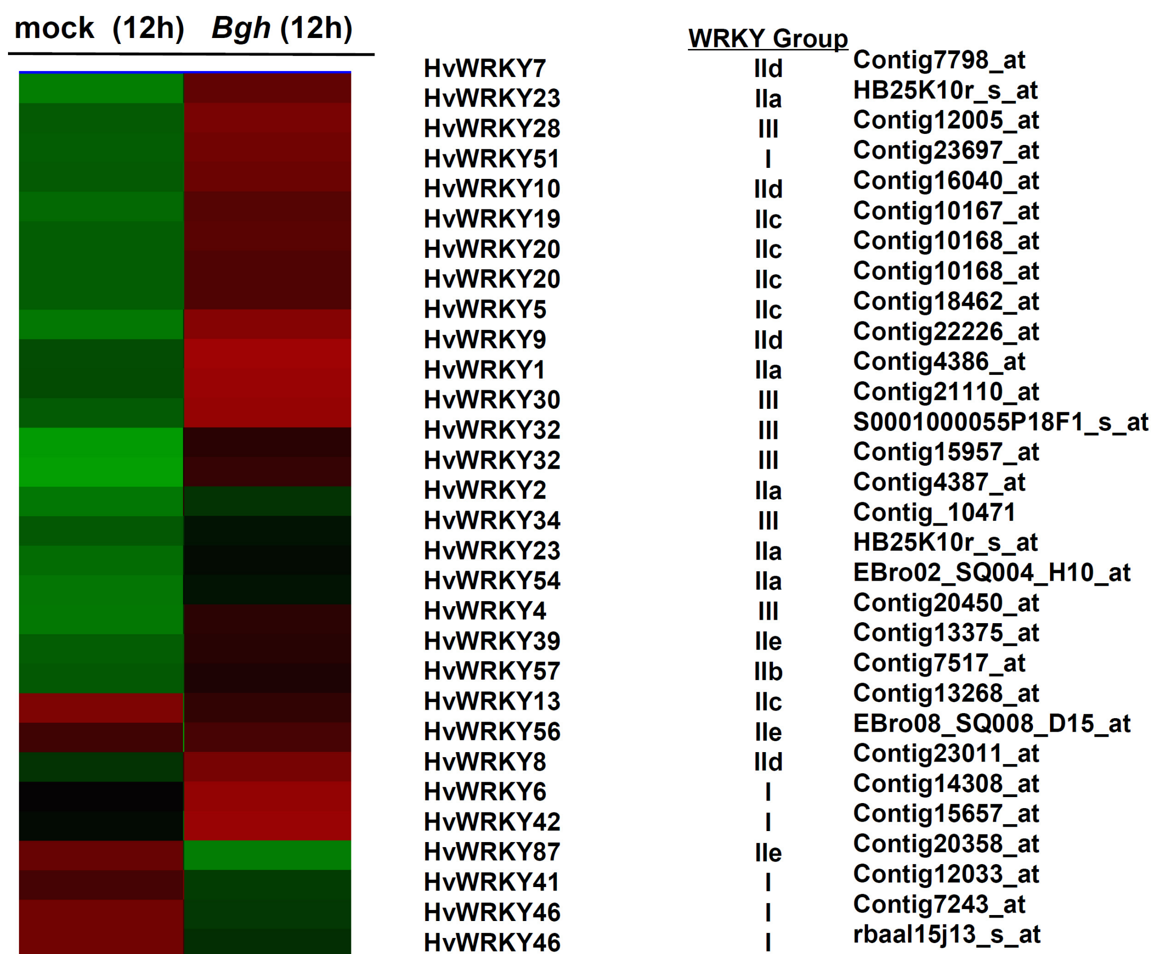

Figure 3. 9 Expression of putative WRKYs present on the Barley1 GeneChip. Gene expression profiles of 26 putative WRKYs identified on the Affymetrix Barley1 GeneChip in wild-type barley cv. Golden Promise 12 h after *Bgh* inoculation are shown in the Eisen plot after hierarchical clustering with dChip. Note that there are two probes for *HvWRKY20*, *HvWRKY23* and *HvWRKY32*.

From previous microarray data, the expression profile of barley WRKY proteins were shown in Figure 3. 9 (Langen *et al.*, unpublished). Most identified members of WRKY groups II and III present of Affymetrix Barley1 GeneChip respond to *Bgh* infection. The prominent up-regulated barley WRKYs include *HvWRKY1,*

HvWRKY5, HvWRKY6, HvWRKY8, HvWRKY 9, HvWRKY19, HvWRKY20, HvWRKY23, HvWRKY28, HvWRKY30, HvWRKY32, HvWRKY51 and *HvWRKY56*. Remarkably, several members of group I are down-regulated after *Bgh* challenge. For example, *HvWRKY41, HvWRKY42, HvWRKY46* and *HvWRKY87* were down-regulated after *Bgh* attack (Figure 3.9).

Figure 3. 10. Pathogen induction of *HvWRKY1* (A, contig4386_at) and *HvWRK2* (B, contig4387_at). Shown is induction pattern of *HvWRKY1* and *HvWRKY2* after the infection of *B.graminis* or *F.graminearum*. The experiments showing the highest induction ratio relative to non-inoculation control are selected from Genevestigator database (Zimmermann *et al.*, 2004).

3.2.2 Gene structure of *HvWRKY1* and *-2*

To elucidate the gene structure of *HvWRKY1* and *HvWRKY2*, a blastn search was performed using their cDNA sequence (Genebank Accessions, AJ536667 and AJ853838) as query sequence against the barley genome draft assembly (http://webblast.ipk-gatersleben.de/barley/index.php). Genomic sequence Contig_1006384 and Contig_2162573 perfectly match *HvWRKY1* and *HvWRKY2* respectively. Based on comparison of cDNA sequences with the genomic contig sequences, the online tool GeneSeqer (http://www.plantgdb.org/cgi-

bin/GeneSeqer/index.cgi, Usuka *et al.*, 2000) allowed the identification of exons and introns. Both genes contain one intron 109 bp in length at the N-terminal side of the conserved WRKY DNA-binding domain (Figure 3. 11). The distances from the intron to the WRKY domain are different for HvWRKY1 and HvWRKY2. The intron of HvWRKY1 is 418 bp upstream of the WRKYGQK-encoding region, whereas the HvWRKY2 intron is directly (19 bp) adjacent to the region for the conserved WRKYGQK signature (Figure 3. 11). According to blastn search in ESTdb, no alternative splice variant was found for either gene.

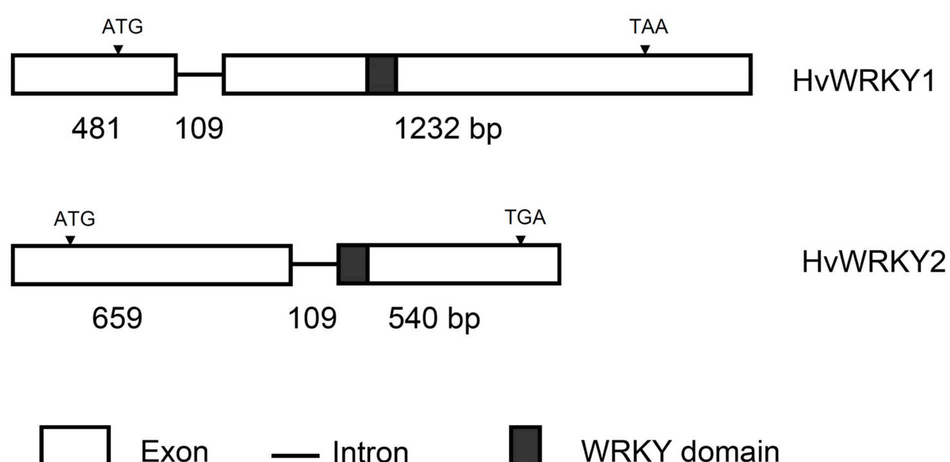

Figure 3. 11. Gene structure of HvWRKY1 and HvWRKY2. Sequence comparisons of cDNA sequences and genomic sequences are performed using the online tool GeneSeqer (Usuka *et al.*, 2000). Exons and introns are shown in scale. WRKY domains are represented with black boxes. Start codons and stop codons are indicated by arrow heads above the plot.

3.2.3 Artificial microRNA-based silencing of *HvWRKY2*

In a previous study, transient overexpression of *HvWRKY2* resulted in enhanced susceptibility whereas virus-induced gene silencing (VIGS) of *HvWRKY2* enhanced resistance to *Blumeria graminis* f.sp. *hordei* (*Bgh*) (Shen *et al.*, 2007). The VIGS approach might have some problems, such as compromised specificity due to the silencing of sequence-related non-target genes. Moreover, the virus infection could negatively interfere with the real effect of target gene during the study of plant-pathogen interaction. A novel gene silencing strategy was established based on the use of artificial microRNA (amiR) in plants and promised to provide higher specificity and less side effect in reverse genetics studies. Artificial microRNA-based efficient gene silencing has been successful used on both rice and *Arabidopsis* (Schwab *et al.*, 2006; Warthmann *et al.*, 2008). The Web

MicroRNA Designer (WMD) tool allows researchers to design amiRNAs against the specific targets, either individual genes or groups of sequence-related genes, in a highly specific and efficient way.

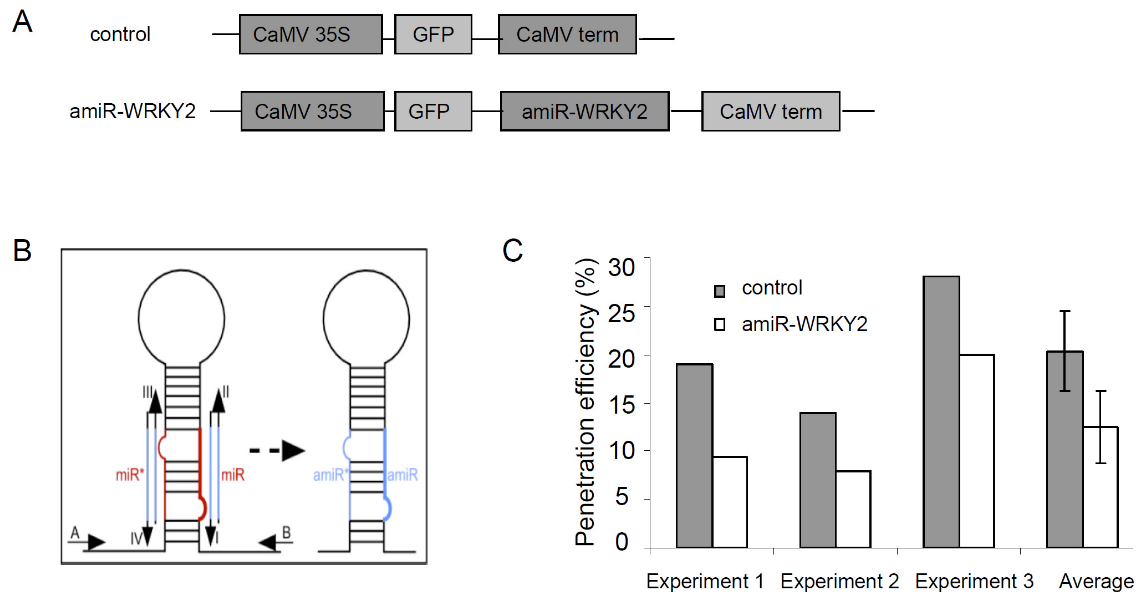

Figure 3. 12. Artificial microRNA-based silencing of *HvWRKY2*. **A**, Schematic diagram of the constructs for control and artificial microRNA of *HvWRKY2 (*amiR-WRKY2*)*. **B**, Procedure for the generation of amiR-WRKY2 construct. Three overlapping PCR were performed with the primer combinations A+IV, III+II and I+B using the rice miRNA precursor osa-MIR528 stem loop in pNW55 as template (Warthmann *et al.*, 2008). A final PCR fragment was amplified by primer pair A+B using the mixture of the three PCR products as template to yield amiR-WRKY2. **C**, amiR-WRKY2 overexpression enhanced resistance in barley epidermal cells infected with *Blumeria graminis* f.sp. *hordei* (*Bgh*). Either the control plasmid or amiR-WRKY2 plasmid was bombarded in seven-day-old primary leaves of cv. Golden Promise. After transformation, leaves were densely inoculated with *Bgh* conidia. Determination of *Bgh* penetration efficiency in GFP transformed cells was performed under fluorescence microscopy 2 days post inoculation. Data from three independent experiments are shown. The last column represents mean values of penetration efficiency (PE) from the three experiments. Bars represent standard errors.

To test the feasibility of amiR-approach for specific and efficient *HvWRKY2* silencing, an artificial microRNA was designed and cloned using the rice osa-MIR528 precursor backbone (accession number: MI0003201) in pNW55 as template (Warthmann *et al.*, 2008). The amiR fragment targeting *HvWRKY2* was double digested by *EagI* and *SpeI*, thereafter cloned downstream of GFP in pGY1-GFP, which contains the fluorescence reporter GFP under the control of CaMV 35S promoter (Figure 3. 12, A; section 2.5). To analyze the silencing effect, the

construct was used for barley transient transformation through particle bombardment (section 2.6). Penetration efficiency of powdery mildew fungus was examined in the transformed epidermal cells in both the amiR-WRKY2 treatment and the control bombardment (pGY1-GFP). From three biological replicates, the HvWRKY2 silencing construct resulted in lower penetration efficiency than that of control (Figure 3. 12). The result is in agreement with the previous study using double-stranded RNA interference (dsRNAi) approach, which revealed that silencing of *HvWRKY2* enhanced the barley resistance to *Bgh (*Leib, 2005*)*. To our knowledge, this is the first report that the rice microRNA precursor osa-MIR528 functions properly in barley. The result confirmed the role of HvWRKY2 as negative regulators in barley-*Bgh* interaction.

3.3 *HvGER4c* promoter as a target of HvWRKY1 and -2

3.3.1 Repression of the *HvGER4c* promoter by HvWRKY1 and -2

The Germin-like protein (GLPs) family in barley basal defense has been well documented previously and several members are strongly expressed upon powdery mildew infection in leaf epidermal cells (Zimmermann *et al.*, 2006). One member of the barley GLP family, *HvGER4c*, was identified as a factor of basal defense showing a linear correlation between protein accumulation and *Bgh* resistance. Transient silencing of *HvGER4* induced hypersusceptibility, while overexpression of *HvGER4* increased the plant resistance (Zimmermann *et al.*, 2006). Numerous W-boxes are present in the *HvGER4c* promoter and were functionally identified to be required for pathogen inducibility (Himmelbach *et al.*, 2010) but specificity of WRKY binding proteins were still unclear.

I speculated that there might be interaction of HvWRKY1 or -2 with *HvGER4c* promoter and tested the possible regulation of *HvGER4* promoter by HvWRKY1 and -2 through the use of transient expression of a GUS reporter gene under control of various *HvGER4c* promoter variants. Co-bombardment of *HvWRKY1* or *-2* overexpression plasmids with pUbi::GUS reporter plasmids had no effect on the GUS activity (Figure 3. 13, A, left; section 2.7 and 2.8). In contrast, the powdery mildew-induced GUS activity in co-bombardment of *pHvGER4c::GUS* with the HvWRKY1 construct was significantly lower than that of control. HvWRKY2 co-bombardment almost abolished the *Bgh*-induced GUS activity of *HvGER4c::GUS*

(Figure 3. 13, A, right). The results indicate that both HvWRKY1 and HvWRKY2 are transcriptional repressors of *HvGER4c* promoter and HvWRKY2 showed a much stronger repression capacity. The fact that they repress *HvGER4c* promoter but not the *Zea mays* ubiquitin promoter demonstrated the transcriptional repression in a highly specific manner.

Previous studies revealed that HvWRKY1 and -2 conferred susceptibility to powdery mildew in wild type plants Ingrid (*Mlo*, *Ror1*) but not in cultivar A89 (*mlo-5, ror1*) (Leib, 2005). The cultivar A89 is a moderately susceptible cultivar which is mutated in *Ror1* (required for *mlo*-specific resistance) in *mlo-5* background. The WRKY1/2-mediated compatibility might require a functional Mlo. In addition, the N-terminal coiled coil (CC) domain of a MLA resistance protein was shown to physically interact with HvWRKY1 and HvWRKY2 in an effector-dependent manner (Shen *et al.*, 2007). I therefore included cultivars harbouring *Mlo*, *mlo-5* and *MLA12* to compare the suppression of *HvGER4c* promoter by HvWRKY2. Interestingly, a similar suppression of *HvGER4*c promoter by HvWRKY2 was observed in the back cross (BC) line Ingrid (*mlo-5*), backcross line Pallas (*mlo-5*) and Sultan 5 (*MLA12,* incompatible interaction with *Bgh* race A6) compared with the wild type Ingrid (*Mlo*) (Figure 3. 13, B).This indicate that the HvWRKY2-mediated repression of *HvGER4*c promoter is independent of Mlo or Mla12.

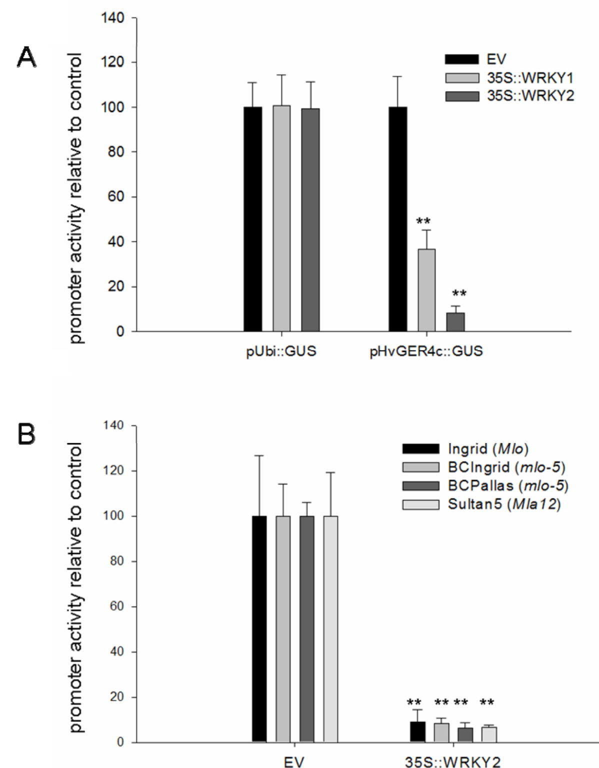

Figure 3. 13. Specific repression of the *Bgh*-induced promoter activity of *HvGER4c* by *HvWRKY1* and *HvWRKY2*. *HvGER4c* promoter was fused to the wheat *WIR1a* intron and the *GUS* reporter gene. The pathogen-dependent activation of reporter constructs was tested by a histochemical GUS assay using a transient expression system in barley leaves. GUS-positive cells were evaluated 48 hours post inoculation with *Bgh*. The *GUS* expression in each experiment was normalised to a co-bombarded pUbi::GFP construct. Mean values ± SE of three independent biological replicates are shown. Statistically significant reduction of promoter activity compared with control is indicated (**, p<0.01, Student's t-test). EV, empty vector. **A**, *Bgh*-induced promoter activity of *HvGER4*c was repressed by 35S::WRKY1 and 35S::WRKY2. **B**, Repression of *HvGER4c* promoter by 35S::WRKY2 was independent of *Mlo* or *Mla12*. BCIngrid (*mlo-5*) and BCPallas (*mlo-5*) are race-nonspecific powdery mildew-resistant back-cross lines from Ingrid and Pallas, respectively.

3.3.2 Repression of truncated promoter *HvGER4c* by HvWRKY1 and -2

The consensus TGAC core sequence (W-boxes) is the potential binding motif recognized by WRKY proteins. In the *HvGER4c* promoter, there are enriched W-boxes and at least four functionally redundant W-boxes are important for high-level pathogen-induced gene expression (Himmelbach *et al.*, 2010). To test if the individual W-boxes have different binding affinities to WRKY proteins or activities, a series of point-mutated *HvGER4c* promoters were further evaluated. Consistent with the recent report (Himmelbach *et al.*, 2010), my results showed that mutation

of individual W-box 1, -2, -5, and 6 in *HvGER4c* promoter resulted in reduced activity compared to wild-type promoter after powdery mildew induction (Figure 3. 14). This result support the notion that each tested W-box can positively modulate expression in compatible interaction and all the four W-boxes appeared to be required for full pathogen-inducibility of *HvGER4c* promoter. After co-bombardment with *35S::WRKY1*, *HvGER4c* promoter and its mutated derivates showed less activity than the empty vector control bombardment (Figure 3. 14). The reduction of *HvGER4*c promoter activity was much stronger in the co-expression with 35S::WRKY2 (Figure 3. 14) demonstrating that both WRKY1 and WRKY2, when transiently over-expressed, negatively modulate *HvGER4c* promoter activity.

