Article Addendum

Downstream targets of WRKY33

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Abbreviations: BTH, benzothiadiazole; SA, salicylic acid

Key words: Arabidopsis, MAPK cascade, defense response, transcription factor, target gene

Innate immunity signaling pathways in both animals and plants are regulated by mitogen-activated protein kinase (MAPK) cascades. In a recent publication we show that MPK4 and its substrate MKS1 interact with WRKY33 in vivo, and that WRKY33 is released from complexes with MPK4 upon infection. Transcriptome analysis of a *wrky33* loss-of-function mutant identified a subset of defense-related genes as putative targets of WRKY33. These genes include *PAD3* and *CYP71A13*, which encode cytochrome P450 monooxygenases required for synthesis of the antimicrobial phytoalexin camalexin. Chromatin immunoprecipitation confirmed that WRKY33 bound the promoter of *PAD3* when plants were inoculated with pathogens. Here we further discuss the involvement of two other targets of WRKY33, *NUDT6* and *ROF2* in defense responses against invading pathogens.

Plants are constantly exposed to external stimuli by a broad range of microbes and have evolved complicated immune systems to defend against invading pathogens.^{1,2} A common mechanism to convert such external stimuli into cellular responses is the activation of mitogen-activated protein kinase (MAPK) cascades. These cascades consist of MAPKKK-MAPKK-MAPK modules that play central roles in signal transduction mechanisms linking upstream receptors and downstream targets and leading to rapid activation of defense responses upon recognition of invading pathogens.^{3,4}

Recently, MPK4 was shown to be part of a MAPK cascade including MEKK1 and MKK1/MKK2.^{5,6} MEKK1 acts downstream of the flagellin receptor FLS2 and is required with MKK1/MKK2 for activation of MPK4 by phosphorylation of conserved Thr and Tyr residues in the kinase activation loop.⁷⁻⁹ Previously, MKS1 was identified as a MPK4 substrate, and analysis of transgenic plants and transcript profiling indicated that MKS1 was required for full

resistance in *mpk4* mutants.¹⁰ MKS1 interacts with two transcription factors, WRKY25 and WRKY33, and may function as an adaptor linking MPK4 activity to WRKY-regulated gene expression. Interestingly, *wrky33* mutants exhibit enhanced susceptibility to two necrotrophs, *Botrytis cinerea* and *Alternaria brassicicola*, whereas overexpression of *WRKY33* increases resistance, suggesting a specific role of WRKY33 in plant defense against necrotrophic pathogens.¹¹

To further understand how WRKY33 regulates gene expression, we employed expression profiling using ATH1 GeneChips (Affymetrix) to screen for putative target genes of WRKY33. From this, we identified four transcripts that accumulated in response to BTH (SA analogue) treatment in wild type, but not in *wrky33*.¹² Two of these, *PAD3* and *CYP71A13*, are strongly co-regulated¹³ and are required for synthesis of the antimicrobial compound camalexin important for resistance against necrotrophic pathogens. Furthermore, we show that MPK4 and MKS1 associate in a complex with WRKY33, and that activation of MPK4 and phosphorylation of MKS1 upon infection leads to a release of MKS1 and WRKY33 from MPK4, followed by recruiting WRKY33 to the promoter of *PAD3*.

Two other putative targets identified from the microarray analysis are *NUDT6*, a member of the cytosolic Nudix hydrolase family¹⁴ and a peptidyl prolyl cis/trans isomerase (PPIase, named *ROF2*)¹⁵ that catalyze the cis/trans isomerization of peptide bonds adjacent to a proline.¹⁶ To further verify and elucidate the biological relevance of these two targets in defense, we performed real-time RT-PCR of wild-type and *wrky33* mutants after infection with different pathogens. In wild-type Col-0 the expression of *NUDT6* is barely detectable, but was found to be highly responsive to the biotrophic pathogen, *Pseudomonas syringae* (Fig. 1, *Pst* DC3000 or *Pst* DC3000 expressing the *avrRpm1* or *avrRps4* effector that triggers resistance through the host resistance genes *RPM1* or *RPS4*, respectively). In contrast, this elevation of *NUDT6* accumulation is impaired in *wrky33* mutants. Expression of *ROF2* is also responsive to *Pst* DC3000 *avrRpm1*, and impaired in *wrky33*, but is barely induced by *Pst* DC3000 or *Pst* DC3000 *avrRps4* (Fig. 1). In contrast to *PAD3* and *CYP71A13*, neither *NUDT6* nor *ROF2* expression is induced by *B. cinerea* infections, suggesting specific roles and potentially different mechanisms of regulating gene targets downstream WRKY33 dependent on different pathogens.

Previously, *NUDT6* was shown to be pathogen responsive, and *NUDT6* induction was found to depend on the defense regulators *EDS1* and *PAD4*.¹⁷ Furthermore, the *nudi7* mutant, a close homologue of *NUDT6*, shows constitutive resistance against *P. syringae*

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Figure 1. Real-time PCR quantitation of *NUDT6* and *ROF2*. Expression in Col-0 and *wrky33* before or after treatment with *Pst* DC3000 for 21 h, with *Pst* DC3000 (*avrRpm1*) for 4 h, with *Pst* DC3000 (*avrRps4*) for 14 h, or with *B. cinerea* for 48 h. Samples were tested in triplicate and normalized to *ACTIN2*. Means $\pm$ SD are shown.

and *Hyaloperonospora parasitica* suggesting these two closely related *NUDTs* function as repressors of the basal defense response.¹⁸ *ROF2* is a member of plant co-chaperones FKBP that function in signal transduction and *ROF2* has chaperone activity. Recent research suggests that *ROF2* interacts with heat shock protein 90 (HSP90).¹⁵ Interestingly, the *hsp90* mutant was shown to be impaired in *RPM1*-specified hypersensitive cell death (hypersensitive response; HR) and disease resistance.¹⁹ These data are in agreement with *ROF2* being only responsive to *Pst* DC3000 expressing *avrRpm1*.

In summary, induction of both *NUDT6* and *ROF2* appear to be dependent on WRKY33 and both seem to be involved in defense responses upon recognition of invading pathogens. However, further studies are required to determine if these genes are direct targets of WRKY33, as was shown for *PAD3*.¹²

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