Transcription factors in Induced Systemic Resistance in *Arabidopsis thaliana*

In search of a molecular link between transcription factors MYB72 and MYC2 through action of ERF2

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Abstract

Understanding the molecular mechanism of how plants regulate their defence mechanism is very important. This to better understand, regulate and grow agricultural crops. Recently, plant defence has been extensively studied; revealing two very important transcription factors involved; MYC2 and MYB72. Both seem to have significant importance in induced systemic resistance (ISR). MYC2 has been previously shown to be important in the regulation

of transcription of genes against wounding and herbivory, so the question arises how this gene is also important in ISR and might there be a molecular link between the resistance against pathogens, herbivory and ISR.

The balance between transcription of genes against wounding and against pathogens is regulated through the balance of MYC2 and ERF1. The expression patterns of multiple homolog of ERF1 and of MYC2 were studied, as well as another related MYB transcription factor. Also the target promoter sequences were studied. Transcription of ERF1 is regulated by EIN3, a close homolog of EIL3. EIL3 is known to interact with MYB72. Here we investigated the predicted expression patterns to establish whether it is plausible that MYB72 regulates the action of MYC2 through action of ERF1.

Based on the analysis of existing data sets, it seems very plausible the interaction between MYB72, EIL3 and possibly MYB10 regulates the transcription of ERF1 to influence the balance of transcription of genes against pathogens or wounding. However, extensive further research is necessary.

Introduction

Transcription factors are very important regulators of various processes in all organisms. Plants and animals share transcription factors that are conserved between them. An example is the E2F transcription factor which is involved in cell cycle functions (Inze, De Veylder 2006). Other transcription factors have diverged in function and are either animal specific or plant specific. Even within species, transcription factors have diverged and in the case of *Arabidopsis thaliana*, about 45% of the transcription factors belong to transcription factors specific to plants. As in animals, within species the transcription factors have expanded enormously, suggesting involvement in clade-specific functions. Families of transcription factor that have expanded largely during the last 100 million years in plants include MADS box proteins, basic region leucine-zipper proteins (bZIP) and the MYB and bHLH families (Becker et al. 2000, Riechmann et al. 2000, Chen, Rajewsky 2007).

Among other processes, all these transcription factors are involved in plant development, biosynthesis, circadian clock, stem cell maintenance, stress responses and defence. All these processes are subject to research at this very moment. The research on how defence

Table 1. Classes of MYB transcription factors.

subgroup	members in *A. thaliana*	repeats	subgroups	encodes proteins involved in
4R	1	4 R1/R2 repeats	-	yet unknown
3R	5-8	R1/2/3 repeat	-	(conserved gene class in eukaryotes) cell cycle control
1R MYB, MYB related and others	64	R1 or partial repeat	• R3 type	• cellular morphogenesis • secondary metabolism control
			• evolutionary older R1/R2	• core components of central circadian oscillator
			• GARP	• morphogenesis • chloroplast development • response to phosphate starvation
R2R3	126	R2/R3 repeat	25 subgroups	• primary and secondary metabolism • cell fate and identity • developmental processes • responses to biotic and abiotic stress

mechanisms in plants against pathogens works is especially important for agricultural uses. It is essential to understand how plant species defend themselves against pathogens present in the soil. One of the least understood mechanisms proves to be the most promising in agricultural use. Induced Systemic Resistance (ISR) was first found in wheat and carnation, but also in *A. thaliana* (van Loon, Bakker & Pieterse 1998). ISR is triggered by beneficial soil-borne microbes, such as *Pseudomonas fluorescens.* The first step in the activation of ISR is the recognition of the beneficial microbes. Compounds on the cell surface play an important role for this recognition. Upon recognition, the transcription factor MYB72 is upregulated in the roots (Van der Ent et al. 2008). Mutant *myb72* plants do not have an ISR response in the leaves, neither upon colonisation by ISR-inducing plant-growth promoting rhizobacteria or fungi. This indicates that the transcription factor MYB72 plays an essential role in the onset of ISR. In addition, systemic activation of ISR in the leaves requires an intact response to the plant hormones JA and ET. The transcription factor MYC2, which plays a key role in the regulation of JA-responsive gene expression, has recently been shown to play an important regulatory role in PGPR-mediated priming for enhanced defence in *A. thaliana* (Pozo et al. 2008). MYB72 and MYC2 play a key role in ISR. These transcription factors are therefore the focus of this paper. Where are these genes expressed? Do they interact? What are the binding sites of the genes?

Here we propose further research which is necessary for complete understanding of how MYB72 and MYC2 function in the ISR of *A. thaliana* and possibly in other plant species. If we understand the interactions and function of both transcription factors, we might be able to use our knowledge about ISR in various crops to increase the production of these crops.

Chapter 1. MYB and MYC transcription factors

A. thaliana contains about ~340 MYB transcription factors, which are involved in almost all processes in the plant, from developmental processes to defence mechanisms (Dubos et al. 2010). MYB transcription factors share the presence of one to four or more imperfect MYB repeats. These repeats function in DNA binding and protein-protein interactions (Kanei-Ishii et al. 1990). DNA binding of MYB transcription factors is mediated by the third α-helix of each repeat, which fits perfectly in the major DNA groove (Jia, Clegg & Jiang 2004, Ogata et al. 1996). MYB transcription factors can be divided into four different classes, based on the number of repeats (summarized in Table 1). The smallest group is the 4R-MYB transcription factor subclass. In *A. thaliana* this group consists of a single member. This putative protein contains four R1 and R2 repeats, but its function is yet unknown (Dubos et al. 2010). The 3R-MYB subclass family members contain 3 R1/R2/R3 repeats. This transcription factor family is conserved throughout all eukaryotic species. The five members all function in cell cycle control (Ito 2005, Haga et al. 2007). The second largest subclass of MYB transcription factors consist of those containing a single or partial R1 repeat. This subgroup includes 62 members and is further divided into three groups; firstly, the R3 type, which functions in cellular morphogenesis (Pesch, Hulskamp 2009, Simon et al. 2007) and in secondary metabolism control (Dubos et al. 2008, Matsui, Umemura & Ohme-Takagi 2008). Secondly, the evolutionary older R1/R2 group, in which the genes encode core components of the central circadian oscillator (Lu et al. 2009). And thirdly, the GARP transcription factor family (Hosoda et al. 2002). These genes function in morphogenesis (Kerstetter et al. 2001), chloroplast development (Waters et al. 2009) and in the response to phosphate starvation (Rubio et al. 2001). The largest subgroup within the MYB transcription factors is the R2R3-MYB group. The 126 members all contain both a R2 and R3 repeat in their sequence and share a N-terminal DNA-binding domain (the MYB domain) and an activation or repression domain located at the C-terminus. R2R3-MYB transcription factors have been found to function in several plant-specific processes including primary and secondary metabolism, cell fate and

identity, developmental processes and responses to biotic and abiotic stress (reviewed by Dubos et al. 2010).

The basic helix loop helix (bHLH) proteins are the second largest group of plant transcription factors, consisting of more than 160 members. The bHLH domain is highly conserved, it is made up of about 60 amino acids encoding two distinct regions. The basic region at the N-terminus contains a basic amino acid region which binds to the DNA-motif CANNTG, where N is any nucleotide. bHLH domains with at least five basic amino acids in this region, combined with the highly conserved HER motif (His5-Glu9-Arg13) are predicted to bind DNA (Atchley, Fitch 1997, Massari, Murre 2000, Toledo-Ortiz, Huq & Quail 2003). The helix-loop-helix region includes two amphipatic α-helices, consisting of mainly hydrophobic amino acids. The helices are connected by a loop of variable length. Proteins that contain the bHLH domain are able to form homo- and heterodimers, which can be required for correct DNA recognition and binding specificity (Brownlie et al. 1997, Carretero-Paulet et al. 2010). The bHLH domain is also known to be able to interact with proteins which do not contain the bHLH domain (Herold et al. 2002, Hernandez et al. 2007). Classification of the bHLH proteins has recently been reorganized, based on evolutionary relationships rather than tissue distribution and dimerisation ability (Pires, Dolan 2010, Murre et al. 1994). After phylogenetic analysis, a classification of 26 subgroups was allowed for (Pires, Dolan 2010), which was further expanded to 32 subgroups after analysis of several atypical bHLH proteins (Carretero-Paulet et al. 2010). The BHLH proteins are involved in a wide range of processes in *A. thaliana*. They are important for processes in development, differentiation and hormone signalling. The wide variety of subgroups and their functions have been nicely described and reviewed by Feller et al. 2011.