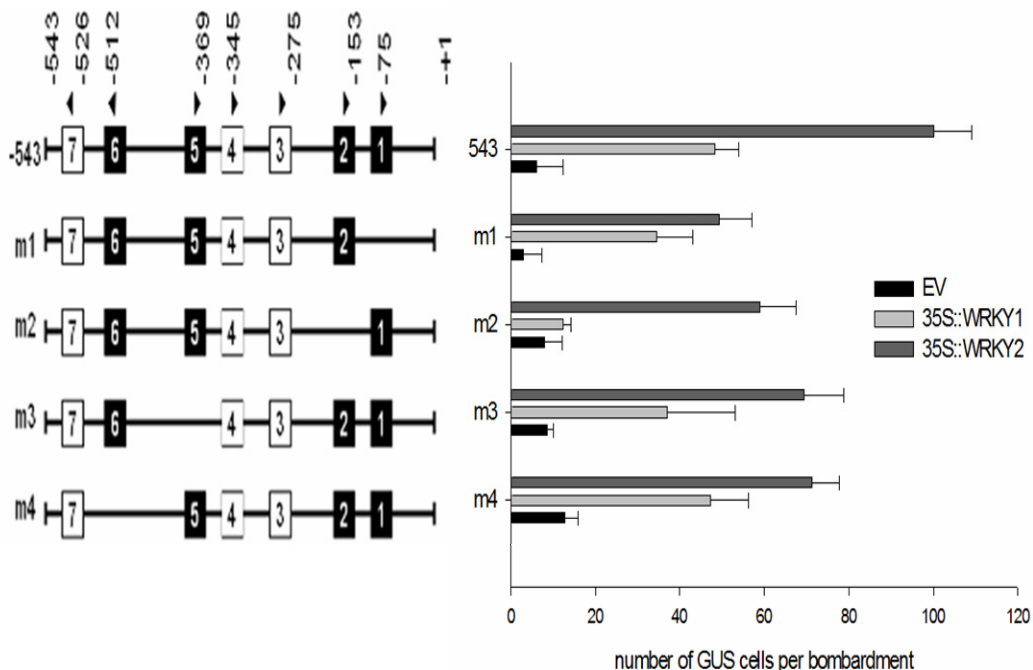

Figure 3. 14. Repression of truncated *HvGER4c* promoter by *35S::WRKY1* and *35S::WRKY2. HvGER4c* promoter derivatives were fused to the wheat *WIR1a* intron and the *GUS* reporter gene. The W-box position in bp relative to the *HvGER4c* transcriptional start (position +1) was indicated by arrowheads and numbers, respectively. Stringent W-box sequences ([C/T]TGAC[T/C]) are shown as closed squares and W-boxes motifs comprising shorter consensus sequences are shown as open squares. The 543-bp-long wild-type *HvGER4*c promoter was labelled -543. The mutant promoters m1, m2, m3 and m4 harbour a point mutation (TGAC to TGAA) in the W-box 1, 2, 5 and 6 respectively. The pathogen-dependent activation of reporter constructs was tested by a histochemical GUS assay using a transient expression system in barley leaves. GUS-positive cells were counted 48 hours post inoculation with *Bgh*. The GUS expression in each

experiment was normalised to a co-bombarded pUbi::GFP construct. Mean values ± SE of three independent biological replicates are shown.

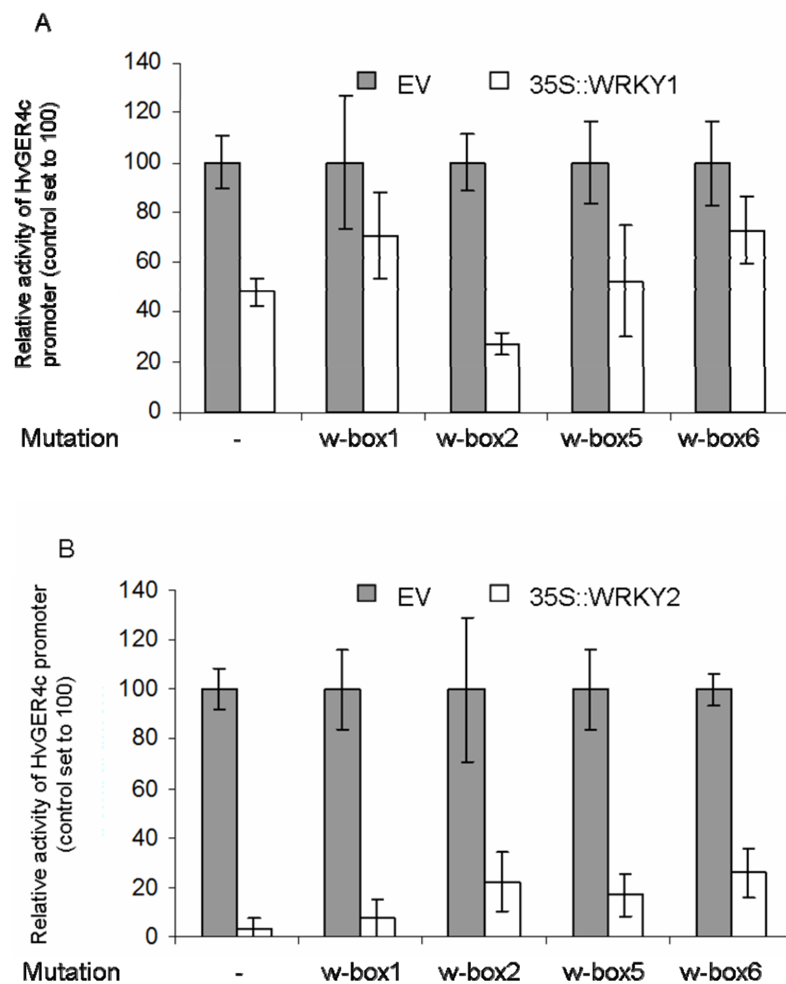

Figure 3. 15. Relative repression of *HvGER4*c promoter activity by 35S::WRKY1 and -2.GUS cells from control bombardments were set to 100. **A**, Co-bombardment of 35S:.WRKY1 had different repression efficacy on the individual versions of mutated *HvGER4*c promoter. **B**, Relative suppression of *HvGER4*c promoter by 35S::WRKY2. Error bar indicates mean values ± SD from three independent experiments.

To analyze the binding affinities of the individual W-boxes 1, 2, 5 and 6, I normalized the GUS activity by setting the GUS cells from control bombardment as 100 (Figure 3. 15). Typically, HvWRKY1 caused approximately 50% of activity reduction in the wild-type *HvGER4*c promoter. Mutation of W-box 5 had almost no influence on its repression by HvWRKY1, which indicates a very weak binding of W-box 5 with HvWRKY1 protein. When the W-box 1 or W-box 6 was inactivated, HvWRKY1 appeared to exhibit less repression capacity on the promoter activity.

This implies a reduced binding affinity of the promoter because of the W-box mutation. Therefore, W-box 1 and W-box 6 might have strong binding affinity with HvWRKY1 protein. Interestingly, mutation in W-box 2 resulted in even stronger repression by HvWRKY1. One explanation could be that there are potential unknown positive WRKY proteins which bind to W-box 2 and activate the *HvGER4c* promoter. Mutation of W-box 2, therefore, facilitated the HvWRKY1 suppression of *HvGER4c* promoter. In the case of HvWRKY2, its suppression capacity is much stronger and it appeared to abolish all the mutated *HvGER4c* promoter activities. However, the strongest repression by HvWRKY2 was observed for the wild-type promoter (Figure 3. 15, B). The truncated *HvGER4c* promoters with mutations in W-boxes showed less repression by HvWRKY2, implicating a requirement of W-boxes in the transcriptional repression.

3.4 Cross activity of AtWRKY40 protein in barley

In *Arabidopsis*, three IIa WRKY members AtWRKY18, -40 and -60 are homologous to HvWRKY1 and HvWRKY2. They function redundantly as negative regulators in the defense towards the bacterial pathogen *Pseudomonas syringae* and the powdery mildew fungus *Golovinomyces orontii* (Pandey *et al.*, 2010; Shen *et al.*, 2007; Xu *et al.*, 2006), with AtWRKY40 playing a central role. Evolutionary conserved functions have been described for sequence-related WRKY genes between monocot and dicot plants (Mangelsen *et al.*, 2008; Prioietti *et al.*, 2011).

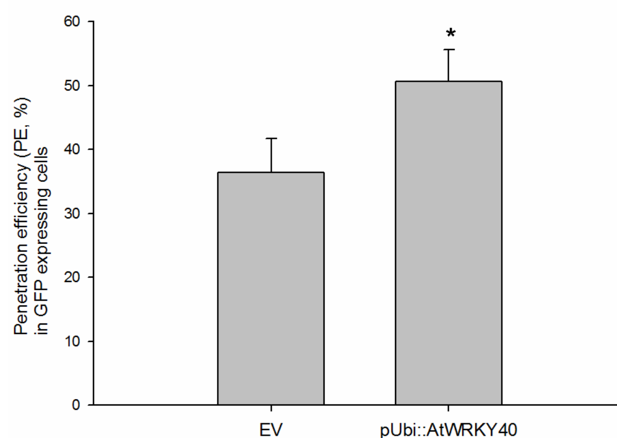

Figure 3. 16 Overexpression of *AtWRKY40* enhanced susceptibility in barley epidermal cells infected with *Blumeria graminis* f.sp. *hordei (Bgh)*. Report construct 35S::*GFP* was co-expressed pUbi::AtWRKY40 (n=3). Control cells were co-bombarded with 35S::GFP and empty vector (EV) pUbi-AB. After transformation, leaves were densely inoculated with *Bgh* conidia. Microscopic

determination of *Bgh* penetration efficiency in GFP-transformed cells was assessed 2 days post inoculation. Columns represent mean values of penetration efficiency from three independent experiments. Bars represent standard errors. Significant enhanced penetration efficiency compared to comtrol (EV) was indicated (*, $p<0.05$, Student's t-test)

To verify possible cross activities of *Arabidopsis* WRKY proteins in barley, AtWRKY40 was selected in the present study to examine its effect in barley-*Blumeria graminis* interaction. The coding sequence of *AtWRKY40* (cDNA clone provided by Imre E. Somssich, Köln) was cloned downstream of the maize ubiquitin promoter to yield *pUbi::AtWRKY40* (section 2.5, Appendix 4.4). The construct was co-bombarded with a reporter construct 35S::GFP in barley epidermal cells. Microscopical evaluation showed that the transient AtWRKY40 overexpression in barley leaves resulted in significantly higher penetration efficiency of the barley powdery mildew fungus as compared with the empty vector control pUbi-AB ($P < 0.05$, Student's t-test). The results indicated that AtWRKY40 was facilitating the *Bgh* infection similar as HvWRKY1 and HvWRKY2, whose transient overexpression enhanced susceptibility to *Bgh* in barley epidermal cells (Shen *et al.*, 2007). The cross-activity of the dicot-derived AtWRKY40 in monocot crop barley provides additional evidence supporting the evolutionary conserved functions of sequence-related WRKY genes.

3.5 Interaction of HvWRKY2 protein with *HvGER4c* promoter

3.5.1 Production of His-WRKY2 recombinant protein

To reveal the possible physical interaction of HvWRKY2 protein with *HvGER4*c promoter, recombinant HvWRKY2 protein was produced. The complete coding sequence of HvWRKY2 was sub-cloned and fused to a N-terminal thioredoxin(TRX)-*6x*His-*S*-tag (THS) in the expression vector pET32a(+) (section 2.5, Appendix 4.1). The resulted pET32a-HvWRKY2 was electroporated into *E. coli* strain BL21(DE3) pLysS. Induction of His-HvWRKY2 protein by IPTG confirmed the protein accumulation after induction at the calculated size of 52.9 kDa (Figure 3. 17, A).

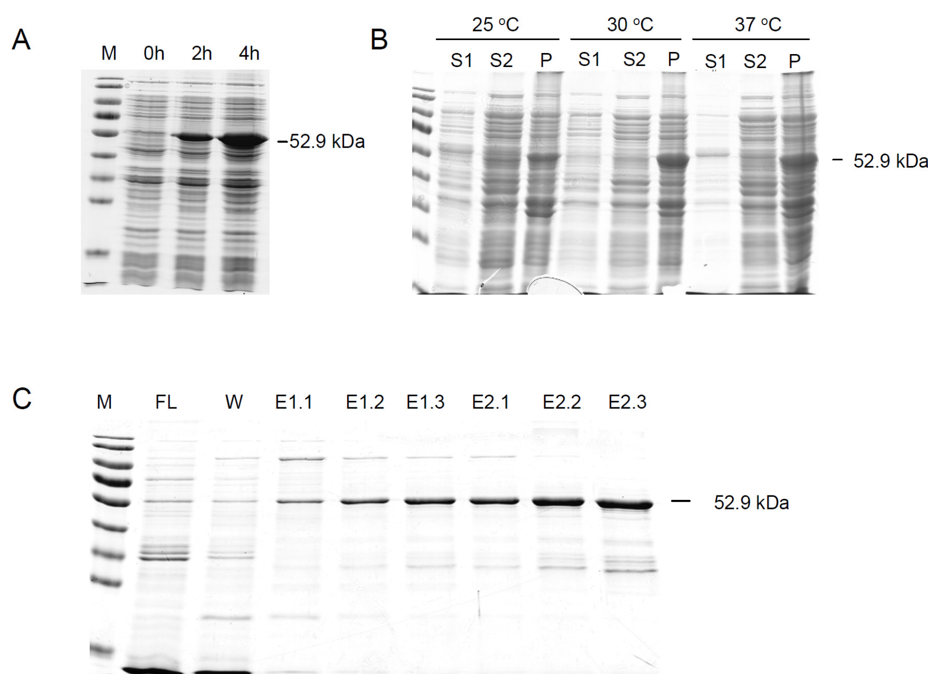

Figure 3. 17. Production of recombinant HvWRKY2 protein. **A**, Induction of the recombinant protein by IPTG. *E.coli* cells were harvested at the indicated time points after addition of 1 mM IPTG to check the abundance of HvWRKY2 recombinant protein. **B**, HvWRKY2 protein was present in inclusion bodies. Several temperatures (25 ^{o}C, 30 ^{o}C and 37 ^{o}C) were tested for bacterial culture. *E. coli* cells were harvested after IPTG induction and disrupted by TissueLyser (Qiagen) or sonication. S1, supernatant from TissueLyser, S2, supernatant from sonication, P, pellet. **C**, Purification of recombinant HvWRKY2 protein. Protein samples were purified using Ni-NTA column under denaturing condition with the presence of 8 M urea. M, protein marker; FL, flow through; W, wash; E, elution. The calculated size of Thioredoxin-6xHis-S tagged HvWRKY2 protein is shown on the right side.

As the recombinant protein was present in the form of inclusion body (Figure 3. 17, B), the purification of recombinant His-HvWRKY2 was performed under denaturing conditions (8M urea) using Ni-NTA columns (Figure 3. 17, C). Afterwards, the protein was refolded using a dilution method (Maidment *et al.*, 1999), concentrated, aliquoted, and stored at -80 ^{o}C for further use.

3.5.2 Binding of HvWRKY2 protein to *HvGER4c* promoter

To test the hypothesis on the negative regulation of *HvGER4*c promoter by HvWRKY1 and HvWRKY2 protein, electrophoretic mobility shift assay (EMSA) was performed to assess the potential binding of HvWRKY2 protein to *HvGER4*c promoter. DIG-labeled probes containing the wild-type and point-mutated W-boxes

1, 2, 5 and 6 in the proximal 543 bp of *HvGER4*c promoter were generated and tested in the EMSA assay with recombinant thioredoxin-6xHis-S (THS)-WRKY2 protein (section 2.10). From the EMSA assay, I observed that the occurrence of retarded bands is dependent on the presence of HvWRKY2 protein (Figure 3. 18). It seems promising that the band shifts are specific reactions but the specificity should be further examined based on two unexpected observations. Firstly, point mutation of TGAC to TGAA in the mutated probes mW1, mW2, mW5 and mW6 showed very similar binding with WRKY2 protein as the corresponding non-mutated probes. Secondly, the specific competitors at 50 folds excess appeared to have no competition with the W-box probes (Figure 3. 18).

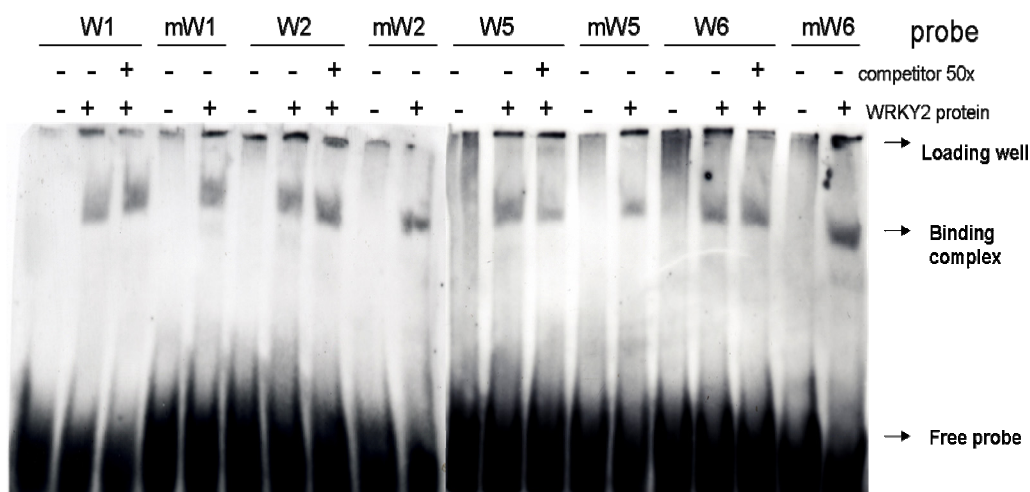

Figure 3. 18. Binding of W box elements in the *HvGER4*c promoter with HvWRKY2 protein. The presence or absence of the reaction components are indicated by + or -. Wild-type W-boxes (W1, W2, W5 and W6) and the corresponding point-mutated W-boxes (mW1, mW2, mW5 and mW6) with the TGAA replacing TGAC in the core sequences are used as probes in the electrophoretic mobility shift assay (EMSA).Recombinant Thioredoxin-His-S tagged WRKY2 protein was purified from *E.coli* cells and used for DNA binding assays with the above mentioned probes. The binding reactions (20 µL) contained 2 ng labelled oligo DNA and 2 µg recombinant protein. The non-labelled double stranded oligos were used as competitors in 50x excess amounts.

3.6 Promoter analysis of *HvWRKY1* and *-2*

3.6.1 In *silico* analysis of cis-elements in the *HvWRKY1* and *-2* promoter

Auto-regulation as a feature for WRKY factors was described as regulation of its own promoter by the WRKY protein, i.e. because of the presence of W-boxes (Miao *et al*., 2008). Another possibility is that other activated WRKY factors induce the promoter. To gain insight into the possible self/auto-regulation of *HvWRKY1*

and *-2*, I searched for the putative W-box elements in the *HvWRKY1* and *-2* promoter regions. From the recently release barley genome database, a fragment of 1.9 kb and 3 kb for *HvWRKY1* and *-2* promoter region is available. The transcription start site is predicted at position -149 (relative to start codon ATG, +1) and -111 for *HvWRKY1* and *HvWRKY2* respectively. Six and eight W-boxes are present as putative WRKY-binding sites in *HvWRKY1* and *-2* promoter (Figure 3. 19). Within the proximal 1 kb region, there are two and one stringent W-boxes. They may be conditionally occupied by HvWRKY1/2 themselves or other WRKY proteins.

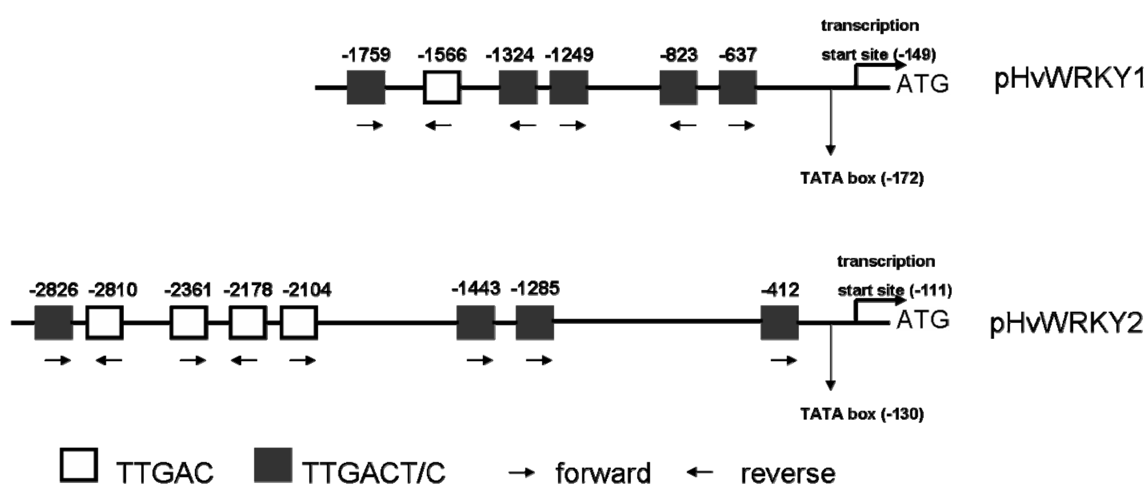

Figure 3. 19 *In silico* analysis of *HvWRKY1* and *HvWRKY2* promoter. The distribution pattern of W-box elements in 1.9 kb and 3 kb region upstream of the start codon ATG (position +1) for *HvWRKY1* and *HvWRKY2* were shown. Black boxes and white boxes indicate stringent W-boxes (TTGACT/C) and invariant core W-boxes (TTGAC) respectively. Arrows represent the orientation of W-boxes. W-box scanning was performed by PLACE.

