WRKY transcription factors involved in salicylic acid-induced defense gene expression
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Summary
SUMMARY

As plants are constantly challenged to cope with a broad variety of stresses, they have developed sophisticated mechanisms that control a range of defense responses. Defense programs against pathogens are regulated through three important signaling pathways, the salicylic acid (SA), jasmonate (JA) and ethylene (ET) signaling pathways. The current knowledge of SA, JA and ET biosynthesis and the transcriptional regulation of defense responses mediated through these signal molecules is discussed in Chapter 1.

The SA signaling pathway triggered by attack of biotrophic pathogens leads to broad spectrum resistance against a plethora of pathogenic fungi, bacteria and viruses and is known as systemic acquired resistance (SAR). One of the hallmarks of SAR is the accumulation of PR proteins and the induced expression of the PR-1 gene is often used as a marker for SAR.

In Chapter 2 we used a cDNA library from tobacco mosaic virus (TMV) infected tobacco plants to screen in a yeast one hybrid assay for proteins that can bind to the tobacco PR-1a promoter. This screening resulted in the identification of NtWRKY12, a protein belonging to the group of WRKY transcription factors. Detailed expression studies of the NtWRKY12 gene revealed that induction of NtWRKY12 coincides with the expression of PR-1a, suggesting a regulatory link between NtWRKY12 and PR-1a. The expression of NtWRKY12 was induced after exogenous application of SA, infection with Tobacco mosaic virus and upon leaf infiltration with Agrobacterium tumefaciens or Escherichia coli.

To elucidate the binding site of NtWRKY12 in the PR-1a promoter, we employed Electromobility shift assays (EMSA) with PR-1a promoter fragments. Through mutational analyses the binding site was narrowed down to the sequence TTTTCCAC. This sequence differs significantly from the consensus WRKY protein binding site TTGAC[C/T] (W-box), and was designated as the “WK-box”.

The WK-box occurs in close proximity of an as-1-like element, which is a binding site for TGA transcription factors, and an MBSII element shown to bind a Myb transcription factor. Further upstream in the PR-1a promoter another WK-box is present. The downstream and upstream WK-boxes are referred to as WK₁-box and WK₂-box, respectively.

The functional importance of the WK-box, as-1 element and MBSII site was analyzed using PR-1a promoter::GUS reporter genes. The effects of mutations in the binding sites were studied in stably transformed tobacco plants sprayed with SA, or in leaves agroinfiltrated with A. tumefaciens carrying PR-1a::GUS constructs. Mutations in the WK₁-box resulted in a major reduction of the SA- or elicitor-induced GUS expression, whereas mutations in the WK₂-box, as-1-like element or MBSII box had little or no effect on the induction of GUS expression. Combined mutations in the WK-box and the as-1-like element completely abolished PR-1a promoter-induced GUS expression. A more direct proof that NtWRKY12 was necessary for induction of PR-1a promoter activity came from transactivation assays in Arabidopsis protoplasts using 35S::NtWRKY12 and PR-1a promoter::GUS
constructs.

The close proximity of the *as-1*-like element and the WK₁-box in the *PR-1a* promoter suggested that protein-protein interactions could occur between NtWRKY12 and TGA transcription factor. This possibility was further investigated in Chapter 3. Using *in vitro* pull down assays and *in vivo* Fluorescence Resonance Energy Transfer analyses it was shown that NtWRKY12 specifically interacts with TGA2.2. No interaction could be found between the closely related TGA2.1 and NtWRKY12.

Further analyses in Arabidopsis protoplasts confirmed that NtWRKY12 and TGA2.2 have an additive effect on *PR-1a::GUS* expression. Current models for transcriptional activation of the *PR-1* gene of Arabidopsis imply the binding of co-activator NPR1 to TGA proteins on the promoter. However, co-expressed tobacco NPR1 did not further enhance reporter gene expression, and transactivation assays in protoplasts from *npr1-1* plants demonstrated that activation of the tobacco *PR-1a* promoter is independent of endogenous NPR1. Furthermore, assays in protoplasts lacking four functional Arabidopsis TGAs revealed that NtWRKY12-mediated activation of the *PR-1a* promoter is independent of endogenous TGAs, supporting the notion that NtWRKY12 is the main transcriptional activator of *PR-1a* expression.

Accumulating genetic data indicate that WRKY transcription factors function in the regulation of defense responses acting along SA, JA and ET signaling routes. Although for most of these transcription factors their involvement in defense has been deduced from gain or loss of function mutants and no direct target genes have been indentified. The close temporal correlation of the induced expression profiles of *NtWRKY12* and *PR-1a* prompted a bioinformatics approach to prospect for other links between transcription factors and genes involved in the biosynthesis of SA, JA and ET and their signaling pathways in Arabidopsis. In Chapter 4 we used publicly available datasets derived from micro arrays related to stress. First, the optimal Pearson Correlation Coefficient (PCC) cutoff was determined to enable identification of biologically relevant co-expression data. Using this PCC cutoff, a co-expression network was constructed comprising the genes involved in SA, JA and ET biosynthesis and signaling that were described in Chapter 1, complemented with a large set of genes encoding members from various classes of transcription factors. The co-expression data derived from the network indicated several links between transcription factors and signaling components that were previously reported in literature, underscroing the validity of the constructed network. In addition, we found many previously unknown links between genes, which may help future research to further unravel the complex pathways and regulatory mechanisms in stress responses.

One of these new links reflected the closely co-regulated expression of the genes encoding transcription factor WRKY28 and isochorismate synthase 1, a key enzyme of SA biosynthesis. A second link indicated co-regulation of *WRKY46* and *PBS3*, which encodes another enzyme involved in the biosynthesis of SA and its derivatives. In Chapter 5, transactivation assays in Arabidopsis protoplasts were used to study expression of promoter::*GUS* fusions with *ICS1* and *PBS3* promoter sequences of 1 kb by transiently expressed WRKY28 and WRKY46. In addition, expression of endogenous *ICS1* and *PBS3* genes by WRKY28 and WRKY46 in these protoplasts was analyzed by qRT-PCR. The results showed that WRKY28 can induce *ICS1* expression whereas WRKY46 can activate *PBS3* expression. Subsequent EMSA binding studies and chromatin immunoprecipitation
analyses revealed that WRKY28 binds to sites in the *ICS1* promoter that are only remotely similar to the consensus W-box.