Chapter 1.1 A single MYB transcription factor, MYB72, is required for ISR

The MYB transcription factor MYB72 is directly involved in ISR. MYB72 was found to be upregulated upon colonization of the *A. thaliana* root by the non-pathogenic rhizobacterial *Pseudomonas fluorenscens* WCS417r (*Pf* WCS417r) (Van der Ent et al. 2008). Moreover, plants with a T-DNA insertion in *MYB72* were unable to mount ISR against the bacterial leaf pathogen *Pseudomonas syringae* pv *tomato* DC3000 (*Pst* DC3000) after colonization of the roots with the ISR-inducing *Pf* WCS417r (Van der Ent et al. 2008). Also, MYB72 is not specifically essential in ISR against *PSt* DC3000, but against a broader spectrum of

pathogens. *MYB72* transcripts accumulate in the root upon colonization of *Pf* WCS417r, but is not activated in response to hormones or biological agents tested (Van der Ent et al. 2008, Kranz et al. 1998). Together this indicates that MYB72 is likely to have an important role in the early signalling of rhizobacteria-induced ISR. Evenmore, MYB72 has been shown to have an important role in fungi (*Thricoderma asperellum*) induced ISR (Segarra et al. 2009). MYB72 functions as an early signalling node, required for the expression of ISR triggered by different beneficial soil microorganisms (Segarra et al. 2009).

Not a lot is known about MYB72; it belongs to the MYB-R2R3 subgroup of the MYB transcription factors, with the highest homology to MYB10, MYB58 and MYB63 (Kranz et al. 1998, Stracke, Werber & Weisshaar 2001). Its amino acid sequence shows a high homology with MYB-like transcription factors from rice (*Oryza sativa*) and from maize (*Zea mays*) (both 75% homology) (Van der Ent et al. 2008). Only the latter transcription factor has been the subject of recent research; it has been shown that the maize transcription factor MYB-Related Protein-1 (ZmMRP1) is expressed in so-called transfer cells. These cells have specializations that facilitate the transport of solutes across plant exchange surfaces. Also, ZmMRP1 target genes that are presumably involved in defence (Barrero et al. 2009, Gomez et al. 2009).

Secondly, MYB72 has been found to interact with EIL3 *in vitro* (Yeast 2 Hybrid system) (Van der Ent et al. 2008). Since overexpression of the MYB72 gene did not result in enhanced resistance, another factor must be required for proper ISR. Finding that MYB72 physically interacts with EIL3, it has been proposed EIL3 is a significant second requirement for ISR (Van der Ent et al. 2008). EIL3 is a member of the EIN3 family of transcription factors. EIN3 and its closest paralogs, EIL1 and EIL2, are essential transcription factors in ethylene regulated gene expression, through binding of ETHYLENE RESPONSE FACTOR1 (ERF1) (Solano et al. 1998). Ethylene signalling in the roots is necessary for ISR in the leaves (Knoester et al. 1999). This shows a direct link between transcription of MYB72 and hormonal pathways, since a fast onset of ISR throughout the plant would require a small mobile molecule which is able to communicate between the soil-borne and above-ground parts of the plant.

Recently, MYB72 was found to interact with MYB10 (Pieterse, personal communication). MYB10 also belongs to the MYB-R2R3 subgroup of the MYB transcription factors. Together

with MYB58 and MYB63, it was found to be closely related to MYB72 (Kranz et al. 1998, Stracke, Werber & Weisshaar 2001). Interaction of MYB10 and MYB72 provides another clue in how MYB72 would be able to activate ISR upon pathogen induction.

Chapter 1.2 MYC2 functions in ISR

A second transcription factor proved to be functionally important in the defence of plants is MYC2. MYC2 belongs to the large family of bHLH transcription factors, in *A. thaliana* there are more than 160 bHLH transcription factors. Like most transcription factors, MYC2 is involved in several processes ranging from stress responses to development (Toledo-Ortiz, Huq & Quail 2003, Heim et al. 2003). MYC2 is involved in blue light mediated photomorphogenic growth (Yadav et al. 2005), but more importantly MYC2 is involved in several hormonal switches, regulating abscisic acid (ABA), ethylene and jasmonic acid (JA) responses (Lorenzo et al. 2004, Dombrecht et al. 2007, Anderson et al. 2004). These hormones play a central role in the highly interconnected signalling networks controlling induced plant defences (Durrant, Dong 2004) (systemic acquired resistance) (Pozo et al. 2008) (jasmonates); (van Loon, Geraats & Linthorst 2006) (ethylene in disease resistance); (Asselbergh, De Vleesschauwer & Hofte 2008) (ABA in plant defence). MYC2 action is regulated by the degradation of repressor protein; JASMONATE ZIM domain (JAZ) proteins. At low levels of JA-Ile (JA conjugated with amino acid isoleucine (Ile), the only known JA derivative needed for JA signalling (Katsir et al. 2008)), MYC2 is bound to the promoter region of the JA-responsive gene, but its activity is repressed by homo- or heterodimers of JAZ proteins. However, upon developmental cues or stress, the levels of JA-Ile increase. This results in the binding of JA-Ile with the jasmonate receptor COI1, which is a F-box protein with leucine rich-repeat (LRR) domain. COI1 is part of the SCF-COI1 complex, and binding with JA-Ile leads to the ubiquitination of the JAZ proteins and subsequent degradation by the 26S proteasome. The repression of MYC2 is then released and activates the expression of the JA-responsive genes (as is reviewed by (Wasternack, Kombrink 2010)).

A putative stress signal inducing the increase of JA-Ile levels, is the mechanical wounding of plants; the rubber tree *Hevea brasiliensis* orthologs hblMYC1 and hblMYC2 respond to tapping, a process in which the tree bark is cut in order to release the rubber flow (Zhao et al. 2011). The expression levels of *hblMYC1* significantly increased, whereas tapping had little effect on the expression levels of *hblMYC2* (Zhao et al. 2011). This shows a

diversification in the function between hblMYC1 and hblMYC2. hblMYC1 and hblMYC2 share a 88.66% identity in amino acid sequence with *A. thaliana* AtMYC2, and studies in *A. thaliana* showed that transcript levels of *MYC2* were raised upon *Pf* WCS417r induction. Also the MYC2-impaired *jin1* mutants failed to display functional ISR upon WCS417r induction (Pozo et al. 2008). Previously, it had already been shown that MYC2-impaired *jin-1* mutants have an increased pathogen resistance, but a reduced insect pest resistance (Dombrecht et al. 2007). Interestingly, the genes involved in defence responses against pathogens are suppressed by MYC2, but are positively regulated by ERF1 and the genes involved in JA-mediated systemic responses to wounding are induced by MYC2, but repressed by ERF1 (Lorenzo et al. 2003). An interplay between these transcription factors enables the plant to effectively select the correct response to either soil-borne pathogen attack or above-ground wounding.