3.6.2 Cloning of *HvWRKY1* and *HvWRKY2* promoter

Two fragments of HvWRKY1 promoter were PCR amplified from barley genomic DNA (Figure 3. 20, A). One 1940 bp fragment contains six W-box elements and the second fragment is 954 bp in length with two stringent W-boxes. These fragments were cloned upstream of GUS coding sequence in pGusi-AM and yield pWRKY1::Gusi-AM constructs. The schematic graph of the resulted construct (1940 bp) is shown in Figure 3. 20, C. One fragment of 2876 bp in length for HvWRKY2 promoter was amplified as well (Figure 3. 21, A). However, the subcloning of HvWRKY2 promoter fragment into pGusi-AM to yield

pWRKY2::Gusi-AM failed (Figure 3. 21, B). These constructs can be used as tools for uncovering the potential auto/cross-regulation of WRKY proteins.

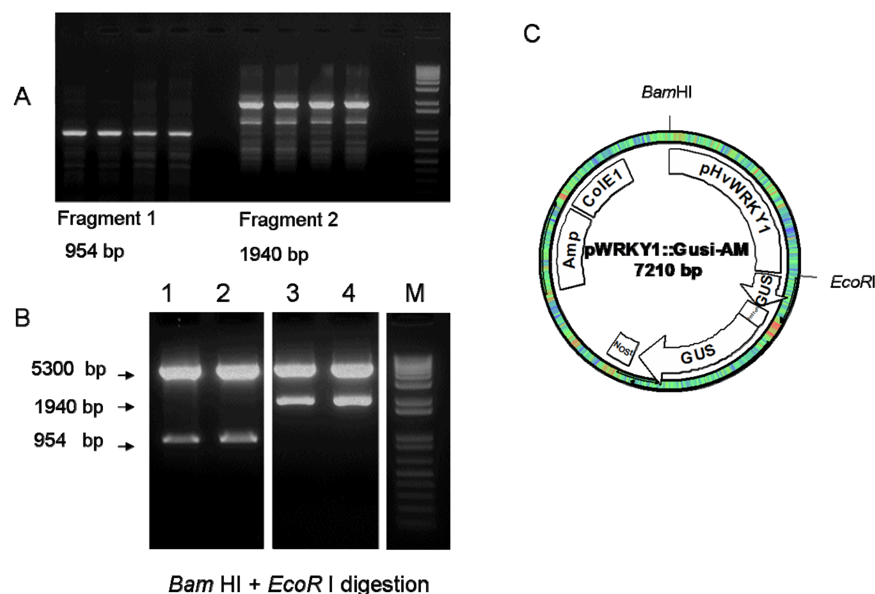

Figure 3. 20. Cloning of HvWRKY1 promoter. **A**, PCR amplification of 954-bp and 1940-bp fragment of HvWRKY1 promoter. **B**,Double digestion of pW1::Gusi-AM with *Bam*HI and *Eco*RI. 1-2, pW1(954 bp); 3-4, pW1 (1940bp), M, 1 kb plus DNA ladder .C, Schematic representation of pWRKY1::Gusi-AM construct (1940 bp promoter fragment).

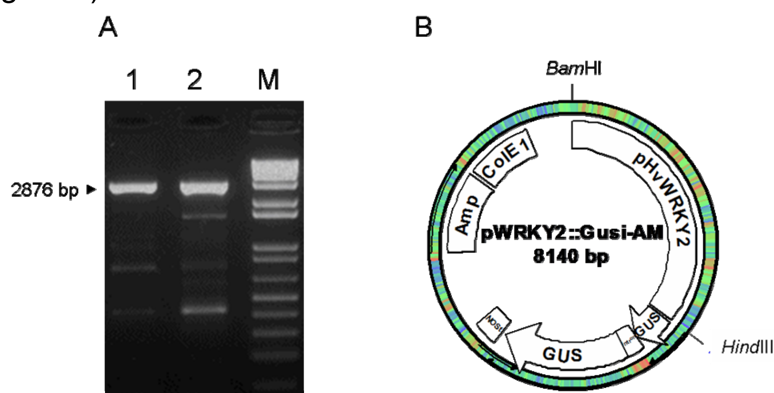

Figure 3. 21. Cloning of HvWRKY2 promoter. **A**, PCR amplification of 2876-bp fragment of HvWRKY2 promoter. M, 1 kb plus DNA ladder. **B**, Schematic representation of pWRKY2::Gusi-AM construct.

3.7 Analysis of HvWRKY2 overexpression (OE) lines

3.7.1 Transgene identification

Stable transgenic barley plants overexpressing HvWRKY2 under the strong maize ubiquitin promoter (pUbi::WRKY2) were generated and propagated at IPK Gartesleben. The F2 generation of pUbi::WRKY2 plants available was a segregating population and thus a reliable selection of the real transgenic

individuals was a prerequisite for further analysis. I optimized a leaf tip selection method based on the presence of hygromycin resistance marker gene (section 2.2).

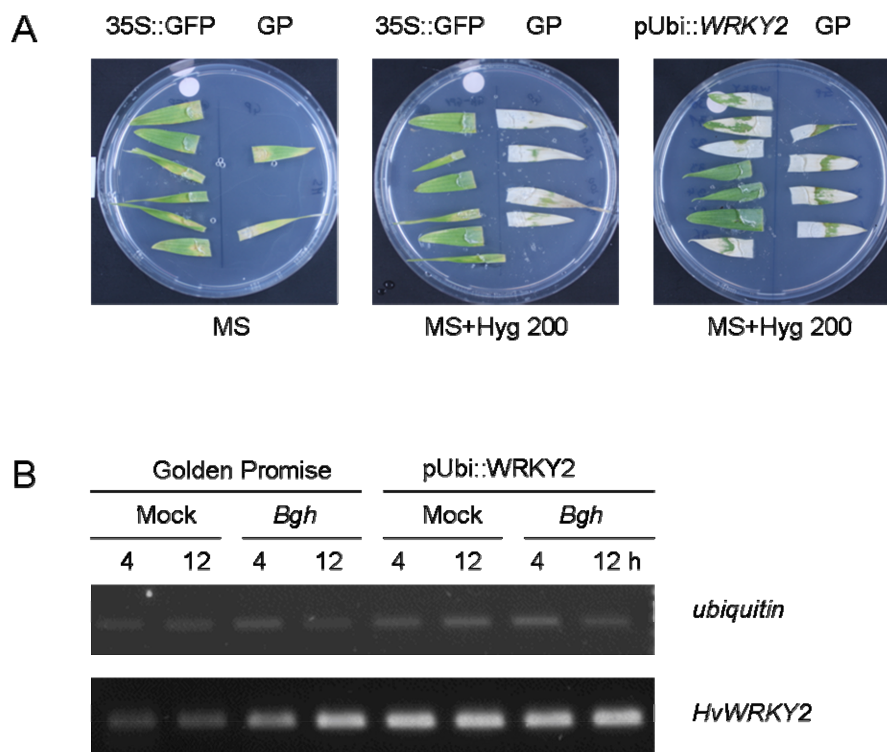

Figure 3. 22. Transgene identification of pUbi::*WRKY2* plants. **A**, Selection of transgenic plants based on the hygromycin-resistance. Leaf segments from the wild-type cv.Golden Promise (GP), 35S::GFP (hygromycin-resistant, in the background of GP) and pUbi::WRKY2 plants (in the background of GP) placed on MS medium with or without 200 mg/L hygromycin for 7 days are shown. **B**, Elevated background expression of *HvWRKY2* in pUbi::*WRKY2* plants. Total RNA was isolated from mock or *Bgh*-inoculated barley leaves that were harvested from one-week-old Golden Promise and hygromycin-resistant pUbi::WRKY2 plants (line E40b, F2 generation) at indicated time points. Gel photographs after ethidium bromide staining of two-step reverse-transcription polymerase chain reaction (RT-PCR) products are shown. Amplification of *ubiquitin* served as control for constitutive gene expression.

As shown in Figure 3. 22, the leaves of homozygous 35S::GFP exhibited a complete resistance to 200 mg/L hygromycin and the wild-type Golden Promise (GP) are bleaching in the hygromycin-containing medium. Some pUbi::WRKY2 plants stayed green and alive and the others showed complete bleaching as wild-type, indicating the reliability of the selection method. In addition, the hygromycin-resistant individuals were used to compare HvWRKY2 expression levels. Indeed, the pUbi::WRKY2 transgenic plants (line E40b, F2 generation) are showing an

elevated transcript level of HvWRKY2 as compared with Golden Promise under unchallenged condition (Figure 3. 22, B).

3.7.2 Powdery mildew resistance of *pUbi::WRKY2* plants

Transient overexpression of HvWRKY2 in barley epidermal cells conferred enhanced susceptibility to *Blumeria graminis*. f.sp *hordei* (*Bgh*) (Leib, 2005; Shen *et al.*, 2007). To characterize the powdery mildew resistance of stable transgenic pUbi::WRKY2 plants, they were inoculated with *Bgh* strain A6 (section 2.1). Compared with azygous plants and wild-type plants, no significant increase of fungal growth was observed on pUbi::WRKY2 plants based on ten biological repetitions (Figure 3. 23). In contrast, the mean number of pustules on WRKY2 overexpressing plants is slightly reduced compared with control plants. Thus HvWRKY2-mediated susceptibility was not observed on the stable pUbi::WRKY2 overexpression plants. Moreover, microscopic evaluation of *Bgh* penetration efficiency showed no difference between the pUbi::WRKY2 plants and azygous plants or the wild-type Golden Promise (Figure 3. 24).

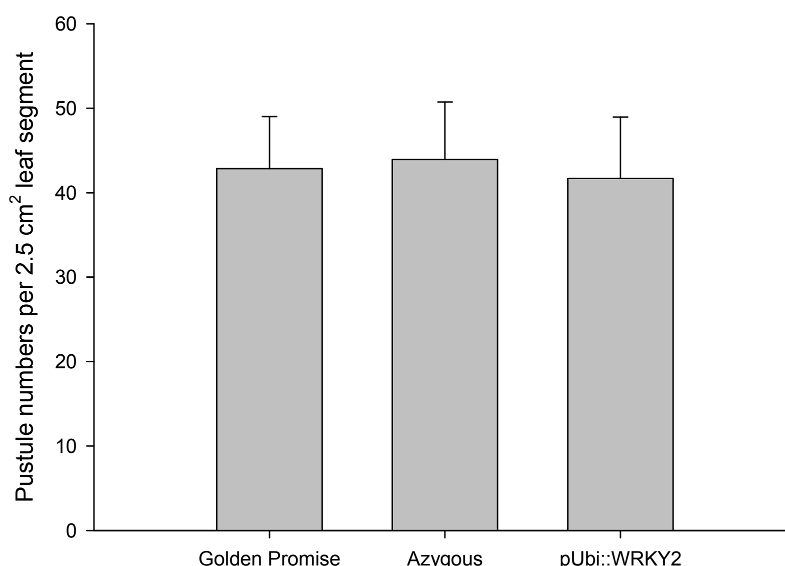

Figure 3. 23. Non-altered response of pUbi::WRKY2 plants against *Blumeria graminis* f.sp. *hordei* (*Bgh*). Primary leaves from one-week-old plants were inoculated with *Bgh*. The second leaves from the segregating pUbi::WRKY2 plants were used in the hygromycin-selection for identification of transgenic individuals. Columns are mean values from ten independent experiments. In each experiment, at least 15 leaves were used for each line. Errors bars represent standard error. No significant difference was observed between the *HvWRKY2* overexpression lines, Golden Promise and azygous plants.

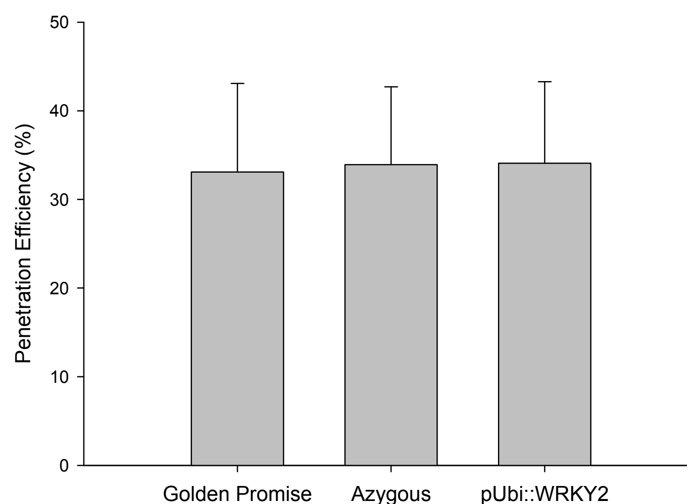

Figure 3. 24. Penetration efficiency of *Blumeria graminis* f.sp. *hordei* (*Bgh*) in Golden Promise, azygous plants and 35S::WRKY2 plants. Columns are mean value from three independent experiments. Errors bars represent standard error. In total, more than 500 interaction sites for each sample were included for the determination of penetration efficiency.

3.7.3 Candidate gene expression in *HvWRKY2* overexpression plants

In this study, *HvWRKY2* overexpression was confirmed in transgenic plants but they did not show expected susceptibility which was observed in single-cell transient transformations (Leib *et al.*, 2005; Shen *et al.*, 2007). To provide insight into the molecular mechanisms and putative altered gene expression in *HvWRKY2* OE plants, expression analysis of some *Bgh*-inducible genes was performed by quantitative real-time PCR (qRT-PCR) (section 2.4). The candidate genes IGS (contig10115), synaptotagmin (contig24167), HvPR2 (contig01637) and HvPR5 (contig02787) were selected from a preliminary microarray experiment (Langen, unpublished). IGS, HvPR2 and HvPR5 (thaumatin-like proteins) appeared to have reduced expression at 12 h post *Bgh* inoculation in pUbi::WRKY2 plants (Figure 3. 25). Especially in the case of PR5, the expression is much lower in pUbi::WRKY2 plants in comparison with Golden Promise at 12 h post inoculation. Synaptotagmin showed minor changes between mock treatment and *Bgh* inoculation. It also showed little difference between wild-type and pUbi::WRKY2 plants. Interestingly, there was an early induction of PR2 expression at 4 hours post inoculation in *HvWRKY2* transgenic plants (Figure 3. 25). It seems that HvWRKY2 has a dual role in both activation and repression of target genes.

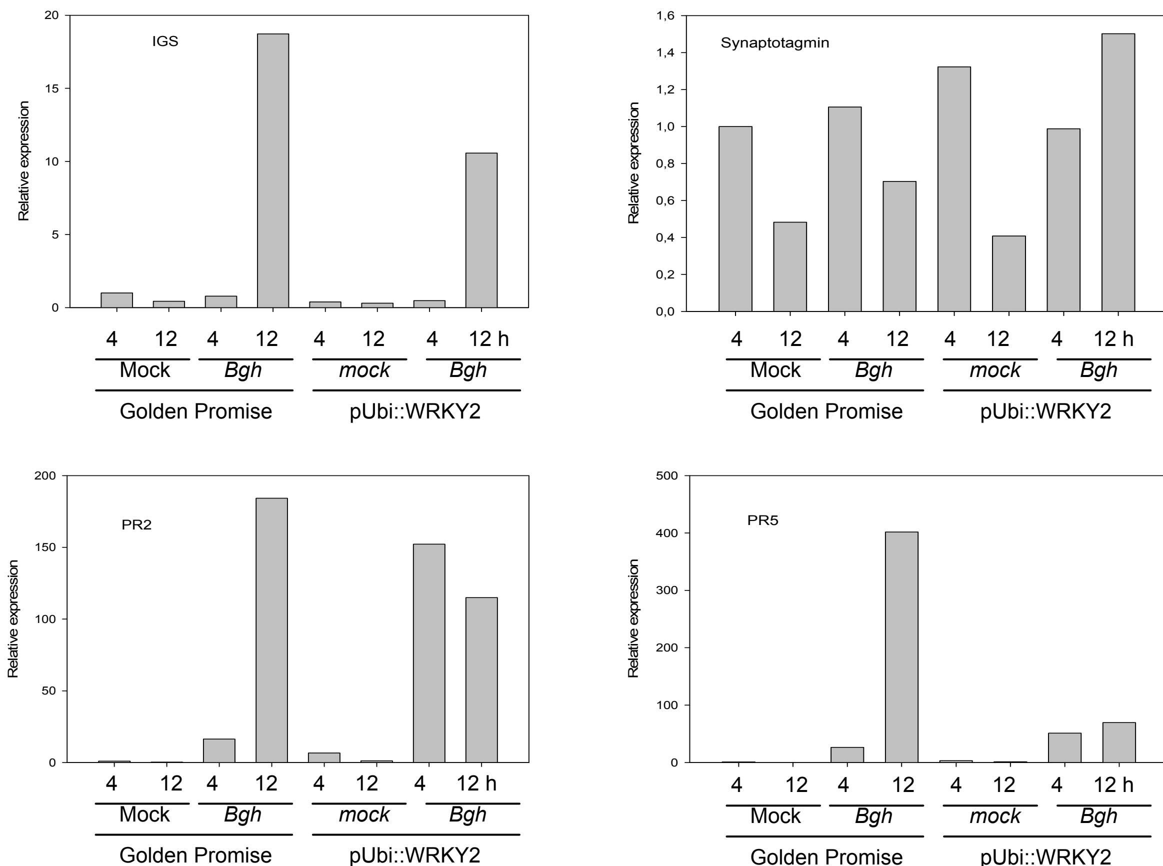

Figure 3. 25. Expression of some candidate genes. Total RNA was isolated from mock or *Bgh*-inoculated leaf samples in the indicated time points. Four candidate genes were selected based on existing microarray expression profile (*IGS*, synaptotagmin, *HvPR2* and *HvPR5*). Quantitative real-time PCR was performed for analysis of the expression. The relative expressions normalized to ubiquitin are shown in the plots (The expression value of Golden Promise mock 4 h was set to 1).

Expression of some other genes including *BAX Inhibitor 1, PR1b, Xylanase inbibitor* and *NH1* were also examined in pUbi::WRKY2 plants. Interestingly, BAX Inhibitor 1 appeared to have higher expression in WRKY2 overexpression plants. Moreover, the early induction of HvPR2 in pUbi::WRKY2 plants observed by quantitative RT-PCR was reproducible in the semiquantitative RT-PCR (Figure 3. 26, A). PR1b displayed no difference between wild-type and WRKY2 transgenic plants. Xylanase inhibitor showed little alteration, however, NH1 expression appears to be slightly enhanced in pUbi::WRKY2 plants.

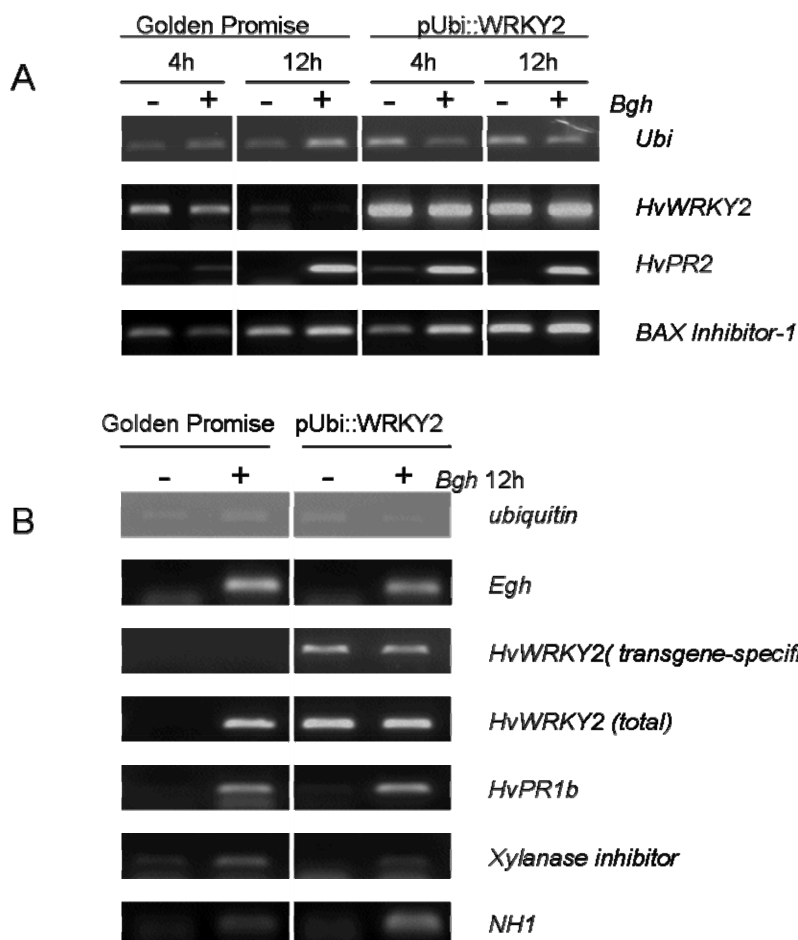

Figure 3. 26. Expression of candidate genes by RT-PCR. **A**, Early induction of *HvPR2* and enhanced expression of *BAX Inhibitor 1* in pUbi::WRKY2 plants. **B**, Expression of *PR1b*, *xylanase inhibitor* and Hv*NH1* (NPR1-homolog1) in Golden Promise and pUbi::WRKY2 plants. PR1b (Genebank: Z21494), xylanase inhibitor (contig04633), HvNH1 (Genebank: AM050559)

3.8 Suggestion of candidate barley WRKY genes for pathogen resistance

Expression profil from publicly available microarry database is a useful tool for gene function prediction. To search for expression data for barley WRKY genes and suggest further candidate WRKYs with a potential role in pathogen responses, I used the cDNA sequences of the putative WRKYs to search for their corresponding probe sets on Barley1 Chip (22k). These probeset IDs were identified from PLEXDB and are listed in Table 3. 3. There are altogether 32 WRKY genes with at least one probeset on Barley1 Chip.