ERF1 is the key factor in the cross talk between ethylene and JA signalling, which determines the activation of genes in the defence against pathogens or herbivores (Lorenzo et al. 2003). It is, however, not the only factor that integrates both these pathways (Solano et al. 1998, Lorenzo et al. 2003). ERF1 activates the defence-related genes, including *PLANT DEFENSIN1.2 (PDF1.2)* (Solano et al. 1998, Lorenzo et al. 2003). Several other ERF transcription factors were found to affect the transcription of *PDF1.2,* but only OCTADECANOIDRESPONSIVE ARABIDOPSIS AP2/ERF59 ORA59 (At1g06160) was found to regulate genes known to be involved in defence. Especially *PDF1.2* was found to be upregulated in plants overexpressing ORA59. In plants where *ORA59* was silenced and the plants were treated with JA, *PDF1.2* induction was severely comprised. Also, plants overexpressing *ORA59* showed enhanced resistance to the necrotrophic fungus *Botrytis cinerea*, whereas plants in which *ORA59* was silenced were less resistant. ERF1 and ORA59 share a 40% amino acid identity over their entire length and both are able to bind to the promoter of *PDF1.2* (Pre et al. 2008, Zarei et al. 2011). Both ERF1 and ORA59 seem to be involved in regulation of defence genes in an antagonistic manner to MYC2.

In a transcript profiling experiment, approximately 25% of the ~1900 putative methyl jasmonate (MeJA)-responsive genes were found to be differentially expressed in ISR-induced plants, compared to non-induced plants. Analysis showed an overrepresentation of the G-

box-like motif in the promoter region of these genes, implying an important role of MYC2 in the regulation of the expression of the MeJA-responsive genes. Also, MYC2 expression was found to be upregulated upon ISR induction (Pozo et al. 2008). Taken together, these results show that MYC2 is involved in ISR, most likely induced by soil-borne pathogens to provide an induced priming state against insect pests.

Chapter 1.3 Closest paralogs of MYC2 are likely to act redundantly

The MYC2 transcription factor binds to the G-box-like motif 5'-CACATG-3' found in the promoter region of its targets. This motif is found in at least 30% of the 5' upstream region of all *A. thaliana* genes. It is possible that many other bHLH proteins bind to this sequence as well, and therefore very unlikely that MYC2 regulates the expression of all these genes (Dombrecht et al. 2007). The closest phylogenetic homologs of MYC2 are MYC3, MYC4 and bHLH028. MYC3 and MYC4 bind to the same JAZ proteins as MYC2, both *in vitro* (yeast two-hybrid screen) and *in vivo* (tandem affinity purification (TAP)). The latter protein does not interact with any JAZ proteins (Fernandez-Calvo et al. 2011). MYC2 shares conserved domains with MYC3 and MYC4, which interact with the JAZ proteins. In addition, the consensus DNA-binding site in the promoter region of putative targets of both MYC3 and MYC4 is strikingly similar to the preferred DNA-binding site of MYC2. The affinity for the consensus was less strong for MYC4, indicating it may recognize a slightly different subset of targets (Fernandez-Calvo et al. 2011). *myc3* and *myc4* mutants have an additive effect on the *myc2* mutant phenotype in regulating the JA-dependent response, mainly in the aerial tissues. This means the genes act redundantly. The expression pattern of *MYC3* and *MYC4* also shows an overlap in both the root and shoot, so it is very likely MYC3 and MYC4 act redundantly (Fernandez-Calvo et al. 2011).

MYC *2,* MYC3 and MYC4 display different expression patterns as well as different homo- and heterodimerization between them (Fernandez-Calvo et al. 2011). This could be the cause of the difference in up- and/or downregulation in expression of the direct downstream targets of MYC2.

Chapter 1.4 Hypothesis: A molecular link between transcription factors MYB72 and MYC2 through action of ERF2

In the defence against pathogens and herbivory there is a tight balance between the

regulation of the defensive genes. This is mediated by the transcription factors MYC2 and ERF1. MYC2 activates genes involved against wounding and herbivory and suppresses of genes involved against pathogens, whereas ERF1 regulates these genes antagonistically from MYC2. *MYB72* was found to be upregulated in ISR, causing a primed state against both pathogen and insect attack. It was previously shown that MYB72 interacts with EIL3, which in turn is able to bind the regulatory promoter region of *ERF1*. Here we propose a molecular link between the action of MYC2 and MYB72 (Figure 1). MYB72 is able to bind with an ethylene response factor; EIL3 and with another MYB transcription factor; MYB10. However, there are more closely related ethylene response factors in this family with which MYB72 or MYB10 could interact, like EIL1, EIL2, EIN3 and ORA59. These factors could form complexes which could bias the balance of MYC2 and/or ERF1 in the regulation of the set of genes in defence. Therefore, there are three questions that will be discussed here: (1) Do MYB72 and its interaction partner MYB10 interact with the other ethylene response factors EIL1, EIL2, EIN3, ORA59 and ERF1; (2) Does this MYB72-MYB10-ERF complex bind to the promoter region of ERF1 to mediate its expression; and (3) Does the MYB72-MYB10-ERF complex interact with the *MYC* genes?

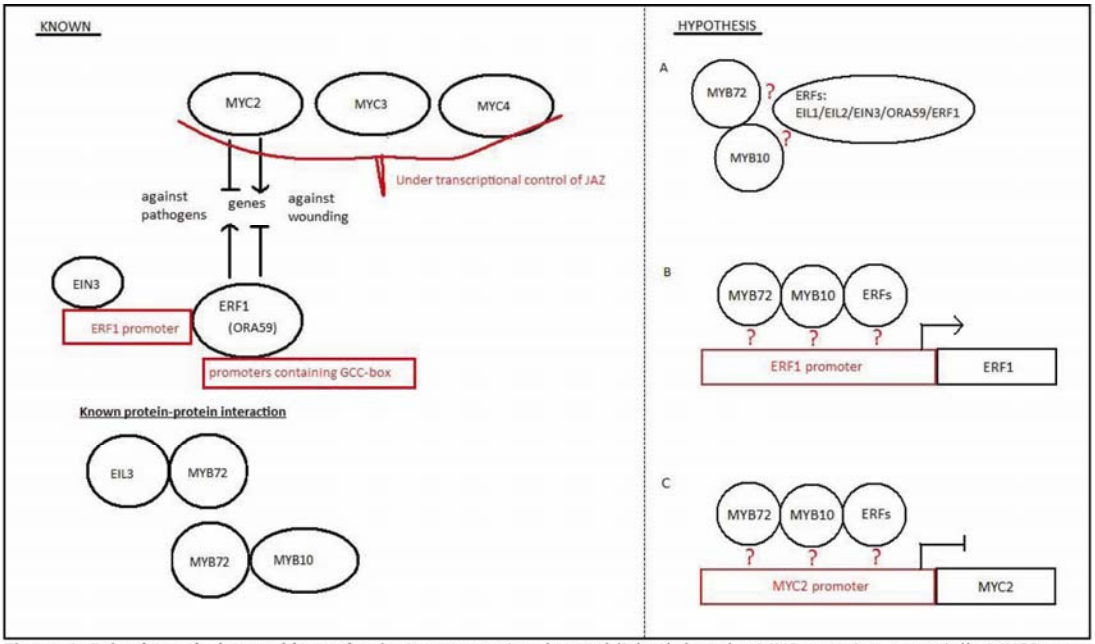


Figure 1. Prior knowledge and hypothesis. It was previously established that the MYC proteins, especially MYC2, regulate genes that play a role in the defence against wouding and pathogens. ERF1 and ORA59 act redundantly. Both are ethylene response factors that regulates the same genes as MYC2, but in an antagonistic manner. Transcription of the *MYC* genes is mediated though action of the JAZ repressors. ERF1 interacts with the GCC-box in the promoters of its target genes. ERF1 itself is regulated by EIN3. MYB72 is known to interact with EIL3 and with MYB10.
Here we have formulated three hypothesises: (A) Do MYB72 and its interaction partner MYB10 interact with the other ethylene response factors EIL1, EIL2, EIN3, ORA59 and ERF1; (B) Does this MYB72-MYB10-Ethylene response factor complex bind to the promoter region of ERF1 to mediate its expression; and (C) Does the MYB72-MYB10-ethylene response factor complex interact with the *MYC* genes?

Chapter 2. Analysis of the predicted expression pattern of the factors involved.