Table 3. 3. List of probes for barley WRKY on Barley1 Chip

HvWRKY	**Barley1 Chip Probeset**	**HvWRKY**	**Barley1 Chip Probeset**
HvWRKY1	Contig4386_at	HvWRKY33	Contig15869_at
HvWRKY2	Contig4387_at	HvWRKY34	Contig10471_at
HvWRKY4	Contig20450_at	HvWRKY35/41	Contig12033_at
HvWRKY5	Contig18462_at	HvWRKY39	Contig13375_at
HvWRKY6	Contig14308_at		Contig15657_at
HvWRKY7	Contig7798_at	HvWRKY45	HVSMEa0001M23r2_at
HvWRKY8	Contig23011_at	HvWRKY46	Contig7243_at RBAAL15J13_S_AT
HvWRKY9	Contig22226_at	HvWRKY51	Contig23697_at
HvWRKY10	Contig16040_at	HvWRKY54	EBro02_SQ004_H10_at
HvWRKY13	Contig13268_at	HvWRKY56	EBro08_SQ008_D15_at
HvWRKY19	Contig10167_at	HvWRKY57	Contig7517_at
HvWRKY20	Contig10168_at	HvWRKY60	HF06A04r_at
HvWRKY23	HB25K10r_s_at	HvWRKY67	EBro03_SQ006_A01_at
HvWRKY28	Contig12005_at	HvWRKY69	S0001000058B11F1_at
HvWRKY30	Contig21110_at	HvWRKY79	HVSMEn0012L05r2_at
HvWRKY32	Contig15957_at, S0001000055P18F1_S_AT	HvWRKY87	Contig20358_at

The probesets listed above is an updated list. It is notable that the previous report from Mangelsen *et al.*, (2008) incorrectly assigned the following probesets, HB25K10r_s_at (*HvWRKY2*, should be *HvWRKY23*), contig21110_at (*HvWRKY23*, should be *HvWRKY30*), contig12005 (*HvWRKY30*, should be *HvWRKY28*), EBro02_SQ004_H10_at (*HvWRKY3*, should be *HvWRKY54*).

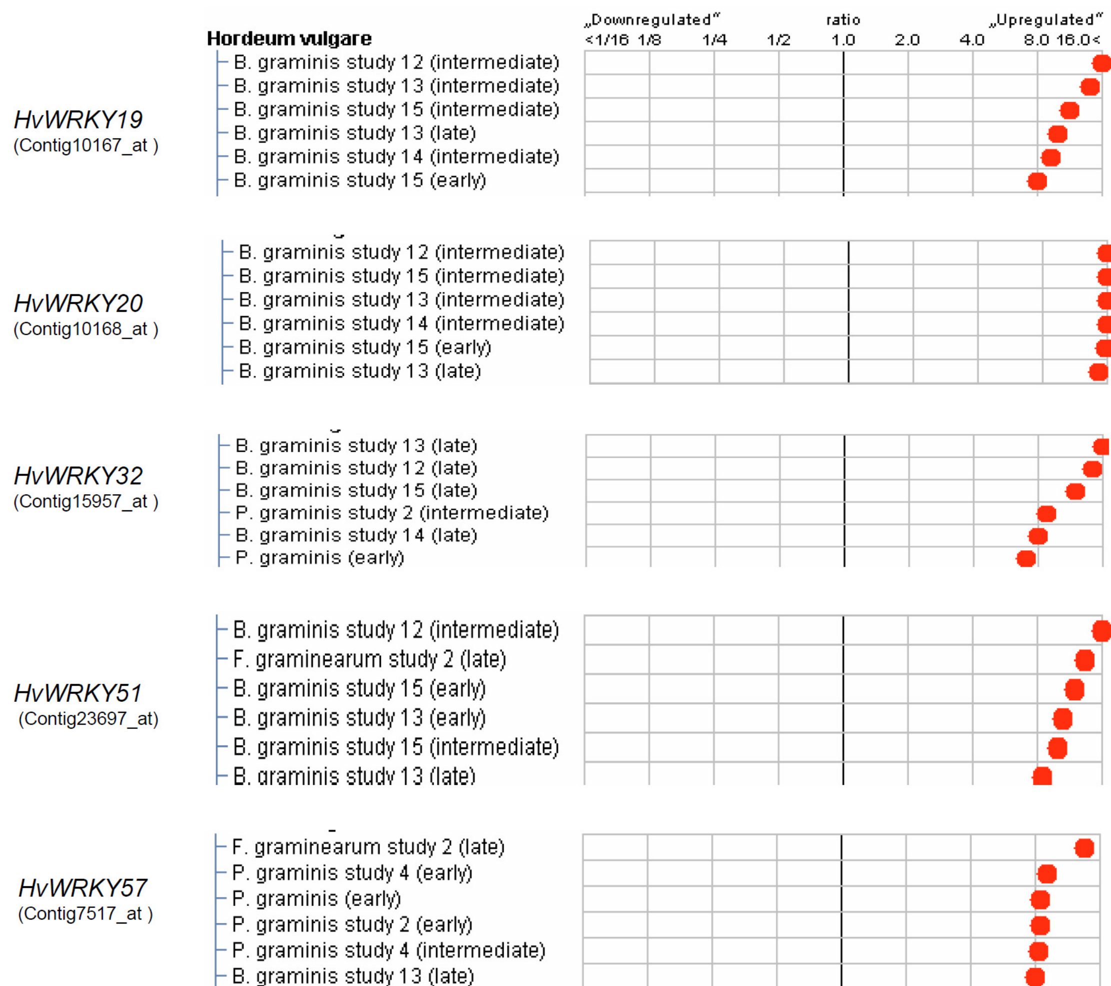

Figure 3. 27. Expression of *HvWRKY19, HvWRKY20*, *HvWRKY32*, *HvWRKY51* and *HvWRKY57* after pathogen infection. Probeset ID on Barley1 Chip was obtained from PLEXDB and used as query for expression data in Genevestigator. Strong upregulation (over eight folds induction relative to control) of the selected genes by pathogen are shown.

Based on their expression data in Genevestigator, many of these WRKYs were found to be up-regulated or down-regulated under biotic stresses. *HvWRKY20* is specifically responsive to *B. graminis* attack through the early and late infection stage. *HvWRKY19* and *HvWRKY51* showed very strong responsiveness to *B. graminis* at intermediate phase and to *Fusarium graminearum* at late phase of infection (Figure 3. 27). *HvWRKY57* can be induced by both *B. graminis* and *F. graminearum* at the late stage of infection. In addition, *HvWRKY57* is up-regulated by the stem rust fungus *Puccinia graminis* over eight folds at early and intermediate time points (Figure 3. 27).

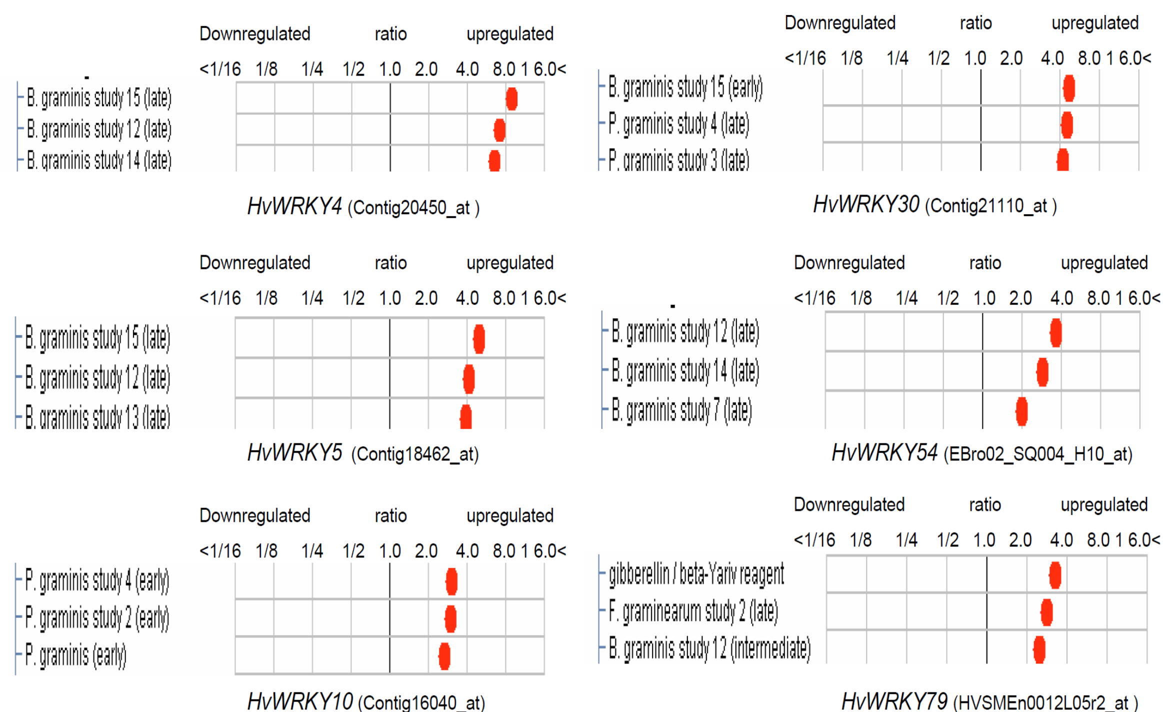

Figure 3. 28. Expression of *HvWRKY4, HvWRKY5, HvWRKY10, HvWRKY28, HvWRKY30, HvWRKY54* and *HvWRKY79* after pathogen infection. Probeset ID on Barley1 Chip was obtained from PLEXDB and used as query for expression data in Genevestigator. Upregulation of the selected genes by pathogen to a less extent (two to eight folds induction) are shown.

Apart from *HvWRKY1* and *-2*, other barley WRKY genes that are less strongly responsive to pathogen attack include *HvWRKY4, HvWRKY5, HvWRKY10, HvWRKY28, HvWRKY54* and *HvWRKY79* (Figure 3. 28). They are induced by pathogens within a range between two to eight folds. Among them, *HvWRKY10* is responsive to *P. graminis* at early stage. *HvWRKY4, HvWRKY5, HvWRKY28* and *HvWRKY54* are induced by *B. graminis* at late infection stage. *HvWRKY30* is up-regulated by *B. graminis* at early stage and *P. graminis* at late stage. *HvWRKY79* is responsive to both *F. graminearum* and *B. graminis*. Analysis of publicly available expression data is restricted to the WRKYs which are present on the 22k Barley 1 microarrays. However, majorities (68 members) of the barley WRKY genes are not represented on the Barley1 Chip. Many of them might also be interesting candidate genes involved in stress response or pathogen resistance.

4. Discussion

4.1 Identification and annotation of WRKY transcription factors in barley

Due to the economic importance of barley crops and the central role of WRKY transcription factors in plant development and stress responses, the function characterization of barley WRKY genes would facilitate the understanding of barley metabolic pathways and application of the knowledge to make plants better in coping with environmental changes and pathogen attack. Genome-wide discovery of WRKY genes in barley and phylogenetic analysis will provide valuable information for the origin and expansion of the superfamily. Recently, the public availability of a large-scale full-length cDNA set and the barley genome draft assembly opens the door to the comprehensive search for WRKY genes in barley (Matsumoto *et al*., 2011; Mayer *et al*., 2011).

In this study, I describe the identification and annotation of 100 members of the WRKY gene family in barley and their classification into group I to III (Table 3.1; Figure 3.3). Around two thirds of them (68 %) are supported by full-length cDNA or EST sequences as expressed genes (Table 3.1). There is no match in the dbEST for the rest 32 predicted WRKY genes. There are several possible reasons. First of all, the dbEST for barley is not saturated yet. It's not unusual that some genes can not be found in the dbEST data set. Secondly, some WRKY genes might only express in a highly temporal and spatial manner or at a hardly detectable level. Last but not least, it's also possible that GENSCAN/FGENESH generate mispredictions from the genomic sequence. Some of these genes could be only pseudogenes that contain WRKY domain sequence in the genomic region. Thus, these 32 putative WRKYs require further experimental verification to confirm the sequence prediction and their expression.

From comparative analysis of WRKY genes in lower and higher plants, it has been demonstrated that there is an expansion of WRKY members during the evolution of plants from simpler, unicellular to more complex, multicellular forms (Zhang and Wang, 2005). In the unicellular green algae (*Chlamydomonas reinhardtii)*, there is only one WRKY protein present in the genome. The number increases to 37 in moss (*Physcomitrella patens)* and nearly 200 in soybean *(Glycine max)* (Rushton *et al*., 2010). In the genome of the japonica group rice (*Oryza sativa* ssp. *japonica*),

the number of WRKY proteins is 102 according to the latest annotation (source: http://systemsbiology.usm.edu/PhytoTech/WRKY07012011/Rice.html). Barley has a much bigger genome than rice and the estimated gene numbers in barley is around 32, 000 which is less than that of rice (41,000) (Jung *et al*., 2008; Mayer *et al*., 2010). If the WRKY proteins in barley have the same richness and gene duplications as in rice, the calculated barley WRKY numbers would be around 80 (80 = 32,000x102/41,000). In an earlier study, 45 members of barley WRKY are identified and they were estimated to cover about 50% of the WRKY family (Mangelsen *et al*., 2008).I have identified 100 members in this family and there might be an overestimation due to the possible presence of pseudogenes. A precise identification may require a better assembly in future. In other monocot plants, the number of putative WRKY genes is close to barley. For instance, there are 91 putative WRKY genes identified in sorghum (*Sorghum bicolor*) and 127 putative WRKY genes in maize (*Zea mays*).

Among the 100 barley WRKY genes identified, both the hallmark WRKYGQK motif and its variants were identified in the WRKY domains. Like in other higher plants, WRKYGQK signature is the dominant motif (97 found out of 114 domains) in barley WRKY proteins (Appendix 2.1). The WRKY motif variants include 4 WRKYGEK, 8 WRKYGKK, 2 WKKYGQK, 1 WSKYGQM, 1 WTKYGQK and 1 WNKYGQK (Appendix 2.1). Notably, the WRKYGEK and WKKYGQK motif are absent in the dicot model *Arabidopsis thaliana* but present in all the monocot plants surveyed (data not shown). The motif WSKYGQM (*HvWRKY24*), WTKYGQK (*HvWRKY89*) and WNKYGQK (*HvWRKY25*) were solely found in barley not in other plant species. These unusual WRKY signatures could be resulted from sequencing errors or misprediction. Recent study indicated that *Arabidopsis* WRKY proteins with WRKYGQK motif exhibit binding site preferences, which are partly influenced by the adjacent DNA sequences spanning the TTGACY-core motif (Ciolkowski *et al*., 2008). WRKY proteins with signature motif other than the canonical WRKYGQK sequence, therefore, may prefer a binding sequence different from W-box element (TTGAC/T). For instance, the soybean WRKYGKK-containing GmWRKY6 and GmWRKY21 lose their binding capability to W-box (Zhou *et al*., 2008). In tobacco (*Nicotiana tabacum*), NtWRKY12 with a WRKYGKK motif is recognizing the WK box (TTTTCCAC), a DNA element distinct

from W-box (van Verk *et al*., 2008). Seven group IIc barley WRKYs (HvWRKY5, --16, -17, -18, -19, -20, -50 and one group I (HvWRKY66N) harbouring the WRKYGKK motif might be able to bind to WK boxes and may have different target genes.

The WRKYGQK motif is found in all the WRKY-containing species including the unicellular green algae *Chlamydomonas reinhardtii* and the slime mold *Dictyostelium discoideum* (Table 1.1). This demonstrates the ancient origin of this motif. The WRKYGEK motif also appears to have an ancient origin due to its presence in the genome of *Chlamydomonas reinhardtii* (Table 1.1; Figure 3.5), which is a single-celled green algae and its evolutionary position is located before the divergence of land plants. Interestingly, this motif is absent in spikemoss *(Selaginella moellendorffii, Sm)* and moss (*Physcomitrella patens, Pp)* but present in all the monocot plants (Figure 3.5).

4.2 Phylogenetic analysis of barley WRKY family

Phylogenetic tree constructed for barley WRKY family using the conserved 60 aa WRKY domains assigns most of the HvWRKYs ambiguously into the predefined seven subgroups I-N, I-C, IIa, IIb, IIc, IId, IIe and III (Figure 3.2). However, HvWRKY36, HvWRKY71 and HvWRKY81 couldn't be clearly assigned to any of the seven subgroups (Figure 3.2). In the study by Mangelsen *et al*. (2008), grouping of HvWRKY36 in group IIc was based on the analysis with sequences extending over the WRKY domain. Using the full-length sequence for a BLASTP search, the most related AtWRKYs identified for HvWRKY36, HvWRKY71 and HvWRKY81 are AtWRKY13, AtWRKY13 and AtWRKY71 respectively. All these AtWRKY members belong to IIc subfamily. Therefore, I also assign HvWRKY36, HvWRKY71 and HvWRKY81 as group IIc members based on the full-length sequence similarity (Appendix 2.1). However, this classification might not necessarily reflect the evolutionary relationships among the groups. From the evolutionary tree constructed, HvWRKY36, HvWRKY71 and HvWRKY81 appeared more close to the group IId+IIe (Figure 3.3).

For group I HvWRKYs bearing two WRKY domains, the phylogenetic analysis clearly distinguishes the N-terminal and C-terminal WRKY domains (Figure 3.2; Appendix 2.2). This clear distinction rules out the possibility that group I proteins originate from recent fusion or duplication of two single-domain WRKY proteins but

indicates a more ancient fusion event. Exceptions from group I members are HvWRKY24 and HvWRKY25, which are placed in group III by phylogenetic analyses (Appendix 2.2). Since both the N-terminal and C-terminal of these two group I WRKYs are clustered in group III, they may result from a recent fusion event of two group III domains. Similar situation was found for OsWRKY41, with both N-terminal and C-terminal WRKY domain clustered in group III. In addition, OsWRKY57 and OsWRKY102 which have a single WRKY-domain are clustered with members in group IN. This might indicate a loss of the C-terminal domain during their evolution.

It was believed that group III WRKYs have most successfully evolved in flowering plants and are greatly expanded in monocot plants (Zhang and Wang, 2005). Therefore, group III members are suggested to be the most advanced and recent in evolution. However, the discovery of group III WRKYs in the genome of moss (*Physcomitrella patens)* is challenging this concept. The presence of group III WRKY members in the moss genome implied its ancient origin (Figure 3.7). Based on comparative genomic observation, I examined the distribution pattern of PpWRKYs and SmWRKYs in the predefined subgroups. It appeared that PpWRKY or SmWRKY are clustering with six out of seven WRKY subfamilies. The only group that lacks a PpWRKY or SmWRKY member is group IIa (Figure 3.8). The absence of group IIa members in moss and spikemoss genome clearly indicate that WRKY IIa was the last group evolved (Figure 3.8). Notably, the WRKY superfamilies are under constant diversification and caution should be taken when comparing the evolution of individual WRKY members from different subgroups. For instance, certain WRKY members such as HvWRKY24 and OsWRKY41 from the most ancient group I may have evolved very recently due to domain duplication or fusion of two individual WRKYs.