In order to investigate whether the hypothesized network of regulation is plausible, the predicted patterns of expression were analyzed. We presume that the genes are only able to interact if they are expressed in the same tissue. As some factors are only upregulated and act upon hormone treatment (e.g. *ERF1* and *ORA59*), or upon pathogen invasion (e.g. *MYB72* and *MYC2*) whereas others do not (that we know of), we only looked at the expression pattern of the transcription factors of interest in the wild type root and of the wild type plant during development. These expression patterns are of non-induced plants. The expression patterns could change upon hormone treatment or hormone changes induced by pathogen inoculation. The expression patterns are basal levels in wild type plants, after a trigger (e.g. environmental or pathogen attack) these patterns could change. It is however a promising indication if the transcription factors of interest are present in similar expression domains in wild type non-induced plants. It must be noted that the predicted expression patterns are shown in absolute expression. The discussed predicted expression patterns are based on two databases; the expression data from Benfey's lab (Birnbaum et al. 2003) and the more recent *Arabidopsis* eFP browser (Winter et al. 2007).

Chapter 2.1 Expression pattern of genes throughout development

Throughout development, the transcription factors of interest are both differentially expressed as well as in a overlapping manner (Figure 2). MYB72 is expressed throughout the whole plant, but especially in the root, the entire rosette and the mature pollen (Figure 2a). MYB transcription factors MYB32 and MYB80 have been reported to be involved in pollen development (reviewed by (Feller et al. 2011)(Dubos et al. 2010, Prouse, Campbell 2012)). MYB72 could potentially function in a redundant manner to establish proper cell wall composition in pollen cell walls. As for expression of MYB72 in the rosette and leaf, this is consistent with the upregulation of *MYB72* upon pathogen or ISR inducing microbe inoculation (Van der Ent et al. 2008). These microbes are soil-borne and their first site of contact with the plant is logically the root and rosette.

The other MYB transcription factor of interest, MYB10, is also predicted to be expressed in the root and rosette of the plant, as well as in the dry seed and during embryogenesis (Figure 2b). Since MYB72 and MYB10 have been found to interact (C. Pieterse, personal communication), it is likely this interaction will be found *in vivo*, since both MYB

transcription factors are predicted to be expressed in the root and rosette. MYB10 is also predicted to be expressed in the dry seed, which is consistent with previous findings of MYB transcription factors to be involved in anthocyanin biosynthesis of the seed coat. In apple fruit, MYB transcription factors cause the red colour in the apple fruit seed coat (reviewed by Feller et al. 2011).

Expression pattern of ERF1 overlaps with that of MYB72

Most of the ethylene response factors of interest in this paper (ERF1, EIN3, EIL1, EIL2 and EIL3) are closely related transcription factors. EIN3 is able to regulate the expression of *ERF1 in vivo*, being able to bind to its promoter region (Solano et al. 1998). Also, EIL3, closely related to EIL1, EIL2 and EIN3, has been shown to interact with MYB72 *in vitro* (Van der Ent et al. 2008). This interaction is only plausible *in vivo* if both transcription factors are co-expressed. This interaction poses a very interesting regulatory pathway. If MYB72 and EIL3 would be able to interact *in vivo*, and if this complex would be able to bind to the promoter region of *ERF1*, this complex could be able to regulate *ERF1* transcription. This way MYB72 would be able to affect the balance between *ERF1*/*MYC2* transcription and thereby the outcome of their downstream targets; the genes against pathogens of the genes against wounding and herbivory.

ERF1 is mostly expressed in the senescent leaf, but in a lesser extent in most other parts of the plant, with the only exception of the early developing flowers (Figure 2c).

Overexpression of *ERF1* showed ERF1 to be involved in the regulation of defence-related genes induced by ethylene and jasmonate as well as late defence response genes (Lorenzo et al. 2003). Logically these genes are found throughout the plant. ERF1 needs to activate the transcription of genes against pathogen attack in the root and ERF1 represses the transcription of genes against wounding and herbivory in the above-ground part of the plant.

Ethylene response factors EIN3, EIL1, EIL2 and EIL2 patterns partially overlap with MYB72

The transcription factor EIN3 is primarily predicted to be expressed in the dry seed and mature pollen. It is also predicted to be expressed in the shoot, between the first and second internode of the shoot (Figure 2d). EIL1 has a broader predicted expression pattern (Figure 2e). The highest predicted expression is found in the later stages of embryogenesis, the dry

seed and the mature pollen. In a lesser extend this transcription factor is predicted to be expressed in the root, rosette and maturing flower.

EIL2 is only predicted to be highly expressed in the mature pollen (Figure 2f). It is predicted to be expressed in several other organs, however this is so little, it could be considered negligible. EIL3 is predicted to be highly expressed, especially in the dry seed, the later stages of embryogenesis and in the mature pollen (Figure 2g). It is also predicted to be expressed in the rosette and the shoot, including the shoot apical meristem (SAM) and developing and mature flowers. EIL3 is able to interact with MYB72 *in vitro* and these predicted expression patterns show that EIL3 and MYB72 are predicted to be co-expressed in the rosette and in the root, where it is most likely to function in the repression of *ERF1* transcription, ERF1 also being expressed there (Figure 2a, 2c and 2g). MYB10 is also known to interact with MYB72 and is predicted to be co-expressed with MYB72, as well as with ERF1 and EIL3. This means it is very likely these factors are to interact *in vivo*.

ORA59 is expressed in the same tissue as ERF1

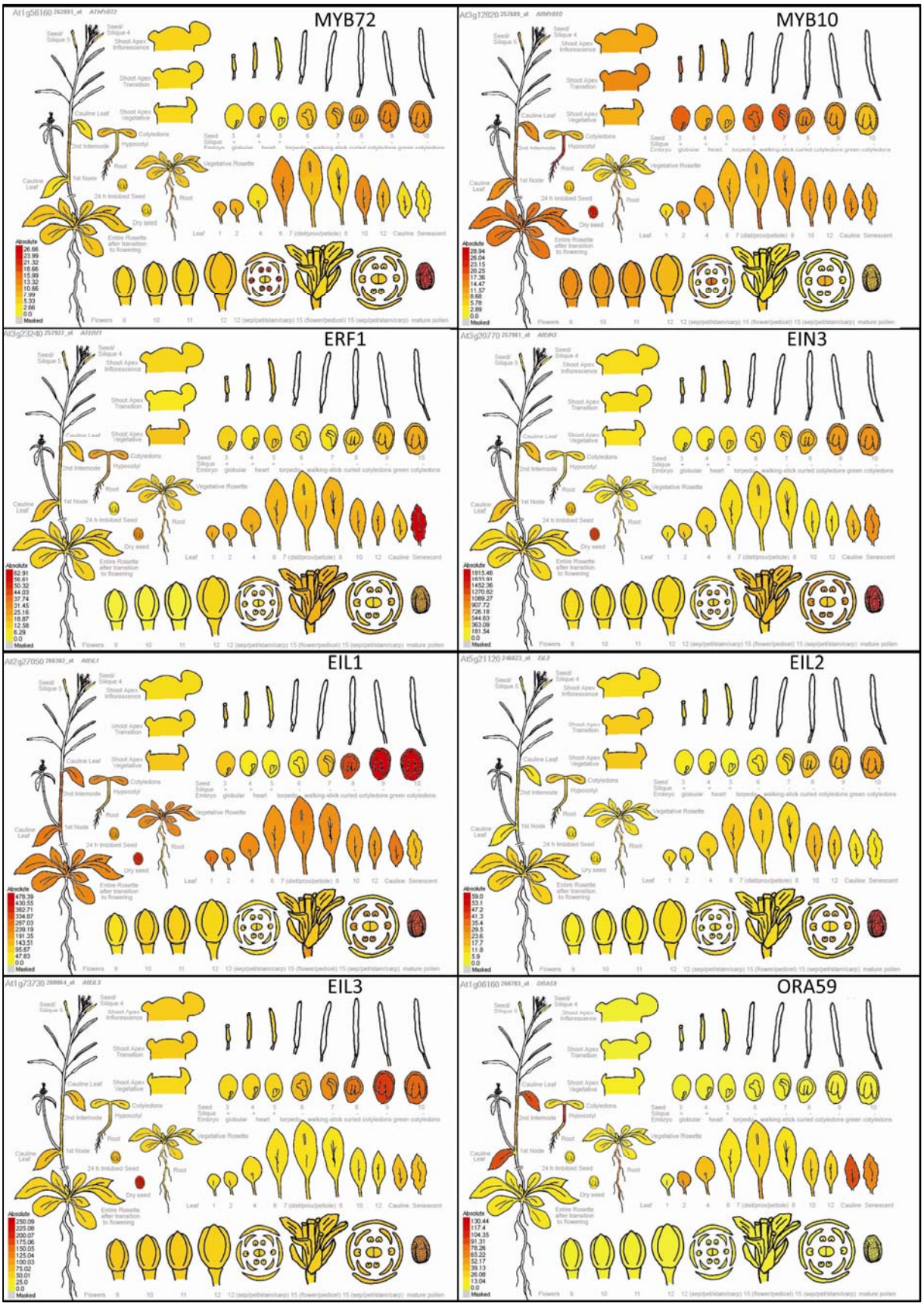
ORA59 is closely related ethylene response factor, which is able to activate the same downstream plant defence targets as ERF1 (Pre et al. 2008). It is thought ORA59 and ERF1 share a redundancy in this activation and therefore it is likely that the MYB72-MYB10-EIL3 complex would be able to regulate the transcription of this factor as well. The predicted expression pattern of ORA59 in development is slightly different from the predicted expression pattern of ERF1 (compare Figure 2h to Figure 2c). In terms of absolute expression, ORA59 is predicted to be more expressed than *ERF1*, but they are expressed in similar domains. Both are mostly expressed in the senescent leaf. Where ERF1 is expressed to a lesser extend throughout the plant, ORA59 has a more specific predicted expression pattern. ORA59 is predicted to be highly expressed in the cauline leaf, the hypocotyl, the root and in the second internode between the cauline leaves. The predicted expression pattern of ORA59 overlaps the predicted expression pattern of ERF1, EIL1, MYB72 and MYB10 with respect to the internode between the second internode of the shoot and with respect to all the factors being present in the root.

MYC2 expression pattern overlaps with ERF1 expression

Not surprisingly, MYC2 is predicted to be expressed in similar domains throughout the plant (Figure 2i) as ERF1. MYC2 is predicted to be expressed everywhere in the plant with the exception of embryogenesis. Since MYC2 would activate the downstream targets involved in defence against wounding and herbivory, the highest expression is predicted to be found in the SAM, the developing flower and in the leaves. However, it is also predicted to be expressed in the root, just like ERF1, MYB72, MYB10, EIN3, EIL3 and ORA59. Regulation of transcription through the previously proposed transcription factors *in vivo* is not unlikely, since they are all predicted to be expressed in similar domains.

***MYC2*, *MYC3* and *MYC4* share an overlapping expression domain**

MYC3 and MYC4 are known to be able to interact *in vivo*, forming homo- and heterodimers with themselves and with MYC2. Also, MYC3 and MYC4 act additively with MYC2 to regulate specifically different subsets of the JA-dependent transcriptional response in defence (Fernandez-Calvo et al. 2011). Naturally, these transcription factors share expression domains within the plant, since they have been proven to be able to interact *in vivo*. Since it is most likely the transcription factors have evolved to have their separate functions as well, the three transcription factors also have their own distinct predicted expression patterns. MYC3 is proposed to be expressed mostly in the senescent leaf, the SAM and the cauline leaves. It can also be found in the developing flower, the root and the rosette leaves. It is predicted to be absent from embryogenesis (Figure 2j). MYC4 is predicted to be expressed mostly in the internode between the cauline leaves. It is also predicted to be expressed in the cauline leaves, the root and in early stages of embryogenesis (Figure 2k). These differences in predicted expression patterns clearly show overlapping areas of expression (all three are expressed in the first internode between the cauline leaves, as well as in the cauline leaves and the root). MYC2 and MYC3 share their expression pattern in the maturing flower, whereas MYC4 is not expressed in this developmental stage. MYC2 and MYC3 seem to be absent from embryogenesis, while MYC4 is proposed to be expressed early embryogenesis and most likely will have a function in development there.


MYB72
MYB10
ERF1
EIN3
EIL1
EIL2
EIL3
ORA59

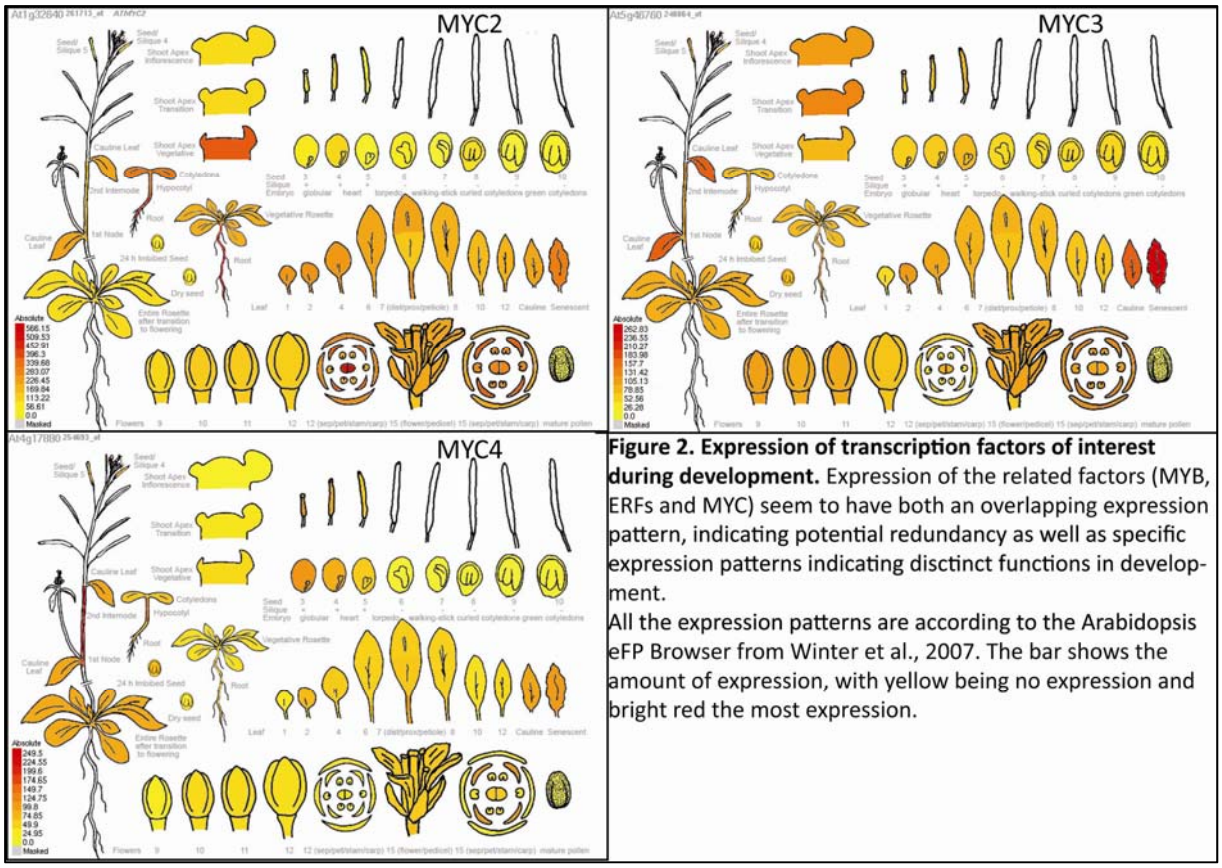


Figure 2. Expression of transcription factors of interest during development. Expression of the related factors (MYB, ERFs and MYC) seem to have both an overlapping expression pattern, indicating potential redundancy as well as specific expression patterns indicating disctinct functions in development.
All the expression patterns are according to the Arabidopsis eFP Browser from Winter et al., 2007. The bar shows the amount of expression, with yellow being no expression and bright red the most expression.