I observed a great expansion of group III WRKYGEK members in monocot plants (Figure 3.2). These members are found in all monocot plant species surveyed but absent from most of the dicot genomes (Figure 3.2). To confirm its unique feature for monocot plant species, BLASTP search was performed using the WRKYGEK signature as query. WRKY proteins with this non-canonical motif were found in the genome of a few dicot plants as well. As example, one in *Lotus japonicus*, three in soybean (*Glycine max*), one in sunflower (*Helianthus Annuus*) and one in tobacco

(*Nicotiana tabacum*) (data not shown). However, none of these dicot-derived WRKYGEK members could be classified into group III (Figure 3.6). On the other hand, most of the WRKYGEK members from monocots are clustered in group III and distant from dicots-derived group III members (Figure 3.6). The expansion of WRKYGEK-containing WRKY members in group III appeared to occur exclusively in monocot plants. The divergence of this monocot-specific group is therefore postulated to happen after the split of dicot and monocot lineage. Though it is not known how these unique members have been evolved, I speculate they resulted from a recent mutation event in group III members. Based on two reasons, it is less likely that they evolved from the ancient WRKYGEK-motif members such as the one found in N-terminal WRKY domain of *Chlamydomonas reinhardtii*. Firstly, the other residues in the ancient WRKYGEK domain shared low sequence similarities with the WRKYGEK-containing members in monocots. Secondly, WRKYGEK domains were completely absent in the spikemoss (*Selaginella moellendorffii)*, moss (*Physcomitrella patens*) and dicot plants which were considered to have earlier origin than monocot plants. Likewise, species-specific WKKY domain was reported recently for *Asteraceae* (Giacomelli *et al*., 2010). The WKKY group contains a WKKYGEK motif and was mainly found in *Asteraceae* plants such as sunflower (*Helianthus spp*) and lettuce (*Lactuca spp*). Though a few WRKY proteins with the WKKYGEK motif were found in genomes of *Sorghum bicolour*, *Zea mays*, *Oryza sativa* and *Vitis vinifera*, they contain the WKKY amino acid residues but not the additional conserved sequences. I also found a WRKY member containing WKKYGEK sequence in the genome of *Medicago truncatula*, however, phylogenetic analysis placed it distantly from the WKKY group in *Asteraceae*. The presence of monocotylenoneae-specific WRKYGEK group and Asteraceae-specific WKKYGEK group strongly indicates a general diversification of functions associated with distinct WRKY proteins. Functional characterizations of these proteins would enable researchers to better understand the diversification events.

4.3 Role of WRKY transcription factors in plant immunity

WRKY transcription factors have been extensively studied in the aspects of senescence, development, abiotic stresses and plant immunity (Pandey and

Somssich, 2009; Rushton *et al*., 2010). Some barley WRKY transcription factors have been shown to involve in starch synthesis, drought and cold tolerance, and innate immunity (Eckey *et al*., 2004; Mare *et al*., 2004; Sun *et al*., 2003; Xiong *et al*., 2010). *HvWRKY1* and *HvWRKY2* were identified as the targets of effector-activated R protein MLA (Shen *et al*., 2007). The interaction between the resistance protein MLA10 and HvWRKY1/2 is dependent on the recognition of a corresponding fungal-derived avirulent protein AVR10. Following the activation by AVR10, a portion of cytoplasm-localized MLA10 protein translocates into the plant nucleus and physically interacts with HvWRKY1/2. Transient virus-induced silencing of *HvWRKY1/2* enhanced resistance towards *Blumeria graminis* f .sp. *hordei* and overexpression of HvWRKY1/2 facilitate the fungal penetration. Thus they are regarded as negative regulators in basal defense. The excellent work from Shen *et al*., (2007) provided a link between basal defense and race-specific defense via HvWRKY1/2.

However, there are some questions remaining to be answered. The concept that HvWRKY1/2 act as negative regulators in basal defense towards *Blumeria graminis* is based solely on transient expression assays. This has to be verified in stable transgenic plants. In addition, the target promoters of HvWRKY1/2 and downstream elements are largely unknown regardless of compatible or incompatible interactions.

Analysis of *HvWRKY1* and *-2* gene structure demonstrated the presence of one intron in each gene (Figure 3.11). The intron size for *HvWRKY1* and *-2* is the same and the intron position is conserved similar as described previously for many WRKY proteins in other species such as rice (Zhang and Wang, 2005). Both the *HvWRKY1* and *HvWRKY2* gene expressions are inducible by powdery mildew fungus according to the microarray data (Figure 3.10) and previous reports (Eckey *et al*., 2004; Leib, 2005; Shen *et al*., 2007). Such a pathogen-inducibility is likely attributed to the cis-elements (i.e, W-boxes) in their promoter sequences (Figure 3.19). For instance, three tandem W-box elements in OsWRKY53 promoter were essential to the elicitor-responsiveness (Chujo *et al*., 2009). Cloning of the promoters might facilitate the functional analysis of these cis-acting regulatory DNA elements. In addition, the fusion constructs of WRKY promoter and reporter

gene can be a useful tool to uncover the auto- or cross-regulation of HvWRKY1 and HvWRKY2 proteins (Figure 3.21).

Based on a recently available silencing tool using artificial microRNA (amiR), I was able to discover that *HvWRKY2* silencing enhanced the resistance to *Blumeria graminis* in barley epidermal cells (Figure 3.12). The result is consistent with the previous findings using dsRNAi or virus-induced gene silencing (VIGS) (Leib, 2005; Shen *et al*., 2007). It is notable that the amiR-based silencing of *HvWRKY2* is a good proof of concept for the further use of the silencing system in cereals especially in barley. The rice microRNA precursor osa-MIR528 was used to generate the microRNA silencing construct in barley. As a first report, the successful application of artificial microRNA in *HvWRKY2* silencing confirms the conservation of microRNA processing machinery between rice and barley, which are believed to have colinearity in the genome. amiR-based gene silencing has been successfully used in a number of plants including *Arabidopsis*, rice, moss and green algae (Khraiwesh *et al.*, 2008; Molnar *et al.*, 2009; Schwab *et al.*, 2006; Warthmann *et al.*, 2008). Compared to the traditional RNAi approach using long dsRNA, amiR approach overcomes the self-silencing problems and promises to be more specific in the gene targeting (Molnar *et al*., 2009). Thus, amiR approach can be used as a high-throughput and high specific silencing system. In this study, the amiR-WRKY2 fragment was placed downstream of GFP coding sequence (Figure 3.12). Such a design combines the reporter gene and the silencing fragment in the same construct and allows simutaneous and precise monitoring of targeted cells compared with the traditional two-plasmids co-expression approach. However, there might be some drawbacks. Because the expression of amiR-WRKY2 could initiate the inhibition of the upstream GFP transcription, those cells highly expressing amiR-WRKY2 thus may have very weak green fluorescence.

To verify the enhanced susceptibility due to over-expression of *HvWRKY2*, transgenic barley lines were generated (Langen, unpublished). Under my tested conditions, barley lines constitutively expressing *HvWRKY2* under the control of strong maize ubiquitin promoter showed no alteration in growth behaviour or plant morphology compared with the wild type Golden Promise and azygous plants. Surprisingly, the stable transgenic plants also showed no altered resistance towards *Blumeria graminis* f.sp. *hordei* (*Bgh*) compared with control plants (Figure

3.23; Figure 3.24). This is an unexpected result as the transient overexpression of *HvWRKY2* resulted in enhanced penetration of the fungus (Leib, 2005; Shen *et al*., 2007). I checked the construct sequence which was used for barley transformation. There was no sequence error or frame shift in the coding region. The transgenic population can be clearly selected on hygromycin-containing medium and they indeed proofed to have elevated transcript levels of *HvWRKY2* under unchallenged condition (Figure 3.22). Notably, the total *HvWRKY2* expression level 12 h after *Bgh* infection is very close to each other in control plants and overexpression lines (Fig. 3.22, B). In this regards, the endogenous *HvWRKY2* expression was negatively regulated by the overrepresented HvWRKY2 protein in the transgenic plants. Therefore, it is interesting to analyse the promoter of *HvWRKY2* and examine the potential self-regulation behaviour *in vitro* and *in vivo*.

4.4 Targets of WRKY transcription factors

As demonstrated previously, WRKY proteins have a binding preference for the consensus sequence TTGACC/T (W-box) (Eulgem *et al*., 2000). Those genes with enriched W-box sequences in the promoter region are considered as potential targets for WRKY proteins. For example, wheat TaWRKY78 was shown to target at PR4 gene by binding directly to the W-box element in the promoter (Proietti *et al*., 2010). In barley, the germin-like protein HvGER4c plays a positive role in the basal defense against *Blumeria graminis* f.sp. *hordei* (Zimmermann *et al*., 2006; Himmelbach *et al*., 2010). In the promoter of HvGER4c, there is an enrichment of W-box elements. Therefore I tested the possible interaction of WRKY proteins and *HvGER4c* promoter. The powdery mildew-induced promoter activity of *HvGER4c* was substantially suppressed by co-expressed *HvWRKY1* or *HvWRKY2*, the latter with a much stronger suppression capacity (Figure 3.13; Figure 3.14). This suppression is a specific effect as *HvWRKY1* and *HvWRKY2* did not exhibit any repression on the activity of the maize ubiquitin promoter (Figure 3.13, A left). In addition, those HvGER4c promoter derivates with single W-box mutation are also shown to be repressed by co-expressed HvWRKY1 and HvWRKY2 (Figure 3.14.). Interestingly, the repression appeared to be independent of Mla12 or Mlo (Figure 3.13, B). In the previous study using transient overexpression assay, it was implied that the *HvWRKY1/2*-mediated susceptibility might require a functional Mlo (Langen, unpublished). Moreover, there was an effector-dependent physical

interaction between *HvWRKY1/2* and the R protein MLA10 (Shen *et al*., 2007). In my experiments, I found that the in the resistant cultivar Sultan5 (*Mla12*) and BCPallas (*mlo*-5) which harbours a functional *Mla12* gene and recessive *mlo*-5 respectively, the *Bgh*-induced promoter activity of HvGER4c was as strongly repressed as in the wild type plants (Mlo).

HvWRKY1/2 targets the *HvGER4c* gene by repression of the transcription. This might explain on the molecular level at least partially the mechanism of HvWRKY1/2-mediated susceptibility. It was found that members of the *HvGER4* famly were the most abundant pathogenesis-related proteins in *Bgh*-infected epidermal cells (Zimmermann *et al*., 2006; Himmelbach *et al*., 2010). This cluster of PR protein is positively involved in basal defense towards *Blumeria graminis*. Hence, suppression of their transcription would reduce the abundance of the accumulated protein and negatively influence the plant resistance status. Many WRKY proteins were demonstrated as negative regulators in plant innate immunity. For example, the HvWRKY1/2 homologous genes in *Arabidopsis*, AtWRKY18, -40 and -60 are known to function partially redundantly as negative regulators in the resistance to the powdery mildew fungus *Golovinomyces orontii and* bacterial pathogen *Pseudomonas syringae (*Pandey *et al*., 2010; Shen *et al*., 2007; Xu *et al*., 2006*)*. Mutation of *AtWRKY18* and *AtWRKY40* resulted in enhanced resistance to powdery mildew *G.orontii (*Shen *et al*., 2007*)*. Other examples include AtWRKY7, -11 and -17 which act redundantly in *Pseudomonas syringae* resistance (Journot-Catalino *et al*., 2006; Kim *et al*., 2006). AtWRKY38 and -62 also negatively regulate the basal resistance to *P. syringae* and they are under the regulation of a SNF1-related kinase (Kim *et al*., 2008; Xie *et al*., 2010). In rice, the IIa member OsWRKY62 negatively regulates XA21-mediated immune response (Peng *et al*., 2008). Rice plants overexpressing OsWRKY62 are compromised in XA21-mediated immunity to *Xanthomonas oryzae* pv.*oryzae* (*Xoo*) and suppressed the activation of defense-related genes including *OsPR1* and *OsPR10* (Peng *et al*., 2008).

OsWRKY28, OsWRKY71, OsWRKY62 together with OsWRKY76 are clustered in the rice WRKY IIa subfamily (Peng *et al*., 2010).Transgenic rice plants overexpressing all the four genes enhanced resistance against *Xoo* and showed activation of OsPR10 expression. These results indicate a functional interaction

between WRKY IIa members in regulating plant innate immunity. Based on phylogenetic tree of the WRKY IIa subfamily, HvWRKY1,-2, -3, -23, -54 and -65 are within IIa subgroup (Figure 4. 1). How the other IIa HvWRKY members are involved in the innate immunity remains to be assessed. Goup IIa WRKY members have putative leucine zipper motifs at the N-terminus, suggesting potential dimerizations between proteins. Studies from Xu *et al*., (2006) have revealed the importance of leucine zipper motifs for the physical interaction of WRKY IIa proteins AtWRKY18, -40 and -60 in *Arabidopsis*.

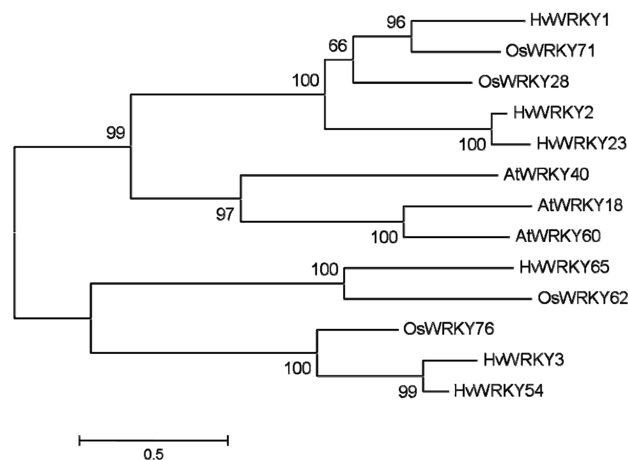

Figure 4. 1 Phylogenetic tree of Group IIa WRKY members from *Arabidopsis*, rice and barley. Thirteen sequences of conserved WRKY domains from *Arabidopsis*, rice and barley were aligned using MUSCLE with default parameters. The evolutionary distances were computed using the JTT matrix-based method. Neighbor-Joining (NJ) tree was constructed using MEGA 5.0 with pairwise deletion. The scale bar represent 0.5 substitutions per site and the values next to the nodes are bootstrap values from 1000 replicates.

The HvGER4 gene cluster contains a dense cluster of tandemly duplicated genes (GER4a-h) that resulted from several cycles of duplication (Himmelbach *et al*., 2010). W-boxes are overrepresented in the promoters of all these duplicated genes. In the present study, HvGER4c was selected as a representative to study the targeting from HvWRKY1/2. However, HvWRKY1/2 might act on the W-box elements of all these GER4c promoters. This hypothesis requires further verification.

Through the WRKY signalling network, there is co-presence of positive regulators and negative regulators in the process of transcriptional regulation. For instance, in rice aleurone cells, OsWRKY24 and OsWRKY45 were found to repress ABA-inducible promoter whereas OsWRKY72 and OsWRKY77 were shown to act as

activators of the same promoter (Xie *et al*., 2005). Regarding the promoter of defense-related gene HvGER4c, there are potentially positive WRKY factors or other types of transcription factors acting as transcriptional activators. To search for potential transactivators, I performed homology search for barley WRKYs using the known activators from rice WRKYs. HvWRKY12 and HvWRKY5 were found to be the homologous gene for OsWRKY72 and OsWRKY77. They might act as activators in the similar manner as OsWRKY72 and OsWRKY77, however, not necessarily acting on the corresponding ABA-inducible promoter in barley. The wheat and *Arabidopsis* WRKY factors TaWRKY78 and AtWRKY20 are known to be activators of PR4 promoters (Proietti *et al*., 2010). To verify their possible activation on HvGER4c, these two WRKY constructs together with its barley homolog HvWRKY46 (also known as SUSIBA2) were used in co-bombardment with *pHvGER4c::GUS* constructs and they were found to be unable to activate pHvGER4c (data not shown).

4.5 Gene expression in *HvWRKY2* overexpression lines

HvWRKY2 overexpression plants in the present study did not display enhanced susceptibility under the experimental conditions. Expression analysis of some *Bgh*-inducible genes might provide additional information as explanations. The candidate genes were selected from a primary microarray experiments. *IGS*, *HvPR2* and *HvPR5* (thaumatin-like proteins) appeared to have reduced expression at 12 h post *Bgh* inoculation (Figure 3.25; Figure 3.26). However, there was an early induction of *HvPR2* expression at 4 hours post inoculation in *HvWRKY2* transgenic plants. Therefore, HvWRKY2 might have a dual role in both activation and repression of target genes either directly by itself or indirectly through other transcription factors.

4.6 Conserved activity of WRKY proteins across species

Due to the high conservation of WRKY protein family, the homologous WRKY genes from distinct plant species may retain their functions in the context of cross-species comparison (Mangelsen *et al*., 2008). Recently, it was reported that wheat TaWRKY78 showed cross-activity with the *Arabidopsis* AtWRKY20 (Proietti *et al*., 2011). In wheat, TaWRKY78 was able to bind to and positively regulate the wPR4e promoter. In *Arabidopsis*, the promoter of PR4-type AtHEL was activated

by AtWRKY20, the orthologue of TaWRKY78. Interestingly, both WRKY factors were shown to activate the orthologous cognate promoters of wPR4e and AtHEL, suggesting a conserved mechanism across monocot and dicot species (Proietti *et al*., 2011). In my study, the *Arabidopsis* orthologue AtWRKY40 was transiently overexpressed in barley epidermal cells and resulted in enhanced susceptibility to *Blumeria gramini* f.sp. *hordei* (*Bgh*) (Fig. 3.16). This indicated that AtWRKY40 has a similar function in barley as its orthologs HvWRKY1 and HvWRKY2 and retained its function in another species as a negative regulator in the basal defense to powdery mildew fungus. The results support the notion from Mangelsen *et al*. (2008) that gene orthology implies similar gene function and diversification between monocot and dicot has most likely occurred after the specialization of some WRKY proteins.

4.7 Suggestion of candidate WRKY genes for disease resistance

The WRKY transcription factors, like the NF-kB factors in animals, are the central regulators of immune responses in plants (Eulgem, 2005). Such a superfamily is a valuable pool of disease resistance-related factors. During pathogen attack, many of the genes encoding WRKY transcription factors could be up-or down-regulated. Though the up- or down-regulation by pathogens does not necessarily indicate their direct role in conferring resistance to the pathogen, the expression profile provide precious information about the related signalling cascade and potential involvement in plant immunity. The expression patterns of barley WRKY genes from microarray database together with the orthologs based comparison would be a powerful tool to preselect some candidates WRKY genes. In further studies, researchers and breeders may give priority to these candidate genes in the sense of gene cloning, functional validation and application in crop improvement practice. From Genevestigator data set, five barley WRKY genes were found strongly (over eight folds induction) responsive to pathogen infection (Figure 3.27) and seven additional WRKYs were induced to a less extent (two to eight folds) (Figure 3.28).
HvWRKY19 and *HvWRKY20* are the orthologs of *AtWRKY50*, *AtWRKY51* and *OsWRKY7*. These orthologous genes contain the WRKYGKK motif and belong to group IIc subfamily. Both *HvWRKY19* and *HvWRKY20* are strongly up-regulated after *Blumeria graminis* infection with the latter showing a stronger induction (Figure 3.27). Their *Arabidopsis* ortholog AtWRKY50 and AtWRKY51 were

recently found to be mediators of SA and low oleic acid-dependent repression of JA signalling and function positively in basal defense to biotrophic pathogens (Gao *et al*., 2011). Both *AtWRKY50* and *AtWRKY51* are SA-inducible and they suppress the JA-mediated induction of *PDF1.2* and *VSP1* expression. In rice, the orthologous gene *OsWRKY7* was not characterized yet but the expression profile of *OsWRKY7* (Os.8961.1.S1_s_at) showed an eight folds induction 2 days after inoculation with the rice blast fungus *Magnaporthe oryzae*. RT-PCR experiment confirmed the significant induction of OsWRKY7 after blast fungus infection between 24 and 48 hours (Ryu *et al*., 2006). Moreover, *OsWRKY7* transcript was increased rapidly at early time points (4–12 h) after infection with the bacterial blight pathogen *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) (Ryu *et al*., 2006). In addition, the mock treatment also induced *OsWRKY7* expression and suggested a possible role in abiotic stress as well. With K to Q substitution in the signature motif, *AtWRKY50* protein was still able to bind W-box elements and even the mutated W-box (Brand *et al*., 2010).

The group III member *HvWRKY32* is orthologous to *AtWRKY70* and *OsWRKY45*. It is highly induced by *Blumeria graminis* infection at late stage and responsive to stem rust fungus *Puccinia graminis* at early-intermediate stage (Figure 3.27). Therefore, *HvWRKY32* might be involved in the resistance to the biotropic pathogen *B. graminis* and *P. graminis*. The *Arabidopsis AtWRKY70* has been well-known as a mediator in the cross-talk between SA and JA signalling as well as being crucial for R-gene mediated resistance (Li *et al*., 2006; Knoth *et al*., 2007). *AtWRKY70* overexpressors showed enhanced resistance to *E. cichoracearum* while the wrky70 mutants are impaired in resistance to *E. cichoracearum* (Li *et al*., 2006). It is also required for full RPP4-mediated resistance and basal defense against *H. parasitica* (Knoth *et al*., 2007). Likewise, the rice orthologous gene *OsWRKY45* plays an important role in resistance towards bacterial and fungal pathogens (Shimono *et al*., 2007, 2011; Tao *et al*., 2009). Overexpression of *OsWRKY45* conferred extremely strong resistance to the fungal pathogen *M. grisea* and bacterial pathogen *Xoo*. However, OsWRKY45 overexpressors are susceptible to the necrotrophic pathogen *Rhizoctonia solani* (Shimono *et al*., 2011), which might limit further use of this gene in resistance breeding.