Table 2. Table of expression of the transcription factors of interest in the root. Based on Arex database (Birnbaum et al. 2003) and *Arabidopsis* eFP browser (Winter et al. 2007). +: expressed in this layer; RH: only expressed in the root hair cells of the epidermal layer.

					stele				stem cells				
	epidermis	cortex	endodermis	pericycle	phloem	xylem	procambium	QC	columella	CEI	stele	columella	LRC
MYB72	RH						+						
MYB10							+					+	+
ERF1	+	+		+									
EIN3		+	+	+	+	+		+	+				+
EIL1	+/RH	+							+				
EIL2							+						
EIL3	+		+	+								+	
ORA59							+					+	
MYC2		+	+	+	+								
MYC3	RH	+	+	+	+							+	+
MYC4	RH		+	+		+							

Chapter 2.2 Expression pattern of genes in the wild type root

Since the onset of ISR is mediated in the root, due to soil borne microorganisms, it is essential to study the predicted expression pattern of the transcription factors of interest in the root. These expression patterns are based on the Arex database (Birnbaum et al. 2003) and on the more recent established *Arabidopsis* eFP browser (Winter et al. 2007) and summarized in Table 2. Unfortunately, the results from the *Arabidopsis* eFP browser are not as clear as the developmental expression pattern (Figure 3).

In the root, MYB72 is expressed in the root hair cells of the epidermal layer and in the procambium. MYB72 is co-expressed with ERF1, EIL1, EIL3 and both MYC3 and MYC4 in the root hair cells of the epidermis. The interaction between MYB72 and EIL3 is very likely to happen *in vivo* in this cell type. However, what this functionally means with respect to the induced state of ISR and defence targets is yet unknown.

EIL3-MYB72 and EIN3-MYB10 complexes could be formed *in vivo*

MYB10 and MYB72 are only co-expressed in the procambium, together with EIL2 and ORA59. In this cell file, MYB72 could be able to manipulate the transcription of defence target genes, since ORA59 and ERF1 partially regulate the same subset of genes involved in defence, including the activation of *PDF1.2* (Pre et al. 2008). Interaction between MYB72 and EIL2 has not yet been established, either *in vitro* or *in vivo*. However, it is likely these factors are able to interact, since EIL2 is closely related to EIL3 (Chao et al. 1997). Analysis of the EIL1, EIL2, EIL3 and EIN3 transcription factors revealed they share a predicted amino acid sequence and structural similarity. This sequence similarity ranges from 59% to 85%, with EIL1 being the most related transcription factor and EIL3 the least related to EIN3 (Chao et al. 1997). Also, EIL1 and EIL2 are able to complement the *ein3* single mutant, providing evidence of their functional similarity, making it quite possible EIL2 and MYB72 could interact *in vivo*. The same holds for MYB10 and EIN3, both these genes are predicted to be expressed in the lateral root cap cells (LRC).

Regulation of ERF1 through EIN3 is mediated by a shared expression domain in the root

ERF1 is primarily expressed in the pericycle cells, but is also expressed in the epidermis cells and in the cortex cells of the maturation zone of the root (Figure 3c). Since it is previously established that *ERF1* transcription is regulated by transcription factors EIN3 and EIL1, it is worth looking at their expression. EIN3 is expressed in the undifferentiated columella stem

cells. It is also expressed, though in a lesser extent, in the cortex cells, pericycle cells and the xylem cells of the maturation zone (Figure 3d). EIL1 on the other hand, is expressed in the cortex and epidermis cells throughout the root, from the meristematic zone, in the elongation zone to the maturation zone (Figure 3e). EIL1 is not expressed in the pericycle cells, where both ERF1 and EIN3 are expressed. Therefore, in the pericycle cells, *ERF1* could be regulated by solely EIN3 and in the epidermis cells redundantly bij EIL1. In the cortex cells *ERF1* transcription could be regulated redundantly by both EIN3 and EIL1. Also in the columella stem cells, EIN3 and EIL1 are expressed together. Since ERF1 is not expressed in these cells, it is possible that EIN3 and EIL1 and a yet unknown transcription factor form a complex there in order to establish the columella stem cell fate. The unknown transcription factor is not one of the transcription factors of interest, since no other of these factors is expressed in the columella stem cells. How ethylene would be able to establish the columella stem cell fate is unknown.

Expression domains of ORA59 and ERF1 do not overlap

ORA59 and ERF1 act on similar target transcription factors, being a crosslink between the JA and ET hormonal pathways in plant defence (Lorenzo et al. 2003, Pre et al. 2008). It was previously thought these factors have similar expression patterns, even though this was only based on the response of transcription of these genes in several mutant backgrounds and the knowledge that both these genes partially regulate the same subset of genes involved in defence, including the activation of *PDF1.2* (Lorenzo et al. 2003, Pre et al. 2008). The predicted expression patterns of ERF1 and ORA59 are quite different (Figure 3c and figure 3h). There are no overlapping expression domains. ERF1 is predicted to be expressed in the epidermis, the cortex cells and in the pericylce cells, whereas ORA59 is predicted to be expressed in the procambium and in the columella cells. However, the predicted expression domains of both genes do complement each other, making it very plausible the genes have similar functions in different expression domains. Since the ethylene response factors, to which ORA59 also belongs, are closely related, it is possible that ORA59 is diverged directly from ERF1, sharing the same function, simply in a different expression domain. This would make sense, especially in the defence against pathogens. If the first boundary, set by the expression of ORA59 in the columella cells, located at the very tip of the root, has been

overcome by the pathogens, the second boundary is still intact, regulating the same defence pathway. However, this is only a hypothesis on which no research has been done.

Redundancy between the three *MYC* genes

The three *MYC* genes show a similar predicted expression pattern (Figure 3i, 3j, and 3k). MYC2, MYC3 and MYC4 are predicted to be co-expressed in the endodermis layer and in the pericycle cells. There they could function in a redundant manner, making sure the early JA-dependent defence genes are being activated. Also, in several other layers in the root at least two of the MYC genes are predicted to be co-expressed. In the root hair cells of the epidermal layer, both MYC3 and MYC4 are expressed and MYC2 and MYC3 are predicted to be co-expressed in the cortex and the phloem. MYC4 is also expressed alone in the xylem, which could indicate a separate independent function of MYC4 which is yet unknown. The same holds for MYC3. This transcription factor is expressed without its family members in the columella cells and in the lateral root cap cells. Since this expression domain is completely different from the domain in which the three *MYC* transcription factors are co-expressed.