HvWRKY51, similar to *AtWRKY33* and *OsWRKY24*, belongs to the group I subfamily. It is highly responsive to *B. graminis* at early and intermediate infection phase but highly accumulated after *P. graminis* inoculation at late time point (Figure 3.27). Its *Arabidopsis* ortholog *AtWRKY33* was known to confer resistance to the necrotrophic pathogens *Botrytis cinerea* and *Alternaria brassicicola* (Zheng *et al*., 2006). In contrast, the *AtWRKY33* overexpressor displayed susceptibility to the hemibiotrophic pathogen *Pseudomonas syringae* DC3000 (Zheng *et al*., 2006). In rice, *OsWRKY24* (LOC_Os01g61080) was known to be a repressor of ABA and GA signalling (Zhang *et al*., 2009). Though its role in defense has not been described, expression data supports its potential participation in rice disease resistance. For instance, *OsWRKY24* (Os.31521.1.S1_at) was induced by *M. grisea* 16 folds at 2 and 4 days after inoculation. In addition, *OsWRKY24* was down-regulated by abiotic stresses such as drought stress in 2-week-old seedlings, indicating a role in the drought tolerance as well (Ramamoorthy *et al*. 2008).
HvWRKY57 belongs to group IIb and is similar to *AtWRKY6* and *OsWRKY1*. *HvWRKY57* is highly accumulated by *Fusarium graminearum* at late time point and induced by *P. graminis* at early and intermediate infection phase (Figure 3.27). It is also responsive to *B. graminis* at late stage of infection. The Arabidopsis orthologous gene *AtWRKY6* was known to be associated with senescence and defense (Robatzek and Somssich, 2001; Robatzek and Somssich, 2002**).** In rice, *OsWRKY1* (OsAffx.11050.1.S1_x_at) was not characterized but the high induction (8-16 folds) by *M. grisea* at late infection phase hints a possible function in pathogen resistance.
Other *HvWRKY* genes that are inducible by pathogens include *HvWRKY4*, *-5, -10*, *-28*, *-30*, *-54* and *-79*. Among them, *HvWRKY4* and *-28* might be specifically involved in *B. graminis* resistance as both of them are induced nearly eight folds at late stage of *B. graminis* infection. They are similar to *AtWRKY70*, a known regulator of SA and JA signalling and R-gene mediated resistance in *Arabidopsis* (Li *et al*., 2006; Knoth *et al*., 2007). *HvWRKY30* is homologous to *AtWRKY41*, which is flagellin-inducible and also suggested to be a regulator during the cross-talk of SA and JA signalling (Higashi *et al*., 2008). *OsWRKY69* (Os.11945.1.S1_at), the rice ortholog of *HvWRKY30*, is induced by *M. grisea* at 2 and 4 days after inoculation. From the microarray data, *HvWRKY54* is only weakly induced by *B.*

graminis. Together with other IIa group WRKY members, it might act in a similar way as its orthologous genes *AtWRKY40* and *OsWRKY76* (Pandey *et al*., 2010; Peng *et al*., 2010; Xu *et al*., 2006).

Taken together, the interspecies retained functions of sequence-related WRKY genes would aid future functional analysis of the understudied barley WRKY family. On the basis of expression profiling, the selected candidate genes can be further verified through knock-down or overexpression approach either in transient system or stable genetic transformation. The elucidation of signalling transduction pathways such as the upstream and downstream components and the interaction partners will help to better understand the plant transcriptional regulation network during biotic and abiotic stresses.

5. Summary

WRKY transcription factors are a conserved superfamily distributed in eukaryotes and extremely expanded in flowering plants. They are central regulators of diverse plant cellular responses such as biotic and abiotic stresses. Despite the economic importance of the crop plant barley (*Hordeum vulgare* L.) and the significance of WRKY transcriptional network, the barley WRKY transcription factors are largely understudied and undetermined. The present work aimed to perform a whole-genome gene discovery of barley WRKY family and functionally characterize two of the important genes *HvWRKY1* and *HvWRKY2* in the interaction with barley powdery mildew fungus *Blumeria graminis* f.sp. *hordei* (*Bgh*).

Based on the NCBI databases and the draft sequence of barley genome, blastp, blastn and tblastn searches were performed to find putative barley WRKY sequences. As a result, 100 unique WRKY members containing at least one WRKY domain were found in the barley genome. Apart from the previously annotated 45 HvWRKY members, the novel sequences were designated HvWRKY47-HvWRKY102. Phylogenetic analysis of the conserved WRKY domain spanning about 60 amino acids using MEGA5 clearly placed them into the predefined subgroups I, IIa+IIb, IIc, IId+e, and group III. The homologous WRKY members between barley, Arabidopsis and rice were assigned according to the phylogenetic analysis.

Expansion of group III members was found in barley compared with the model plant *Arabidopsis thaliana*. Within the group III members, WRKYs with a non-canonical WRKYGEK motif were proven to be a monocot-specific group and this diversification implicates special functions in monocot plants. In addition, phylogenetic analysis using WRKY sequences from 22 plant species clearly indicate that IIa group is the last group evolved.

Expression of two IIa members *HvWRKY1* and *HvWRKY2* was transiently and rapidly up-regulated in response to powdery mildew challenge. Using artificial microRNA-based transient silencing of *HvWRKY2*, I observed on single-cell level enhanced resistance to *Bgh*. Together with previous findings this result confirmed its nature as negative regulator in barley-*Bgh* interaction. Whereas the R-protein Mla10 was identified as an upstream factor for HvWRKY1 and HvWRKY2,

downstream target promoters are unknown for barley WRKYs. HvGER4c is one of the most abundant pathogenesis-related (PR) genes induced in *Bgh*-infected barley leaf epidermal cells and contains enriched WRKY-binding sites in the promoter. The wild-type and W-box mutated versions of HvGER4c promoter GUS fusion constructs were used in the co-bombardment assay with *35S::HvWRKY1/2* plasmids to verify their possible interaction. Co-expression of 35S::*HvWRKY1/2* constructs with *pHvGER4c::GUS* significantly and specifically suppressed the *Bgh*-induced activity of *HvGER4c* promoter, with *HvWRKY2* showing stronger repression activity than *HvWRKY1*. The finding indicates a negative transcriptional regulation of HvWRKY1/2 on the defense-related gene *HvGER4c*. In addition, it was observed that the HvWRKY2-mediated repression of *HvGER4c* was independent of Mla12 or Mlo, which were previously identified as an effector-dependent interaction partner of HvWRKY1/2, or assumed to be required for HvWRKY2-mediated compatibility respectively. Moreover, electrophoretic mobility shift assay (EMSA) was used to analyze the binding of HvWRKY2 recombinant protein with W-box elements in HvGER4c promoter. HvWRKY2 protein showed binding affinity to all the tested W-boxes but with questionable specificity. Transient overexpression of the Arabidopsis homologue AtWRKY40 resulted in enhanced susceptibility to *Bgh* in barley. The retained activity of AtWRKY40 between dicot and monocot indicated evolutionary conserved functions of IIa WRKY factors as negative regulators in basal defense.

Furthermore, the promoters of *HvWRKY1* and *HvWRKY2* were isolated through PCR amplification and two constructs of pHvWRKY1::Gusi-AM were successfully generated. These constructs might be deployed as a useful tool for studies on spatial and temporal activation of HvWRKY1/2, functional analysis of *cis*-elements and auto/cross-regulation of WRKY factors.

HvWRKY2 overexpression plants were analysed regarding the resistance towards *Blumeria graminis*. Unexpectedly, the transgenic plants exhibited wild-type resistance to *Bgh* according to the development of powdery mildew pustules and microscopical evaluation. Expression studies of the candidate genes *HvPR1*, *HvPR2*, *HvPR5* and *HvBI-1* using quantitative RT-PCR indicated conditional suppression and activation of marker genes in *HvWRKY2* overexpression plants.

The outcome of barley-*Bgh* interaction might be dependent on the sophisticated balancing of integrated activation and repression of gene sets.

Taken together, HvWRKY1 and HvWRKY2 act as negative regulators largely dependent on their transcriptional repression of the downstream target genes, including the verified HvGER4c gene. Some further candidate WRKY genes involved in plant immune responses were suggested based on the microarray expression profile after pathogen challenge. Whole-genome annotation and phylogenetic analysis of barley WRKY transcription factors might provide insights into further characterization and cross-species comparison of the conserved family.

6. Zusammenfassung

Die konservierte Superfamilie der WRKY Transkriptionsfaktoren findet sich in Eukaryoten, besonders in blühenden Pflanzen ist diese Gruppe sehr ausgedehnt. In Pflanzen sind WRKY Transkriptionsfaktoren zentrale Regulatoren von verschiedenen zellulären Antworten, zum Beispiel auf biotischen und abiotischen Stress. Trotz der ökonomischen Bedeutung von Gerste (*Hordeum vulgare* L.) und der zentralen Rolle des WRKY- Transkriptionsnetzwerks sind die Gersten-WRKY-Transkriptionsfaktoren kaum erforscht. Das Ziel der vorliegenden Arbeit war die Identifizierung der WRKY- Familie aus dem gesamten Gerstengenom und die funktionelle Charakterisierung von zwei bedeutsamen Genen, *HvWRKY1* und *HvWRKY2*, in der Interaktion mit dem Gerstenmehltau *Blumeria graminis* f. sp. *hordei* (*Bgh*).

Unter Nutzung der Datenbanken des NCBI und der vorläufigen Sequenzierung des Gerstengenoms wurde eine Suche mit den Programmen blastp, blastn und tblastn durchgeführt, um putative WRKY-Sequenzen der Gerste zu finden. Es konnten 100 einzelne WRKY- Mitglieder mit mindestens einer WRKY- Domäne im Gerstengenom identifiziert werden. Neben 45 bereits annotierten Mitgliedern der HvWRKY-Gruppe wurden die neuen Sequenzen als HvWRKY47 bis HvWRKY102. Die phylogenetische Analyse der konservierten, etwa 60 Aminosäuren langen WRKY Domäne mittels MEGA5 ermöglichte eine klare Zuordnung der WRKY-Sequenzen in die zuvor definierten Untergruppen I, IIa+b, IIc, IId+e und Gruppe III. Die homologen WRKY Mitglieder von Gerste, *Arabidopsis* und Reis wurden gemäß der phylogenetischen Analyse eingeordnet.

Die Gruppe III der WRKY Transkriptionsfaktoren ist in Gerste im Vergleich zu der Modellpflanze *Arabidopsis thaliana* erweitert. Es konnte gezeigt werden, dass die innerhalb der Gruppe III identifizierten WRKYs mit einem nicht-kanonischen WRKYGEK-Motiv eine spezielle Gruppe bei Monokotylen darstellen. Diese Erweiterung deutet auf spezielle Funktionen in monokotylen Pflanzen hin. Zusätzlich ergab die phylogenetische Analyse mit WRKY-Sequenzen von 22 verschiedenen Pflanzenarten, dass sich die Gruppe IIa wahrscheinlich als letzte Gruppe ausgebildet hat.

Als Antwort auf die Inokulation mit Mehltau war die Expression der beiden IIa-Mitglieder *HvWRKY1* und *HvWRKY2* kurzzeitig und schnell hochreguliert. Die transiente Ausschaltung von *HvWRKY2* mittels artifizieller microRNA führte zu einer erhöhten Resistenz gegenüber *Bgh* auf Einzelzellebene. Zusammen mit früheren Erkenntnissen bestätigte diese Beobachtung die Eigenschaft von *HvWRKY2* als negativer Regulator in der Gersten-*Bgh*-Interaktion. Während das R-Protein Mla10 als vorgeschalteter Faktor von *Hv*WRKY1 und *Hv*WRKY2 bereits bekannt ist, konnten nachgeschaltete Ziel-Promotoren für Gersten-WRKYs bisher nicht gefunden werden. *HvGER4c* ist eines der am stärksten induzierten Pathogenese-assoziierten Gene (*pathogenesis-related genes*, PR-Gene) in *Bgh*-infizierten Epidermiszellen der Gerstenblätter. Das *HvGER4c*-Gen hat in seinem Promotorbereich eine Anreicherung von WRKY-Bindungsstellen. Deshalb wurden Wildtyp- sowie W-Box-mutierte Versionen von *HvGER4c*-Promotor-GUS-Fusionskonstrukten in einer Co-Bombardement-Untersuchung mit 35S::*HvWRKY1/2* Plasmiden getestet, um eine mögliche Interaktion zu prüfen. Die Co-Expression von 35S::*HvWRKY1/2* Konstrukten mit *pHvGER4c::GUS* unterdrückte signifikant und spezifisch die *Bgh*-induzierte Aktivität des Hv*GER4c*-Promotors. Hierbei zeigte *HvWRKY2* eine stärkere Unterdrückung als *HvWRKY1*. Dieses Ergebnis deutet auf eine negative transkriptionelle Regulation des Abwehr-assoziierten Gens *HvGER4c* durch *HvWRKY1/2* hin. Zusätzlich konnte beobachtet werden, dass die *HvWRKY2*-vermittelte Repression von Hv*GER4c* unabhängig von *Mla12* oder *Mlo* ist, welche bereits als Effektor-abhängige Interaktionspartner von *HvWRKY1/2* identifiziert wurden oder die als notwendig für die *HvWRKY2*-vermittelte Kompatibilität angesehen werden. Des Weiteren wurde eine EMSA-Untersuchung (EMSA = *electrophoretic mobility shift assay*) durchgeführt, um die Bindung von HvWRKY2 rekombinantem Protein mit W-box-Elementen des *HvGER4c* Promotors zu studieren. Das *Hv*WRKY2 Protein zeigte Bindungsaffinität zu allen getesteten W-Boxen, jedoch konnte die Spezifität nicht abschließend geklärt werden.. Die transiente Überexpression des *Arabidopsis*- Homologs *AtWRKY40* in Gerste führte zu einer erhöhten Anfälligkeit gegenüber *Bgh*. Die Aktivität von *At*WRKY40 sowohl in dikotylen als auch in monokotylen Pflanzen deutet auf evolutionär konservierte Funktionen von IIa WRKY- Faktoren als negative Regulatoren in der basalen Abwehr hin.

Außerdem wurden die Promotoren von *HvWRKY1* und *HvWRKY2* durch PCR Amplifikation isoliert und zwei Konstrukte mit pHvWRKY1::Gusi-AM wurden erfolgreich hergestellt. Diese Konstrukte könnten ein nützliches Hilfsmittel für Studien der räumlichen und zeitlichen Aktivierung von *HvWRKY1/2*, der funktionalen Analyse von *cis*-Elementen und Auto-/Kreuz-Regulation von WRKY-Faktoren verwendet werden.

*Hv*WRKY2 überexprimierende Pflanzen wurden bezüglich ihrer Resistenz gegenüber *Blumeria graminis* untersucht. Unerwarteter Weise zeigten die transgenen Pflanzen keine veränderte Resistenz gegenüber *Bgh*, die anhand der Entwicklung von Mehltaupusteln und mikroskopischer Studien untersucht wurde. Expressionsstudien mit den Genen *HvPR1*, *HvPR2*, *HvPR5* und *HvBI-1* mittels quantitativer RT-PCR wiesen auf eine konditionale Unterdrückung bzw. Aktivierung von Markergenen in *HvWRKY2* überexprimierenden Pflanzen hin. Das Ergebnis der Gerste-*Bgh*-Interaktion könnte von einer ausgeklügelten Balance zwischen Aktivierung und Repression verschiedener Gensets abhängen.

Zusammenfassend lässt sich feststellen, dass *HvWRKY1* und *HvWRKY2* als negative Regulatoren zur transkriptionellen Unterdrückung von nachgeschalteten Zielgenen führen, eingeschlossen das überprüfte *HvGER4c*-Gen. Einige weitere WRKY Kandidaten-Gene, die an der pflanzlichen Immunantwort beteiligt sind, konnten anhand von Expressionsprofilen nach Pathogenbefall identifiziert werden. Die vorgenommene WRKY-Annotation basierend auf dem vorläufig identizierten Gersten-Genom und die phylogenetische Analyse von Gersten- WRKY-Transkriptionsfaktoren können weitere Erkenntnisse für die Charakterisierung und Art-übergreifende Vergleiche dieser konservierten Familie liefern.

7. References

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Appendix 1

Appendix 1.1

Predicted CDS or partial CDS from HvWRKY62 to HvWRKY102

>HvWRKY62_complete CDS
ATGTCTTCTGGTGGTGGCGGGGGAGGGGATCAAGGCCGTCATGGCGTCTACCACCAGCATGGC
CACGGCCAACTCACCCGCAACGATGGCGCCGGTGGCTACGAGTTCAGCAACGACGACATGGAG
AGCTTCTTCTTCAACCAGCCTGAGGGCGTCGTCGGTGGTGTGCGCGCCGACGAGATCGAGCCG
TACTCGAGCCTCACGAGCTACCTGCAGGGCTTCTTGGACCCCACCGGGCTAGCTCGGCATCTC
GACGTGCCGGCCAAGCACGAGCTGTCGGTCGACGTTAGGACCCATGACCAAGACAGCCAGGGC
ACCGGCAGCGCTGCTGGCGAAAGCGCTCCGCTGCTAACACCCAACTCATCCGTATCTTTCTCG
TCCGGAGGCGGGGACGGCGAGGGGAAGTCTCACCGGAGCAAGAAGGGTCGGGCGCAGGAGGCG
GATAACCAGGAGGATGGGGAAAGTTCCAAGAAAGCGAATAACAAACCCAAAAAGAAAGCCGAG
AAGAGGCCGCGTCTTCCCCGCGTCTCCTTCCTCACCAAGAGCGAGGTCGATCACCTCGAGGAC
GGCTACCGCTGGCGCAAATACGGCCAGAAGGCCGTCAAGAACAGCCCTTACCCAAGGAGCTAC
TATCGGTGCACGACGCCAAAGTGCGGGGTGAAGAAGCGGGTGGAGCGGTCGTACCAGGACCCG
TCGACGGTGATCACCACGTACGAAGGGCAGCACACGCACCACAGCCCCGCCAGCTTCCGGGGA
ACCTCAGCGCACCTCTTCATGCCC
CCCGGCGTCCACGGGCTCCCGCCGCCACACCTCATGCCGCCGGGGGTGTTCCACCCGGAGCTG
ATGAGCATGATGCGCATGCCGTACCCCAGCCCTAACATGCACCTGCCGAGTGTGCCACCGCCT
CCCCATCATCATCCAATGGCGGGAACTCCTCTCCAGCAGCACCATTTCACTGACTACGCGCTA
TTGCAAGACCTCTTCCCTTCCACAATGCCCAACAACCCATGA

>HvWRKY63_complete CDS
ATGGCCGTCGACCACATGGGATGCCGCTACGCCACGCATGGGCACGCGGCCGAGGAGCAGTTC
CAGGAGGCCGCTGCCGCGGGGCTCCGCAGCCTCGAGCTCGTCCGCTCGTCCTTCTCCTCCCGT
GCCGGCTGCGCGCCGCCGCCCCTCGGAGAGATGGCCGACCAAGCGGTGTCCAGGTTCCACCGG
GTCATCAACATCCTCGACCGCACCGGCCACGCCCGCTTCCGCCGCGGCCCTGGACCGGCAGTC
TTGCTGCAGCTGGCGCCGCAGAAAAGCATGACGCTGGACTTCACGAAGCCTTTGAAGGCGCCG
GCCGCCCCTTCCGTGACGTCGACGTCCTTCTTCTCGTCCGTGACGGCCGGGGGCGAGGGCAGC
GTGTCCAAGGGCTGGAGCCAGCTCGTCTCCTCCGGCAAGCCGCCGCTCCCAGCTGGGACCAAA
CGCAAGCAACGCCAACAGCAGACGCGCTTCGCGCACTCCGACACCGCTGCCGGTGCCCGGTGC
CATTGTCTGAAGAAGGGCAAGCACCGGGTGAAGTACACGACGCTCGAGCCCACGGTAACCTCG
CGCGCGGTGGACGTCCCCGGCGTCGGCGACAAGTACTCGTGGCGCAAGTACGGGCAGAAGACT
ATCAAGGGGTCCCCTCACCCCCGCTGCTACTACAGGTGCGGCACCGTCAAGGGCTGCCCGGCG
CGGAAGCACGTGGAACGCGCCACCGACGACCCCGCCATGCACCTCGTCACCTACGAGGGCGAC
CACCGCCACGACACTTGGCCGCCG
GCGGCCGCAAATTAA

>HvWRKY64_complete CDS
ATGAGGGGGAGCAGCATGCTCAGCTCCATTGGGAGCAACAAAAGGATGCTGCAGCAGGACTGC
AGTGGCGGCAGCCATGCCCAGGAGCACACCAAGAAGAAGGCACGTGTCGGCATGAGAACAGAC
TACACATATGCACCGTATCATGATGGCTTCCAGTGGAGGAAATATGGGCAGAAGGTGATCCGG
GGCAATGCCTTCCCAAGGTGCTACTACAGGTGCACGTACCACCAAGATCATGGCTGTTCGGCG
AGCAAGCACGTGGAGCAGCACAACTCAGCGGACCCGCCGCTGTTCCGCGTGGTGTACACGAAC

GATCACACATGCAGCGGCGCTGCTGCTGCGGCATCGGACTACATGGCCTCATCCATGCACATC
CAGCAGATCGCCGACGCTTCTCTGAGAAAGGCCGACACGGAACCGGAAAGGCCGCCGCGCCCG
CAGCAGCCTCGCTCCGGCGGTGGTTGCGCCGCAGCGATAAAAGAGGAGAAAGATGTCATCGTC
TCCTCCCTGCTCGCCGTCATCAGAGGCAGCTGCGACGTTGCGAAATCTGACGCTGCGCACCAG
GGCTATAGCAGTGCGTCGTCGTTGGCTAGTGCTAACTGCTATGCGATGTCACCGTCGGTGGCC
GGAGGTAGCCGTGAGGGTAGCAGCAGCTCTTCGGTTTCGCCCGCGGTGCTGCCGGCGCCAGAC
GACATGGGATTGGGACTGGACTTCATGGTGGAGTCCCACTGGTTCGAGCCTTTGGATTTGGGT
TGGTTCGTAGAATAG

>HvWRKY65_complete CDS

ATGGACGACGGTTCGTCATGTCCGACCAACAGCGCCGGGCTTCTGCCGCTCTCCGCCGGCTCC
CCGACGCTGCAGTCGTTGCTGATCACTGCGTTCTTGAACGCGGAGCAGGCTGAGGGTCTCGAA
GAGAAGCTGAGGCGAGTGCGCGAGGAGAACCGGCGGCTGGCCGGCACACTGGGCGCCATACTC
GCCGATCGCCCCGACCTGCGTGCTCTCATGAGGGCGCCAGCGTCAGCTGTCGCCTGCGCTAGG
GCGCCGAGCGGCTCTGCCTCCAATGCAGCGAGGGAGGAGGCCGCCGGCGTGACGGTGGAGCCG
CGGCCCAAGGTCAGGACGGTCTGCGCGCGCGCGGAGCCGGCCGACACCGACGCCAACCTTAGC
GTCAAGGACGGGTACCAGTGGAGGAAGTACGGGCAGAAGGTGACGCGCGACAACCCGCACCCA
AGATCCTACTTCCGCTGCGCGTTTGCTCCATCTTGCCCCGTGAGGAAGAAGGTGCAGAGAGAC
GCGGAGGACACGTCAAAGCTAGTGGCGACGTACGAGGGCGAGCACAACCACGCTAGATCCCCG
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Appendix 1.2 Deduced amino acid sequences of barley WRKYs

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Appendix 2

Appendix 2.1 Multiple alignment of barley WRKY domains.
The alignment of conserved WRKY domains spanning ca. 60 aa is performed by MUSCLE. Subgroups including representatives from AtWRKYs are indicated. 1-N and 1-C represent the N-terminus and C-terminus WRKY domain of group I respectively.

Group IIa

```
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HvWRKY1     DGYQWRKYGQKV-TKDNPCPRAYFRCSFA---PGCPVKKKVQRSAEDKTILVATYE-GEHNHTQ
HvWRKY3     DGYQWRKYGQKV-TRDNPSPRAYFRCAFA---PSCPVKKKVQRSAEDSSMVEATYE-GEHNHPR
HvWRKY54    DGYQWRKYGQKV-TRDNPSPRAYFRCAFA---PSCPVKKKVQRSAEDSSVVEATYE-GEHNHQR
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HvWRKY65    DGYQWRKYGQKV-TRDNPHPRSYFRCAFA---PSCPVRKKVQRDAEDTSKLVATYE-GEHNHAR
AtWRKY18    DGFQWRKYGQKV-TRDNPSPRAYFRCSFA---PSCPVKKKVQRSAEDPSLLVATYE-GTHNHLG
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Group IIb

```
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HvWRKY59    DGCQWRKYGQKV-AKGNPCPRAYYRCTVA---PACPVRKQVQRCQEDMSILITTYE-GTHNHPL
HvWRKY57    DGCQWRKYGQKM-AKGNPCPRAYYRCTMA---TGCPVRKQVQRCAEDRTILITTYE-GTHNHPL
AtWRKY6     DGCQWRKYGQKM-AKGNPCPRAYYRCTMA---TGCPVRKQVQRCAEDRSILITTYE-GNHNHPL
```

Group I-N

```
HvWRKY35N   DGYNWRKYGQKA-VKGGKCPRSYYKCTLN-----CPVRKNVEHSADGR-IIKIVYR-GQHCHEP
HvWRKY49N   DGYNWRKYGQKA-VKGGEYPRSYYKCTQA---S-CPVKKRVEHSAYGQ-ITQIIYR-GQHNHQR
HvWRKY78N   DGYNWRKYGQKV-VKGSDCPRSYYKCTHP---S-CPVKKKVEHAEDGQ-ISEIIYK-GKHNHQR
HvWRKY6N    DGFNWRKYGQKQ-VKSSDNSRSYYRCTNS---S-CLAKKKVEHCPDGR-IIEIIYR-GTHSHEP
HvWRKY42N   DGYNWRKYGQKQ-VKSSEHPRSYYKCTHP---D-CPVKKKVERSQDGQ-ITEIVYK-SSHNHPL
HvWRKY66N   DGYNWRKYGKKQ-VKNSGHPTNYYKCSHQ---N-CPVKKKVEHCQDGD-ITEIVYK-GSHNHPL
HvWRKY40N   DGYNWRKYGQKQ-VKGSEFPRSYYKCTHP---T-CPVKRKVETTVDGQ-IAEIVYN-GEHNHPQ
HvWRKY51N   DGYNWRKYGQKQ-VKGSENPRSYYKCTFP---N-CPTKKKVETSLEGQ-ITEIVYK-GTHNHAK
AtWRKY33N   DGYNWRKYGQKQ-VKGSENPRSYYKCTFP---N-CPTKKKVERSLEGQ-ITEIVYK-GSHNHPK
HvWRKY43N   DGYNWRKYGQKQ-MKGSENPRSYYKCSFA---G-CPTKKKVEQAPDGQ-VTEIVYK-GTHNHPK
HvWRKY46N   DGYNWRKYGQKH-VKGSENPRSYYKCTHP---N-CEVKKLLERAVDGL-ITEVVYK-GRHNHPK
HvWRKY52N   DGYSWRKYGQKQ-VKHSEYPRSYYKCTHP---S-CQVKKKVERSHEGH-VTEIIYK-GTHNHPR
HvWRKY58N   DGYNWRKYGQKQ-LKDAESPRSYYKCTRD---A-CPVKKIVERSFDGC-IKEITYK-GRHTHPR
AtWRKY1N    DGYNWRKYGQKL-VKGNEFVRSYYRCTHP---N-CKAKKQLERSAGGQ-VVDTVYF-GEHDHPK
```

Group I-C

```
HvWRKY35C   DGYRWRKYGQKV-VRGNPHPRSYYKCTYQ---G-CDVKKHVERSSQEPHAVITTYE-GKHTHDV
HvWRKY49C   DGYRWRKYGQKV-VKGNPHPRSYYKCTYQ---G-CDVKKHIERCSQDPTSVITTYE-GKHSHDV
HvWRKY42C   DGYRWRKYGQKV-VKGNPNPRSYYKCTHQ---G-CSVRKHVERASHDLKSVITTYE-GKHNHEV
HvWRKY52C   DGYRWRKYGQKV-VKGNPNPRSYYKCTHP---G-CSVRKHVERASHDLKSVITTYE-GKHNHEV
HvWRKY46C   DGYRWRKYGQKV-VKGNPNPRSYYKCTST---G-CPVRKHVERASHDPKSVITTYE-GKHNHEV
HvWRKY66C   DGYRWRKYGQKV-VKGNPNPRSYYKCTHP---S-CPVRKHVERASNDPKSVITTYE-GRHTHEV
HvWRKY51C   DGYRWRKYGQKV-VKGNPNPRSYYKCTTV---G-CPVRKHVERASHDLRAVITTYE-GKHNHDV
AtWRKY33C   DGYRWRKYGQKV-VKGNPNPRSYYKCTTI---G-CPVRKHVERASHDMRAVITTYE-GKHNHDV
HvWRKY43C   DGYRWRKYGQKV-VKGNPNPRSYYKCTTP---N-CPVRKHVERASQDLRAVVTTYE-GKHNHDV
HvWRKY58C   DGYRWRKYGQKV-VKGNPRPRSYYKCTAE---N-CNVRKQIERASTDPRCVLTTYT-GRHNHDP
HvWRKY78C   DGYRWRKYGQKV-VKGNPHPRSYYKCTFA---A-CNVRKHIERASSDPKAVITTYE-GKHNHEP
AtWRKY1C    DGYRWRKYGQKS-VKGSPYPRSYYRCSSP---G-CPVKKHVERSSHDTKLLITTYE-GKHDHDM
HvWRKY6C    DGYRWRKYGQKI-VKGNPNPRSYYRCTHD---G-CPVRKHVERAPDDINNMVVTYE-GKHNHGQ
HvWRKY40C   AAFRWRKYGQKA-VNGNSFPRSYYRCSTA---R-CNARKFVERSSD--NSLVTTYE-GKHNHAQ
```

Group IIc

```
HvWRKY17        DGFKWRKYGKKA-VKNSPNPRNYYRCSAE---G-CGVKKRVERDRDDPRYVVTTYD-GVHNHAT
HvWRKY50        DGFRWRKYGKKA-VKSSPNLRNYYRCSAD---G-CGVKKRVERDRDDPRYVLTTYD-GVHNHVA
HvWRKY16        DGFKWRKYGKKA-VKNSPNPRNYYRCSTE---G-CNVKKRVERDREDHRYVITTYD-GVHTHPL
HvWRKY5         DGYRWRKYGKKM-VKNSPNPRNYYRCSSE---G-CRVKKRVERDRDDERFVITTYD-GVHNHLA
HvWRKY19        DGYKWRKYGKKS-VKNSPNPRNYYRCSTE---G-CSVKKRVERDRDDPAYVVTTYE-GTHSHAS
HvWRKY20        DGYKWRKYGKKS-VKNSPNPRNYYRCSTE---G-CDVKKRVERDRDDPAYVVTTYE-GTHSHAS
HvWRKY18        DGYKWRKYGKKS-VKNSPNPRNYYRCSTE---G-CNVKKRVERDKDDANYVVTMYE-GVHNHAS
HvWRKY70        DGYKWRKYGQKV-VKNSLHPRSYFRCTHS---N-CRVKKRVERLSTDCRMVITTYE-GRHTHPP
HvWRKY77        DGYKWRKYGQKV-VKNSLHPRSYYRCTHS---N-CRVKKRVERLSEDCRMVITTYE-GRHTHTP
HvWRKY14        DGYKWRKYGQKV-VKNTQHPRSYYRCTQD---K-CRVKKRVERLAEDPRMVITTYE-GRHVHSP
HvWRKY15        DGYRWRKYGQKA-VKNSPFPRSYYRCTNS---K-CTVKKRVERSSEDPTVVITTYE-GQHCHHQ
HvWRKY75        DGYRWRKYGQKA-VKNSPFPRSYYRCTNN---K-CTVKKRVERSSEDPSVVITTYE-GQHCHHT
HvWRKY53        DGYRWRKYGQKA-VKNSPYPRSYYRCTTQ---K-CVVKKRVERSFQDTAVVITTYE-GKHTHPI
HvWRKY79        DGYRWRKYGQKA-VKNSPFPRSYYRCTTQ---K-CPVKKRVERSYQDAAVVITTYE-GKHIHPI
AtWRKY8         DGYRWRKYGQKA-VKNSPYPRSYYRCTTQ---K-CNVKKRVERSYQDPTVVITTYE-SQHNHPI
HvWRKY62        DGYRWRKYGQKA-VKNSPYPRSYYRCTTP---K-CGVKKRVERSYQDPSTVITTYE-GQHTHHS
HvWRKY60        DGYRWRKYGQKA-VKNSSFPRSYYRCTAA---R-CGVKKLVERSQQDPSTVVTTYE-GRHGHPS
HvWRKY12        DGYRWRKYGQKA-VKNNNFPRSYYRCTHQ---G-CNVKKQVQRLSRDEGVVVTTYE-GTHTHPI
HvWRKY13        DGYRWRKYGQKA-VKNSAFPRSYYRCTHH---T-CNVKKQVQRLAKDTSIVVTTYE-GVHNHPC
HvWRKY81        DGYQWKKYGQKF-IKNIQKIRSYFRCRDR---R-CGAKKKVEWQPGDP-SLRVVYD-GAHQHGS
HvWRKY36        DGYRWRKYGQKF-IKNNPHPRSYYKCTSA---R-CSAKKHVEKSTDDPEMLIVTYE-GSHLHGP
HvWRKY71        DGYKWRKYGQKA-IKNSPNPRSYYRCTNP---R-CNAKKQVERAVDEPDTLVVTYE-GLHLHYT
```

Group IId

```
HvWRKY7         DEYSWRKYGQKP-IKGSPYPRGYYKCSTV---RGCPARKHVERALDDPAMLVVTYE-GEHRHSP
HvWRKY10        DDFSWRKYGQKP-IKGSPYPRGYYKCSTV---RGCPARKHVERDPSDPSMLIVTYE-GEHRHSP
HvWRKY9         DEFSWRKYGQKP-IKGSPHPRGYYKCSSV---RGCPARKHVERCVDDPAMLIVTYE-GDHNHNR
HvWRKY61        DEYSWRKYGQKP-IKGSPHPRGYYKCSSV---RGCPARKHVERCVDDPSMLIVTYE-GEHNHTR
HvWRKY8         DEYSWRKYGQKP-IKGSPHPRGYYKCSTV---RGCPARKHVERCVDEPAMLIVTYE-GEHSHNR
AtWRKY7         DEFSWRKYGQKP-IKGSPHPRGYYKCSSV---RGCPARKHVERALDDAMMLIVTYE-GDHNHAL
HvWRKY63        DKYSWRKYGQKT-IKGSPHPRCYYRCGTV---KGCPARKHVERATDDPAMHLVTYE-GDHRHDT
HvWRKY72        DDYSWRKYGQKP-IKGSPYPRCYYRCSTA---KGCPARKHVERATDDPAMLVVTYE-GEHRHDT
HvWRKY11        DNYSWRKYGQKP-IKGSPHPRGYYRCSSI---KDCPARKHVERCRGDAGMLIVTYE-NDHNHAQ
```

Group IIe

```
HvWRKY56        DLWAWRKYGQKP-IKGSPYPRGYYRCSSS---KGCPARKQVERSRTDPNMLVITYT-SEHNHPW
HvWRKY69        DLWAWRKYGQKP-IKGSPYPRGYYRCSSS---KGCPARKQVERSRTDPNMLVITYT-SDHNHPW
HvWRKY68        DLWAWRKYGQKP-IKGSPYPRGYYRCSSS---KGCLARKQVERSRSDPNMLVITYT-AEHNHPW
HvWRKY39        DSWAWRKYGQKP-IKGSPYPRGYYRCSSS---KGCPARKQVERSRTDPTVLLVTYS-YDHNHPW
HvWRKY45        DSWAWRKYGQKP-IKGSPFPRAYYRCSSS---KGCPARKQVERSQADPDTVLITYS-YEHNHSS
AtWRKY29        DAWAWRKYGQKP-IKGSPYPRSYYRCSSS---KGCLARKQVERNPQNPEKFTITYT-NEHNHEL
HvWRKY44        DPWAWRKYGQKP-IKGSPYPRGYYRCSTD---KACEARKMVERCRDDPNSFILTYTGGEHSHPA
AtWRKY22        DVWAWRKYGQKP-IKGSPYPRGYYRCSTS---KGCLARKQVERNRSDPKMFIVTYT-AEHNHPA
AtWRKY27        DLWAWRKYGQKP-IKGSPYPRNYYRCSSS---KGCLARKQVERSNLDPNIFIVTYT-GEHTHPR
HvWRKY55        DLWAWRKYGQKP-IKGSPYPRGYYKCSSM---KGCMARKLVERSPAKPGVLVITYM-AEHCHPV
HvWRKY80        DQWAWRKYGQKP-IKGSPYPRGYYKCSSM---KSCTARKLVERSTAKPGVLLVTYI-ADHCHAV
HvWRKY84        DLWAWRKYGQKP-IKGSPHPRAYYKCSSL---RACTARKLVDRSPAEPEALIVTYI-DDHCHAV
```

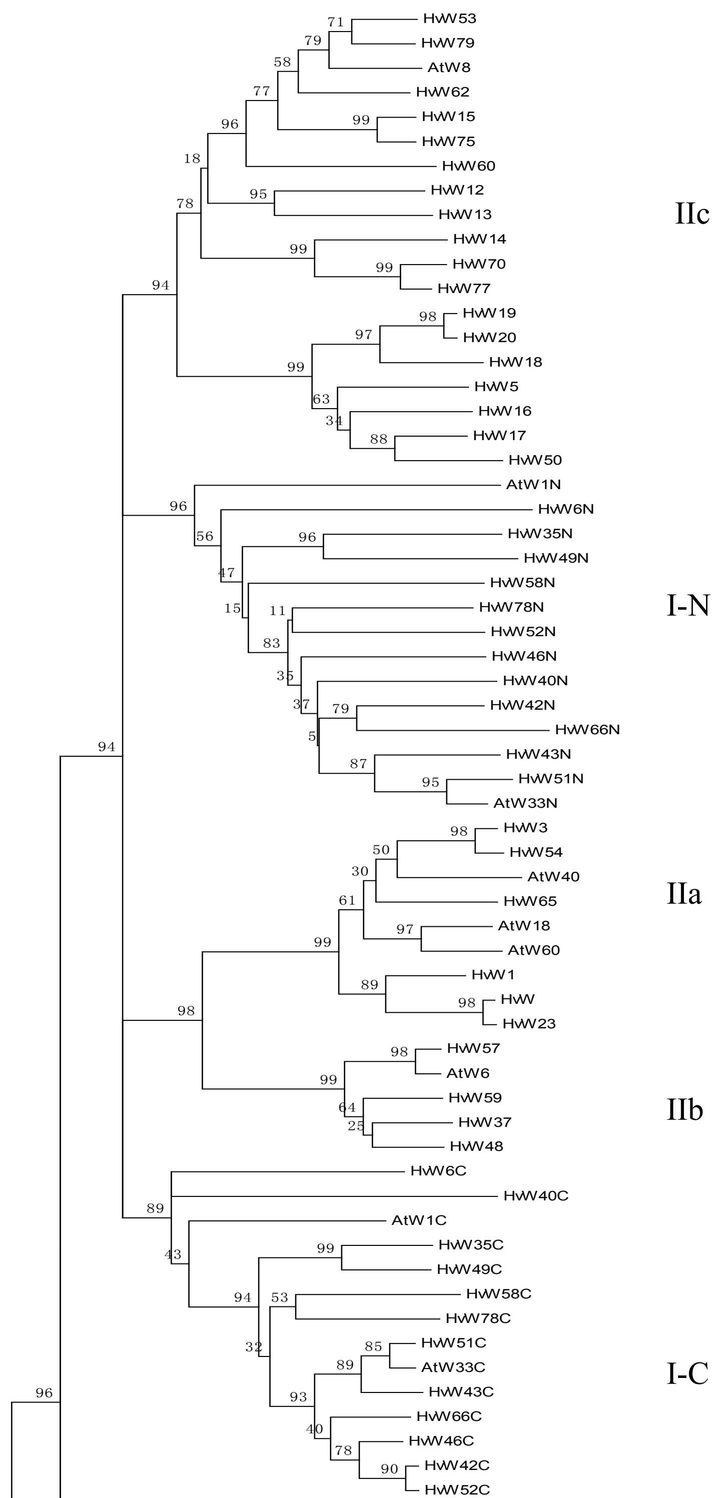
Group III