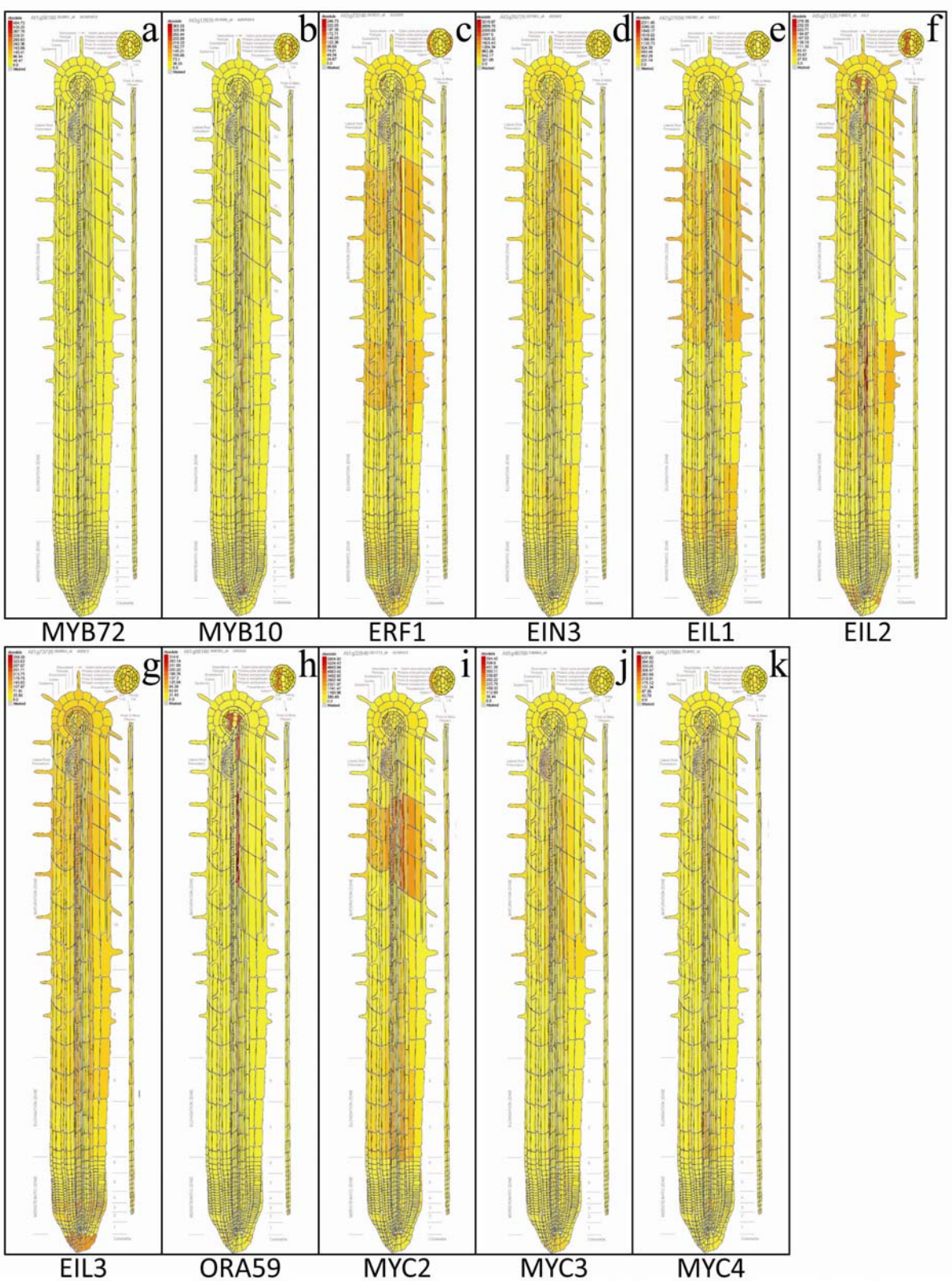


Figure 3. Expresseion pattern of the transcription factors of interest in the root. Absolute expression, based on the Arabidopsis eFP browser (Winter et al., 2007). Scale of expression is from bright yellow (no expression) to bright red (maximum absolute expression. Layers are described in the figure.

Chapter 3. Interaction between the transcription factors of interest

Now we have established transcription factors of interest could interact *in vivo*, because they are expressed in the same tissues, either during development or in the root. The next step, logically, would be to uncover whether these transcription factors are able to interact; we are interested in whether the proteins have known DNA- and protein-interaction sites. However, not all sequences have been studied extensively and DNA-binding and protein interaction sites remain unknown. It is however a very strong tool in the prediction as to whether proteins are able to interact *in vivo*.

Table 3. Known interaction domains and target sequences of the transcription factors of interest.

transcription factor	interaction domain	target gene	target DNA sequence
MYB72		EIL3 (1)	
EIN3/EIL1/EIL2/EIL3		ERF1 (2)	GCC-box (3)
ORA59		PDF1.2	GCC-box (4)
EIN3		SID2	ATGTAC (5)
R2R3 MYB	3 conserved tryptophans (6)		
JAZ	N-terminus ZIM domain	COI1 (7)	
JAZ	C-terminus	MYC2	G-box: CACGTG (7)
JAZ	JAS domain	MYB21/MYB24 (8)	
MYC2/3/4		JAZ (9)	G-box-TTTT (10)

References: (1) Van der Ent et al., 2008; (2)Chao et al., 1997; Solano et al., 1998; (3) Lorenzo et al., 2003; (4) Zarei et al., 2011; (5) Kosugi & Ohashi, 2000; Chen et al., 2009; (6) Kanei-Ishii et al., 1990; (7) Dombrecht et al., 2007; Chini et al., 2007; (8) Song et al., 2011; (9) Niu et al., 2011; (10) Figueroa & Browse, 2012

Chapter 3.1 Target DNA binding sites

When it comes to target DNA binding sites of the predicted regulatory interaction, such as the JAZ-proteins being targeted to the *MYC* transcription factor promoters in order to regulate their transcription, only in the last decade tools have been developed in order to investigate transcription factor binding to DNA sequences. ChIP-sequencing techniques are used to obtain a global idea of the DNA sequence the transcription factor binds *in vivo*. Using this technique, it is now known that several different transcription factor binding sites are present in promoters of genes. For example, EIN3 binds to the ATGTAC-sequence in the promoter of *SID2*; a transcription factor important in the crosstalk between two hormonal pathways; the salicylic acid (SA) pathway and the ethylene pathway (Table 3) (Kosugi, Ohashi 2000).

The GCC-box is a target sequence of the ethylene response factor transcription factors

In our selection of transcription factors of interest, only two target DNA sequences are known. The first is the GCC-box to which the ethylene response factors are able to bind and is found in the promoter region of *ERF1* and *PDF1.2* (Table 3)(Chao et al. 1997)(Solano et al. 1998, Lorenzo et al. 2003, Zarei et al. 2011). The consensus sequence of the GCC-box consists of 6 nucleotides; GCCGCC (Lorenzo et al. 2003). There are two of these GCC-boxes in the *PDF1.2* promoter region; the first is located at -262 to -257 bp. The nucleotides that make up this box are according to the consensus sequence of the GCC-box: GCCGCC. The second GCC-box in the promoter region of *PDF1.2* is slightly different; GCAGCCGCT. ORA59 and ERF1 both bind to these two GCC-boxes. Also, both boxes are functionally equivalent and necessary for JA and/or JA/ET responsive activity of *PDF1.2* (Zarei et al. 2011). This indicates one of the major problems in the prediction of target DNA-sequences of transcription factors that we are interested in, in this study. Since the development of ChIP-sequencing several consensus sequences have been found. However, transcription factors are able to bind sequences that only slightly resemble the consensus sequence, as is described above. So if we search the databases for the consensus sequence, it is very possible that we miss sequences that resemble the consensus sequence, but are essential in binding of the regulating transcription factor and therefore essential for the correct transcription of the target gene.

The G-box sequence is important in MYC regulated transcription

The other target DNA sequences which are known to be involved in our selection of transcription factors of interest, is the G-box sequence. The consensus sequence is CACGTG/T (Chini et al. 2007). This consensus sequence was found in the promoter region of transcription factor *MYC2*. This transcription factor is regulated by action of the JAZ factors (as described in Chapter 1.2). *MYC2* transcription is possible when the JAZ proteins are targeted by the SCF-CO1 complex, the JAZ proteins are ubiquitinated and hence degradated by the 26S proteasome. The repression of *MYC2* transcription is then released and subsequent cascade of MYC2 regulated defence genes is either repressed or activated.

The JAZ genes are targets of the *MYC* genes as well (MYC2, MYC3 and MYC4); however, the G-box sequence alone in the sequence of the promoter region of *JAZ2* is not sufficient to confer specificity of the proposed binding (Figueroa, Browse 2012). The consensus G-box

sequence (that is without considering G-box variant sequences!) is found in more than 5000 genes in the *A. thaliana* genome, but only a fraction of those genes are induced early by JA (Goda et al. 2008). So even if a promoter sequence contains the G-box sequence, it does not necessarily mean these genes are regulated by MYC2 in a JA dependent manner.
Recent research showed that the promoters of genes activated in early response to JA are enriched with a thymidine residue, which are highly conserved, even in related *Brassicaceae* species. A genome-wide search for the G-box-TTTT motif in promoters of *A. thalaiana* genes revealed that 25.0% of these genes are known to be induced within 30 min of JA treatment (Figueroa, Browse 2012).
Knowledge of this additional G-box-TTTT motif creates an opportunity in the search of MYC-regulated genes. However, the focus of this research is not to which factors are MYC-regulated, but rather on how *MYC2* transcription is regulated. It is known that the *JAZ* genes and the *MYC* genes regulate each other (Dombrecht et al. 2007, Chini et al. 2007, Figueroa, Browse 2012). It is therefore very possible that the *JAZ* genes regulate *MYC2* transcription in a similar manner that the *MYC* genes themselves are regulated through binding of the JAZ genes to additional specificity sequences present in the *MYC2* promoter region.