```
HvWRKY4         DGKTWRKYGQKC-IHACTNPRSYYRCSHKPDQ-GCQATRQVQESD-SNPSEYLISYYGQHTCKD
HvWRKY82        DGKSWRKYGQKQ-INDST--RSYYRCTHKPDQ-GCQAKRHVQESE-SNPAEYTIDYYGQHTCRD
HvWRKY88        DGHIWRKYGQKE-IQNSTHPRSYYRCTHKSDQ-GCNAKRQVQICE-THPIKYAVTYYGEHTCKA
HvWRKY31        DGLIWRKYGQKE-IHNSTHPRLYFRCTYKHDS-GCPATRQVQQSE-DDPSLYVITYFGDHTCCQ
HvWRKY67        DGFIWRKYGQKE-ITHSKNPRLYFRCTYKHDS-GCPATRQVQQSD-DDHSLYIITYFGDHTCCQ
HvWRKY32        DGQTWRKYGQKE-IQNSKHSKAYFRCTHKYDQ-QCPARRQAQRCD-EDPDTYRVTYIGVHTCQD
HvWRKY73        DGHAWRKYGQKE-IHNSKHSRAYFRCTHKYDQ-LCAAQRQVQRCD-DDEGMFRVTYIGVHTCRD
AtWRKY70        DAFSWRKYGQKE-ILNAKFPRSYFRCTHKYTQ-GCKATKQVQKVE-LEPKMFSITYIGNHTCNT
HvWRKY26        DGLSWRKYGQKD-ILGATYPRAYFRCTHRHSQ-GCQATKQVQRAH-ADPLLFDVVYHGAHTCAQ
```

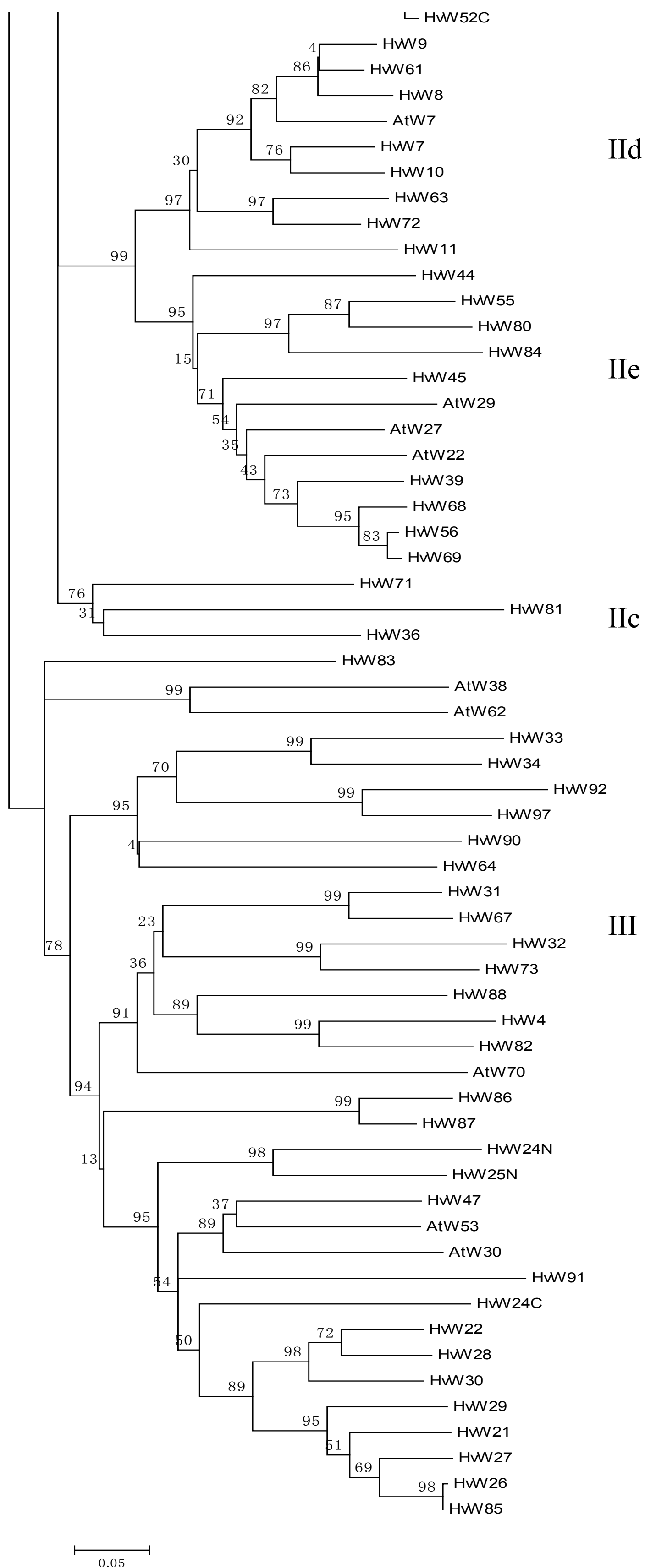
```
HvWRKY85        DGLSWRKYGQKD-ILGATYPRAYFRCTHRHSQ-GCQATKQVQRAH-ADPLLFD-VYHGAHTCAQ
HvWRKY27        DGLSWRKYGQKD-ILGATYPRAYFRCTHRHTQ-GCQATKQVQRTH-ADPLLFEVAYHGEHTCVQ
HvWRKY21        DGFSWRKYGQKD-ILGAMYPRAYFRCTHRHTQ-GCYASKQVQRAH-GDPLLFDVVYHGNHTCAQ
HvWRKY29        DGMSWRKYGQKG-ILGATYPRSYFRCTHRHTQ-GCAATKQVQRAT-ADPLLFDVVYVGAHTCAG
HvWRKY22        DGHSWRKYGQKD-ILGAKHPRGYYRCTHRKSQ-GCAATKQVQRAD-EDPTLFDVIYHGEHTCVH
HvWRKY28        DGHSWRKYGQKD-ILGAHHPRAYYRCTYQKTQ-GCAATKQVQRAD-EDPALFDVIYHGEHTCLH
HvWRKY30        DGHSWRKYGQKD-ILGAKHPRAYYRCTHRNSQ-GCTATKQVQRAD-EDPVLFDVVYHGQHTCRP
HvWRKY24C       DGYSWSKYGQMD-ILGSKHPRCYYRCVHKHDK-GCQATKQVQRSD-SDTQLFDIVYHGEHTCAE
HvWRKY91        DGFSWKKYGQKD-ILGTKHPRGYFRCSRRESE-GCRATKEVQRTD-DCDEIFDFEYKGKHTCTP
HvWRKY47        DGFSWRKYGQKD-ILGAKFPRGYYRCTYRNAQ-GCAATRQVQRSD-ADLAVFDVTYQGAHTCLQ
AtWRKY53        DVFSWRKYGQKD-ILGAKFPRSYYRCTHRSTQ-NCWATKQVQRSD-GDATVFEVTYRGTHTCSQ
AtWRKY30        DGFSWRKYGQKD-ILGAKFPRGYYRCTYRKSQ-GCEATKQVQRSD-ENQMLLEISYRGIHSCSQ
HvWRKY24N       DGFSWRKYGQKD-ILGSRYPRRYYRCKHRLTQ-GCEAVKQLQATD-GDPLLFNAMYVGNHICIQ
HvWRKY25N       DGFSWRKYGQKDNIFGSKYPRGYYKCNH--AR-GCPARKELQATD-GDPLLFDATYVGNHTCAH
HvWRKY86        DGFAWRKYGQKD-INGSNHPRLYYRCAFR-GE-GCAATRRVQRAQ-EEPAAFVIAYYGEHTCGA
HvWRKY87        DGFAWRKYGQKD-INGSNHPRLYYRCSSS-GE-GCAATRRVQRSQ-EDPAAFIIAYYGEHTCGA
HvWRKY33        DGHQWRKYGEKK-INNCNFPRYYYRCTYKDNM-NCPATKQIQQKDHSDPPLYQVTYYNEHSCNS
HvWRKY34        DGHQWRKYGEKK-LSNSNFPRFYYRCTYKTDL-KCPATKQVQQKDMSDPPLFTVTYFNHHSCNT
HvWRKY90        DGHLWRKYGQKN-IKDSVFP----RCSYREDK-QCLASKLVQQENHEDPPLFKVTYTYEHTCNS
HvWRKY92        DRFHWRKYGEKK-ILHADFPRLYYRCGYSDEH-KCPAKKYVQQQNSGDQPMFLVTLINDHTCDT
HvWRKY97        DGFHWRKYGEKK-ILNAVFPRSYYRCGYSDEH-ECPAKKHVQQQNNSDPPMFIVTLFNDHTCSA
HvWRKY64        DGFQWRKYGQKV-IRGNAFPRCYYRCTYHQDH-GCSASKHVEQHNSADPPLFRVVYTNDHTCSG
AtWRKY38        DGYLWRKYGQKS-IKKSNHQRSYYRCSYNKDH-NCEARKHEQKIK-DNPPVYRTTYFGHHTCKT
AtWRKY62        DGFLWRKYGQKQ-IKESEYQRSYYKCAYTKDQ-NCEAKKQVQKIQ-HNPPLYSTTYLGQHICQL
HvWRKY83        DGYTWRKYGQKD-ILGSRFPRSYYRCTHKNYY-GCDAKKKVQRLD-DDPFMYEVTYCGSHSCLT
```

Appendix 2.2

Phylogenetic tree of barley WRKY family The multiple alignment is shown in appendix 2.1. Subgroups are indicated on the right side.



---to be continued---


HW52C
HW9
HW61
HW8
AtW7
HW7
HW10
HW63
HW72
HW11
HW44
HW55
HW80
HW84
HW45
AtW29
AtW27
AtW22
HW39
HW68
HW56
HW69
HW71
HW81
HW36
HW83
AtW38
AtW62
HW33
HW34
HW92
HW97
HW90
HW64
HW31
HW67
HW32
HW73
HW88
HW4
HW82
AtW70
HW86
HW87
HW24N
HW25N
HW47
AtW53
AtW30
HW91
HW24C
HW22
HW28
HW30
HW29
HW21
HW27
HW26
HW85
IId
IIe
IIc
III
0.05

Appendix 3 List of primers used in this study.

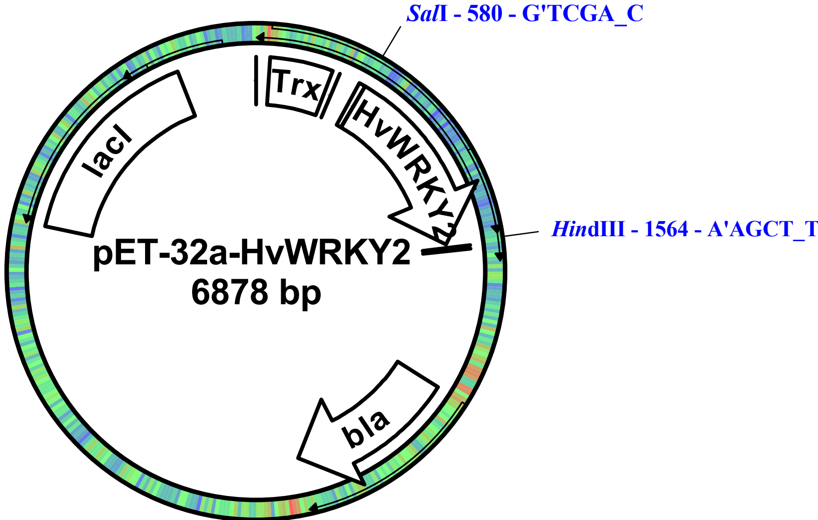
Introduced restriction sites are bold italicized.

Primers	Sequences (5'-3')
HvWRKY2Sal_F	ACGC***GTCGAC***ACATCGAGGAGCAGTGGA
HvWRKY2Hind_R	CCC***AAGCTT***GCTGGGAGATTTCCGATCA-
pET-W1EcoRI5	CG***GAATTC***ATGGATCCATGGATGGGCAG
pET-W1SalI3	ACGC***GTCGAC***TTAATTGATGTCCCTGGTCG
AtW40Bam_F	GCG***GGATCC***ATGGATCAGTACTCATCCTC
AtW40Hind_R	CCC***AAGCTT***CTATTTCTCGGTATGATTC
pHvW1Bam_F1	GCG***GGATCC***AACGTGACGGGGAGGAA
pHvW1Bam_F2	GCG***GGATCC***GCTGAGTGAATTTATTTTGAACG
pHvW1EcoR_R	CCG***GAATTC***TGGTCCCTCAAACACCAC
pHvW2Bam_F	GCG***GGATCC***CGGCCGTTTTGATTAGTGTT
pHvW2Hind_R	CCC***AAGCTT***GTCTTCCTCGAATTCACGGT
HvW3Bam	CGC***GGATCC*** ATGGAAACGG CGCGGTGGT
HvW3EcoRI	CCG***GAATTC***CTAATAATCC GGCAGCTTCC GC
HVW23SmaI	TCC***CCCGGG***ATGGACGAGCAGTGGATGAT
HvW23HindIII	CCC***AAGCTT***TCAGCTATTGGACGGCGACAG
HvW2-394ImiR-s	AGTTCAGACGTAGTCACCGACTACAGGAGATTCAGTTTGA
HvW2-394IImiR-a	TGTAGTCGGTGACTACGTCTGAACTGCTGCTGCTACAGCC
HvW2-394IIImiR*s	CTTAGTCCGTGTCTACGTCTGAATTCCTGCTGCTAGGCTG
HvW2-394IVmiR*a	AATTCAGACGTAGACACGGACTAAGAGAGGCAAAAGTGAA
amiRPCR4_F	ACACGGCCGTATCTAGATACCGCTGCTGATGCTGATG
amiRPCR4_R	GCCGCTCTAGAACTAGTGGATCCC
M13_R	TCACACAGGAAACAGCTATGAC
M13_F	CGCCAGGGTTTTCCCAGTCACGAC
pGY1fwd2	CGTTCCAACCACGTCTTCAA
NosT	ATTGCCAAATGTTTGAACGA
term35S-R	GCTCAACACATGAGCGAAAC
T7Prom	TAATACGACTCACTATAGGG
T7 term	ATCCGGATATAGTTCCTCCTTTC
HvUbi60deg_fF	ACCCTCGCCGACTACAACAT
HvUbi60deg_R	CAGTAGTGGCGGTCGAAGTG
RT04837WRKY2rev	CGTGGACGTAGACCTTGGAG
UBI-Prom5'UTR	AACCAGATCTCCCCCAAATC
HvW2_F	AACAACCACCACCAGTCGTT
HvW2_R	TCACCTTCTGCCCGTACTTC
RT9-10_F	GACGAGGGACCCCAGCTTCAA
RT9-10_R	CATGGAAATTATGGAACGGAACATTTG
HvPR1b-F	GGACTACGACTACGGCTCCA
HvPR1b -R	GGCTCGTAGTTGCAGGTGAT
HvPR2_F	TCTACAGGTCCAAGGGCATC

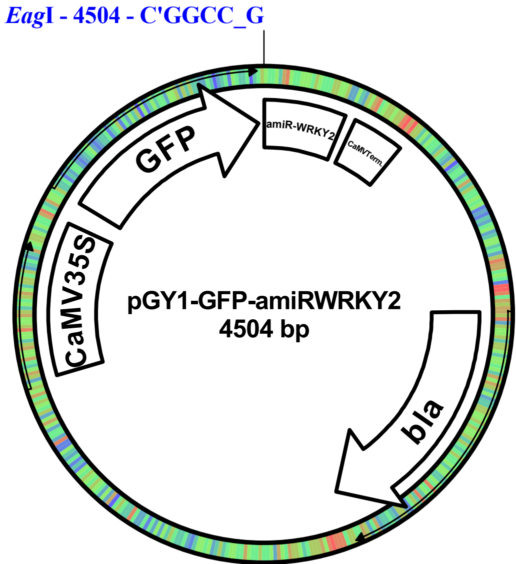
HvPR2_R	GATGTTCACGGCAGGGTAGT
HvPR5_F	CAAATTCACCACCACTGCAC
HvPR5_R	GTGTAGGAGCACCGGTTGAC
HvNH1_F	CAGGTCGACAACCCTTTCAT
HvNH1_R	GGATCCTTGTAGAGCTCGTCCAT
IGS_F	GCTTGCCTTAGCATCCTCAC
IGS_R	GGTAGCACAGCAGCAATCAA
HvBI-1_F	GTCCCACCTCAAGCTCGTTT
HvBI-1_R	ACCCTGTCACGAGGATGCTT
synaptotagmin_F	AGTTCCAGTTCGTGGTGGAC
synaptotagmin_R	CCTGGAGTTGATGAGGTGGT
XI_F	CACAACACAAGCCCAAAATG
XI_R	CGGAGAGGTCAAAGACCAGT
Egh16_for	GTGTGCCGGTACAAATGATG
Egh16_rev	GCTTCCTTTCCAGCTTCCTT

Appendix 4

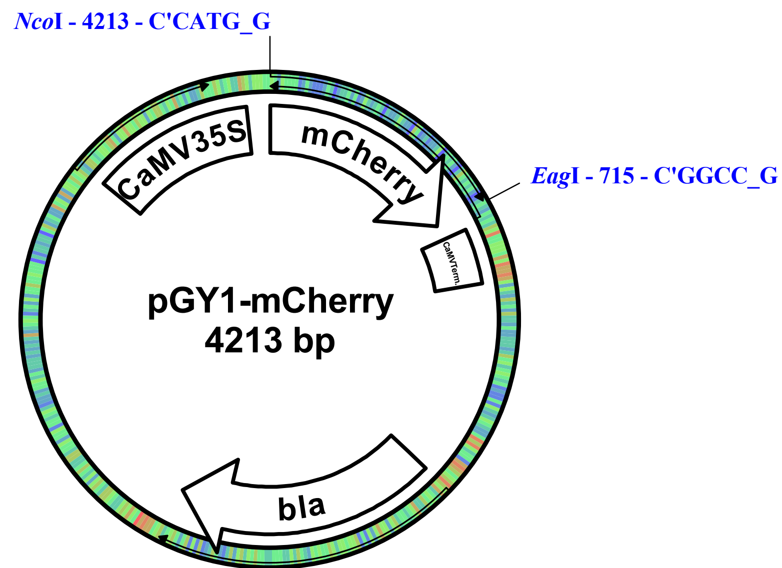
Maps of vectors generated in this study



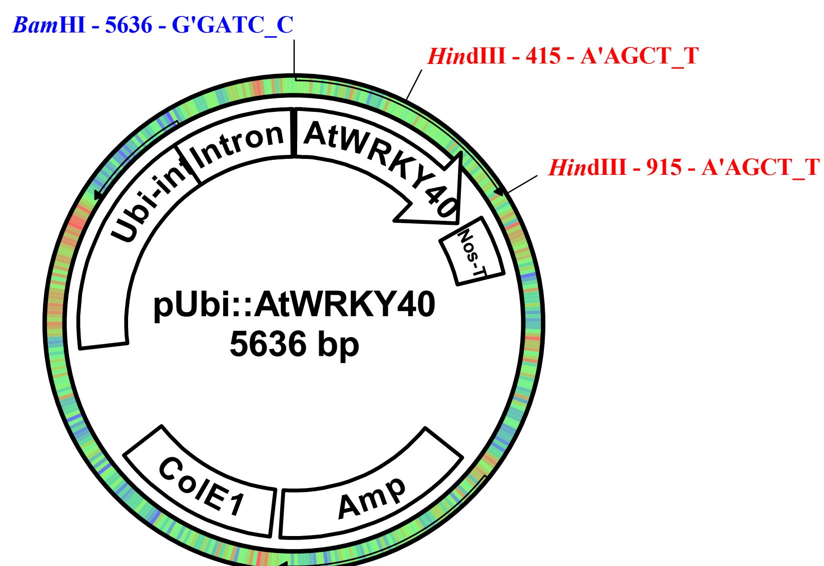
Appendix 4.1 **Vector map of pET32a-HvWRKY2**



Appendix 4.2 **Vector map of pGY1-GFP-amiRWRKY2**



Appendix 4.3 Vector map of pGY1-mCherry



Appendix 4.4 Vector map of pUbi::AtWRKY40
(The plasmid pDONR-AtWRKY40 was kindly provided by Dr. Imre E. Somssich)

Declaration

I declare that the dissertation here submitted is entirely my own work, written without any illegitimate help by any third party and solely with materials as indicated in the dissertation.

I have indicated in the text where I have used texts from already published sources, either word for word or in substance, and where I have made statements based on oral information given to me.

At all times during the investigations carried out by me and described in the dissertation, I have followed the principles of good scientific practice as defined in the "Statutes of the Justus Liebig University Gießen for the Safeguarding of Good Scientific Practice".

Signature:
Date:

Acknowledgements

"Life is a journey, not a destination." When making moves in the scientific path, I realised that I owe a debt to a number of people from whom I have directly or indirectly benefited a lot in this journey.

First of all, I would like to thank Prof. Karl-Heinz Kogel for providing me the opportunity to work with my PhD project under his supervision. His excellent advices, unlimited encouragement and support throughout the graduate study will always be remembered.

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Many thanks to Dr. Patrick Schweizer (IPK, Gatersleben) for offering the *pHvGER4c::GUS* constructs, Dr. Imre E. Somssich (MPIZ, Köln) for providing the AtWRKY40 cDNA clone and Prof. Carla Caruso (Universita' della Tuscia, Italy) for sharing the *35S::TaWRKY78* and *35S::AtWRKY20* plasmids.

I appreciate the kind suggestions, serious comments and enormous help from Dr Adam Schikora, Dr. Patrick Schäfer, Dr. Jafargholi Imani and Drs. Alga Zuccaro during the thesis research. Their efforts and successful experience in scientific path will always encourage me to be a good scientist.

Special thanks to Elke Stein and Martina Claar, from whom I learned a lot for the bench work in the lab and enjoyed a lot from the leisure time outside of the lab.

Der Lebenslauf wurde aus der elektronischen Version der Arbeit entfernt.

The curriculum vitae was removed from the electronic version of the paper.

During the four years graduate study full of trouble and joyfulness, I also appreciate Dagmar Biedenkopf and Christina Neumann and Rebekka Fensch for their technical guidance and practical help.

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