EIN3 possibly binds to the GCC-box found in the promoter of ERF1, but it is also able to bind to another DNA sequence, found in the promoter of transcription factor SID2. EIN3 binds to the ATGTAC sequence, both *in vitro* and *in vivo* (Kosugi, Ohashi 2000). The SID2 transcription factor is involved in the crosstalk between the SA and JA hormonal pathways. The fact that EIN3 is able to distinguish between two different DNA sequences to activate the transcription of target genes in different pathways, shows that additional research to the binding sequences of transcription factors is definitely needed. This is necessary in order to understand the underlying mechanism in the distinction of the transcription factors regulating their downstream targets. EIN3 is not the only transcription factor described in this paper that has multiple different target genes it regulates in different situations, such as functions in development and defence. Somehow the transcription factor is targeted to the promoter region of the specific target. How transcription factors 'know' which target gene to activate or repress under certain circumstances, could be explained by induction of different interaction partners. These could be transcribed as a primary reaction to the environment and interact with the transcription factor. This might even slightly change the conformation

of the transcription factor, in order for it to bind to the correct DNA sequence so it is able to regulate the proper response to the environmental cue. However, this is all still just an hypothesis and should be investigated in more detail.

Chapter 3.2 Protein interaction domains

Despite the knowledge on the target DNA-sequences of the promoter regions that the transcription factors of interest regulate, not much is known about the sequences that are needed for protein-protein interactions. Understanding the sites of protein-protein interactions would provide us with information that could be used in further studies. This information would be useful in the identification of new transcription factors or other proteins involved in defence. The knowledge in protein structure and different interaction domains would facilitate the ease with which putative interactions can be assigned to newly found proteins.

Chapter 4 Discussion

The defence of plants is a very important topic of research. This is not remarkable since the plants are our main resource of food. Agriculture could benefit of the knowledge, since we may be able to enhance plants to produce better crops. Understanding the resistance of plants against pathogens is therefore essential. Our current knowledge shows that plants interact with several rhizobacteria and fungi in the rhizosphere. Beneficial microbes enhance the state of the plant; the so-called primed state of the plant. This enables the plant to react faster upon pathogen attack (van Loon, Bakker & Pieterse 1998, Van der Ent et al. 2008). Also the previously discussed ISR will enable the plant to react faster upon an attack and successfully defeat the pathogen. The molecular mechanisms of both priming and ISR, which partly included priming, are currently studied. These defence mechanisms are unique to plants.

It is already known that transcription factors MYB72 and MYC2 play crucial roles in the defence mechanisms (Van der Ent et al. 2008, Pozo et al. 2008). MYC2 is a key player in regulating the transcription of genes that are needed in the defence against wounding and herbivory (Pozo et al. 2008). MYC2 represses the transcription of genes against pathogens, this action is balanced with the action of *ERF1*. *ERF1* transcription is regulated by EIN3, and it activates the transcription of the genes repressed by MYC2 and represses the transcription of genes activated by MYC2 (Pozo et al. 2008, Lorenzo et al. 2003). This way the plant can 'decide' to put its energy in either defence against pathogens or wounding. When beneficial microbes induce ISR, the transcription factor *MYB72* is induced (Van der Ent et al. 2008). MYB72 is known to be able to interact with MYB10 and with EIL3 (Van der Ent et al. 2008; C. Pieterse, personal communication). ISR also causes a primed state of the plant, causing it to react faster when under attack of pathogens. This raised the question as to whether there is a molecular connection between these two major defence mechanisms. The first indication that there might be a connection is the regulation of *ERF1*; its transcription is regulated by EIN3 (Solano et al. 1998). EIN3 is a transcription factor with close homologs. One of these homologs is EIL3, which is able to interact with MYB72 (Van der Ent et al. 2008). This raised the question whether other homologs are able to interact as well and this way be able to influence the outcome between transcription of ERF1 and MYC2 regulated genes. Here we

have analyzed the predicted expression patterns of the closely related ERFs, MYC2 and its closest homologs and of MYB72 and MYB10.

Looking at the predicted expression patterns, MYB72 and the EIL3 are expressed in the same tissue in the plant. Their expression pattern also overlaps with tissue in which ERF1 is expressed. In the same domain MYB10 is predicted to be expressed, so it is plausible these factors could interact *in vivo* to regulate ERF1 transcription. Expression of ORA59, closely related to ERF1, is predicted to be found in a slightly different pattern, but since ORA59 acts redundantly of ERF1, this would cause a broader range in which the target downstream genes are transcribed and act in defence.

Not surprisingly, ERF1 and MYC2 share an expression domain, regulating the same downstream genes. It would be most plausible that MYB72 forms a complex with MYB10 and EIL3, regulating the transcription of *ERF1* and/or *ORA59* (considering redundancy) and possibly even the transcription of *MYC2*. This, however, is less likely, since there is no evidence at all that *MYC2* transcription is regulated by ERFs. On the contrary, it has been very well established that the transcription of the *MYC* genes are regulated by JAZ repressors (Fernandez-Calvo et al. 2011, Niu, Figueroa & Browse 2011, Demianski, Chung & Kunkel 2012).

Chapter 4.1 Extended research on expression patterns is necessary

Still, all this is only a hypothesis. In order to be sure that this is the complex regulating *ERF1* and/or *ORA59* transcription further research is necessary. The *in vivo* expression patterns need to be established in wild type plants, and in plants that have been treated with pathogens. It is possible that the expression pattern could change upon pathogen treatment. Also experiments need to be conducted in order to establish whether the transcription factors ERF1, EIL3, MYB72, MYB10 and MYC2 are expressed in the same cells *in vivo*.

Chapter 4.2 Further research is needed on the proposed interactions

Also, for the proposed interactions previous researched failed to provide us with evidence. We need to establish firstly whether EIL3, like EIN3, can bind to the promoter of *ERF1* and *MYC2*. If this is not the case, there is no possibility of intercommunication between the two different defence mechanisms. A very simple experiment to find out whether EIL3 can bind

to the promoter of either *ERF1* and *MYC2* is a yeast 1 hybrid experiment. Since it is very likely this regulation will only happen upon pathogen attack, and not during healthy conditions, it would be useful to see whether the interaction is genuine *in vivo* using Chromatin Immunoprecipitation (ChIP).

If we would consider EIL3 indeed interacts with the promoter of either *ERF1* and/or *MYC2*, we still would need to establish whether EIL3 needs to interacts with MYB72 and MYB10 in order to be able to affect *ERF1*/*MYC2* transcription. This is a very important step in order to be able to link the two defence mechanisms. If EIL3 is able to bind the promoter of either *ERF1* and /or *MYC2* upon pathogen attack, this is still separate from the ISR, of which the first responsive gene is *MYB72*. It was previously established that EIL3 and MYB72 are able to interact, just like it is known that MYB72 is able to interact with MYB10. But is this a genuine, *in vivo* interaction? Do these factors interact in a complex? Is MYB10 the intermediate interacter between MYB72 and EIL3? Or is the interaction between EIL3 and MYB72 a direct one? Is MYB10 necessary at all? All these questions would be easily answered firstly by yeast 2 hybrid experiments, to confirm the interaction between the proteins *in vitro*. Then interaction complexes could be isolated before and after pathogen attack using co-immunoprecipitation (Co-IP).

In order to establish whether the previously found mechanisms of defence interact *in vivo*, further research is needed. However, based on what is already found in the research to the molecular mechanism of the separate defence mechanisms of priming and ISR, it is likely the two are linked. Here we propose a new link in the molecular regulatory network which underlies the defence of plants.

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